Conversion of cerebral cortex into basal ganglia in *Emx2*$^{-/-}$ *Pax6*$^{Sey/Sey}$ double-mutant mice

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The molecular mechanisms that activate morphogenesis of cerebral cortex are currently the subject of intensive experimental analysis. Transcription factor genes of the homeobox, basic helix-loop-helix (bHLH) and zinc-finger families have recently been shown to have essential roles in this process. However, the actual selector genes activating corticogenesis have not yet been identified. Here we show that high-level expression of at least one functional allele of either of the homeobox genes *Emx2* or *Pax6* in the dorsal telencephalon is necessary and sufficient to stably activate morphogenesis of cerebral cortex and to repress that of adjacent structures, such as striatum.

The molecular mechanisms that trigger morphogenesis of cerebral cortex instead of adjacent telencephalic structures, such as basal ganglia and fimbria, are still largely unknown. Several transcription factor genes differentially expressed in the early telencephalic wall may be crucial for its later morphological subdivision into cerebral cortex and adjacent structures; recent experimental work on mutant mice has confirmed these predictions. The selector genes that activate corticogenesis in place of other competing processes, however, have not been identified. The restriction of homeobox gene *Lhx2* products to the cortical anlage sets up the boundary between presumptive cortex and cortical hem, namely the forerunner of the fimbria[1,2]. The confinement of homeobox gene *Gsh2* products to basal forebrain fixes the boundary between pallial and striatal fields[3,4]. However, cortical specification is not fully abolished in *Lhx2*$^{-/-}$ mutants and complete striatal-cortical conversion does not occur in *Gsh2* knockouts[1–4]. The bHLH genes *Ngn1/Ngn2* and *Mash1* promote multiple aspects of corticogenesis and basal ganglia morphogenesis respectively, although they do not act as 'master genes'. Rather, they simply link regional patterning to the activation of specific neuronogenetic pathways in these structures[5]. The zinc-finger gene *Gli3* is also crucial for corticogenesis; but the cortex of *Gli3*$^{-/-}$ mutants, ventralized and reduced in size, still retains signs of cortical specification[6,7]. The two homeobox genes *Emx2* and *Pax6*, which are expressed in the dorsal telencephalic wall from early in development[8–10], are involved in multiple aspects of cortical morphogenesis including neuroblast proliferation[10–12], development of radial glia[10,13], neuronal radial migration and lamination[13–15], regionalization and arealization[11,16,17] and histogenesis of archicortex and lateral cortex[11,18–21]. Nevertheless, in the absence of either *Emx2* or *Pax6*, cerebral cortex forms and, even if variously affected, is morphologically and molecularly distinguishable from adjacent structures such as basal ganglia and fimbria.

We have previously observed that some laminar markers expressed throughout the cortical field are downregulated in *Emx2* and *Pax6* loss-of-function mutants, respectively, in regions where ventricular expression of *Emx2* and *Pax6* is more intense. This led us to suggest that *Emx2* and *Pax6* could independently activate key aspects of the pan-cortical morphogenetic program by acting along parallel pathways and leading neuroblasts in the dorsal telencephalon to adopt cortical fates (ref. 13 and unpub. observ.). To test this hypothesis, we generated double-knockout mice for *Emx2* and *Pax6* and analyzed embryonic development of their telencephalon. In these mutants, regionalization of the telencephalic vesicle was dramatically altered. A large set of genes normally restricted to the cortical field were silent, whereas genes normally confined to proliferative layers of the ganglionic field or to the cortical hem were intensely expressed throughout the cortical primordium. This was followed by massive ectopic activation of basal differentiation pathways in the cortical primordium, which finally acquired morphological and molecular features reminiscent of those of the striatum. Robust expression of at least one fully functional allele of either *Emx2* or *Pax6* therefore seems necessary and sufficient to stably activate corticogenesis and to suppress competing neighboring morphogenetic programs.

Results

Mispatterning of *Emx2*$^{-/-}$ *Pax6*$^{-/-}$ telencephalons

By mating mice double heterozygous for *Emx2* and *Pax6* loss-of-function alleles[18,22], we generated embryos mutant for both genes in various allelic combinations. We used these embryos at different gestational ages, up to at least embryonic day (E) 17.5, and analyzed development of the cerebral cortex. Hematoxylin

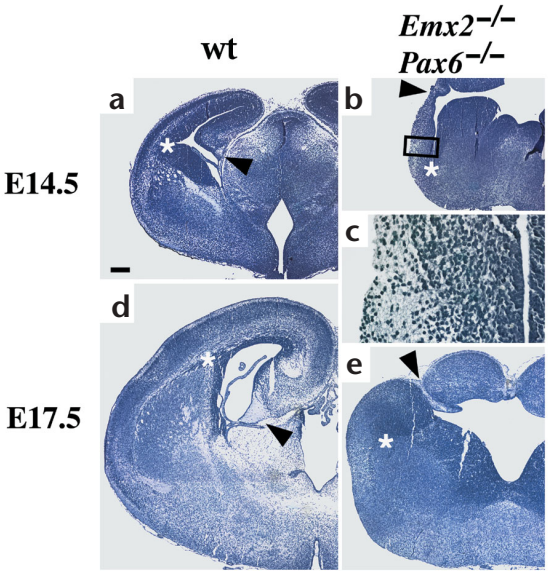

Fig. 1. Hematoxylin staining of *Emx2 Pax6* mutant telencephalons. Mid-frontal sections from E14.5 (**a**, **b**) and E17.5 (**d**, **e**), wild type (wt; **a**, **d**) and $Emx2^{-/-}$ $Pax6^{-/-}$ (**b**, **e**) mouse embryos. (**c**) Magnification of the area boxed in (**b**). Arrowheads point to presumptive cortical hem, asterisks mark putative corticostriatal boundary. Scalebar, 200 μm.

staining showed that morphogenesis of cerebral cortex was impaired in $Emx2^{-/-}$ $Pax6^{-/-}$ embryos at early stages of cortical development (data not shown). At E14.5, their dorsal telencephalic wall was reduced tangentially and composed of an inner, cell body–rich zone that lined the ventricular cavity, and an outer, fiber-rich plexiform layer (**Fig. 1a–c**). By E17.5, this abortive cortical primordium acquired a highly aberrant morphological profile resembling adjacent basal ganglia (**Fig. 1d** and **e**).

To dissect the molecular regionalization of the developing $Emx2^{-/-}$ $Pax6^{-/-}$ telencephalon, we scored mid-neuronogenetic expression patterns of two sets of genes normally confined to the pallial and the subpallial wall. The pallial transcription factor genes *Tbr2*, *Ngn2*, *Ngn1*, *Pax6* and *Emx1*[5,8,23,24] were switched off (**Fig. 2d, h, l, p** and **t**), with the exception of a few EMX1-immunoreactive presumptive neurons that were detectable in the marginal cortex (**Fig. 2u**, arrowheads). The subpallial markers *Gsh2*, *Vax1*, *Islet1*, *Gad65/67*, *Ebf1*, *Six3*, *CRBP-I* and *Calbindin*[4,25–27] were ectopically activated throughout the cortical primordium according to the ventricular-to-marginal progression they exhibit in developing basal ganglia (**Fig. 3a, d, e, h, m, p, q, t, u** and **x** and data not shown). Moreover, the transcription factor gene *Nkx2.1*, normally confined to the medial ganglionic eminence (MGE)[28], was also ectopically expressed in the lateral ganglionic eminence (LGE; **Fig. 3i** and **l**). These patterns suggest that cortex-to-LGE and LGE-to-MGE molecular-specification shifts did occur in $Emx2^{-/-}$ $Pax6^{-/-}$ mutants. To test if cortical hem and choroid plexus fates also spread into the pallial field of these mutants, we analyzed the expression of several genes that demarcate boundaries among pallium, hem and choroid plexus. The genes *Id3*, *Otx2* and *Msx1*, normally restricted to heminested domains including the cortical hem and the choroid plexus[11,29,30], (**Fig. 4m, e** and **i**) were expressed in a large part of the dorsal telencephalon (**Fig. 4p, h, l** and **d**), but the choroid plexus marker *Ttr1*[30] was not (**Fig. 4a**), suggesting that cortical hem identities spread into this structure. However, this phenomenon was largely incomplete. *Lhx2* and *Bf1*, normally detectable throughout the telencephalon except for the cortical hem[1,2,31], were not affected, with the exception of anomalous *Bf1* negative patches in the marginal-dorsal pallium (**Fig. 4u** and **x**, asterisk, and data not shown); the cortical

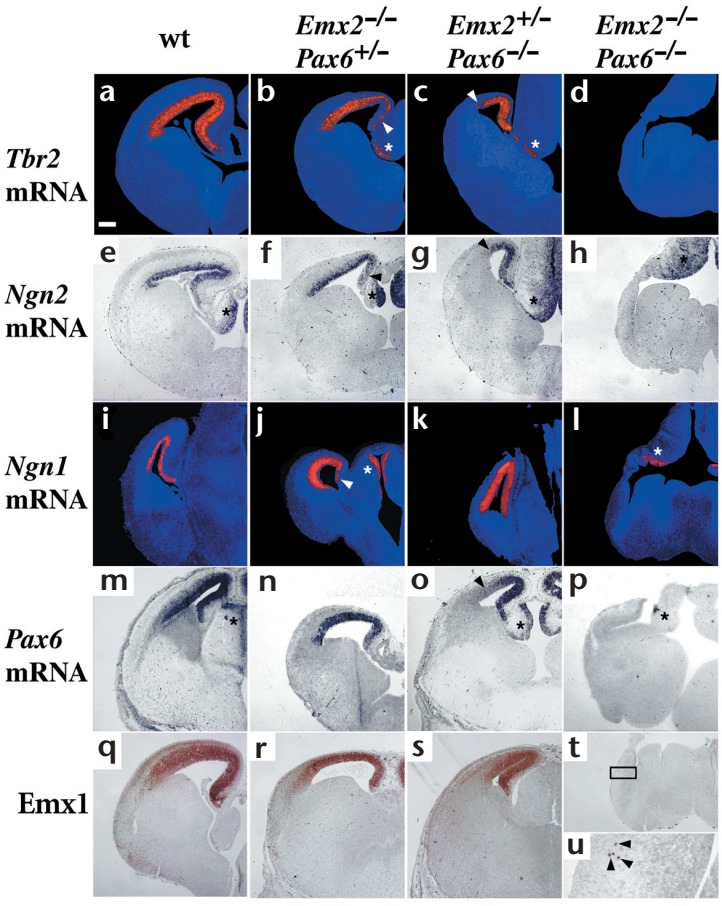

Fig. 2. Downregulation of cortical markers in late gestation *Emx2 Pax6* mutants. *Tbr2* (**a–d**), *Ngn2* (**e–h**), *Ngn1* (**i–l**), *Pax6* (**m–p**) mRNAs and Emx1 (**q–t**) protein in telencephalons of wild-type (wt; **a**, **e**, **i**, **m**, **q**), $Emx2^{-/-}$ $Pax6^{+/-}$ (**b**, **f**, **j**, **n**, **r**), $Emx2^{+/-}$ $Pax6^{-/-}$ (**c**, **g**, **k**, **o**, **s**) and $Emx2^{-/-}$ $Pax6^{-/-}$ (**d**, **h**, **l**, **p**, **t**) E14.5 embryos. (**u**) Magnification of the area boxed in (**t**); frontal sections, medial to the right. Arrowheads point to medial cortex of $Emx2^{-/-}$ $Pax6^{+/-}$ mutants, where *Tbr2*, *Ngn2* and *Ngn1* are downregulated (**b**, **f**, **j**), to lateral cortex of $Emx2^{+/-}$ $Pax6^{-/-}$ mutants; where *Tbr2, Ngn2 and Pax6* are downregulated (**c**, **g**, **o**), to rare Emx1-immunoreactive cells in the putative pallial primordium of $Emx2^{-/-}$ $Pax6^{-/-}$ mutants (**u**). Asterisks mark diencephalic expression domains of *Tbr2* (**b**, **c**), *Ngn2*(**e–h**), *Ngn1* (**j**, **l**) and *Pax6* (**m**, **o**, **p**). Scalebar, 200 μm.

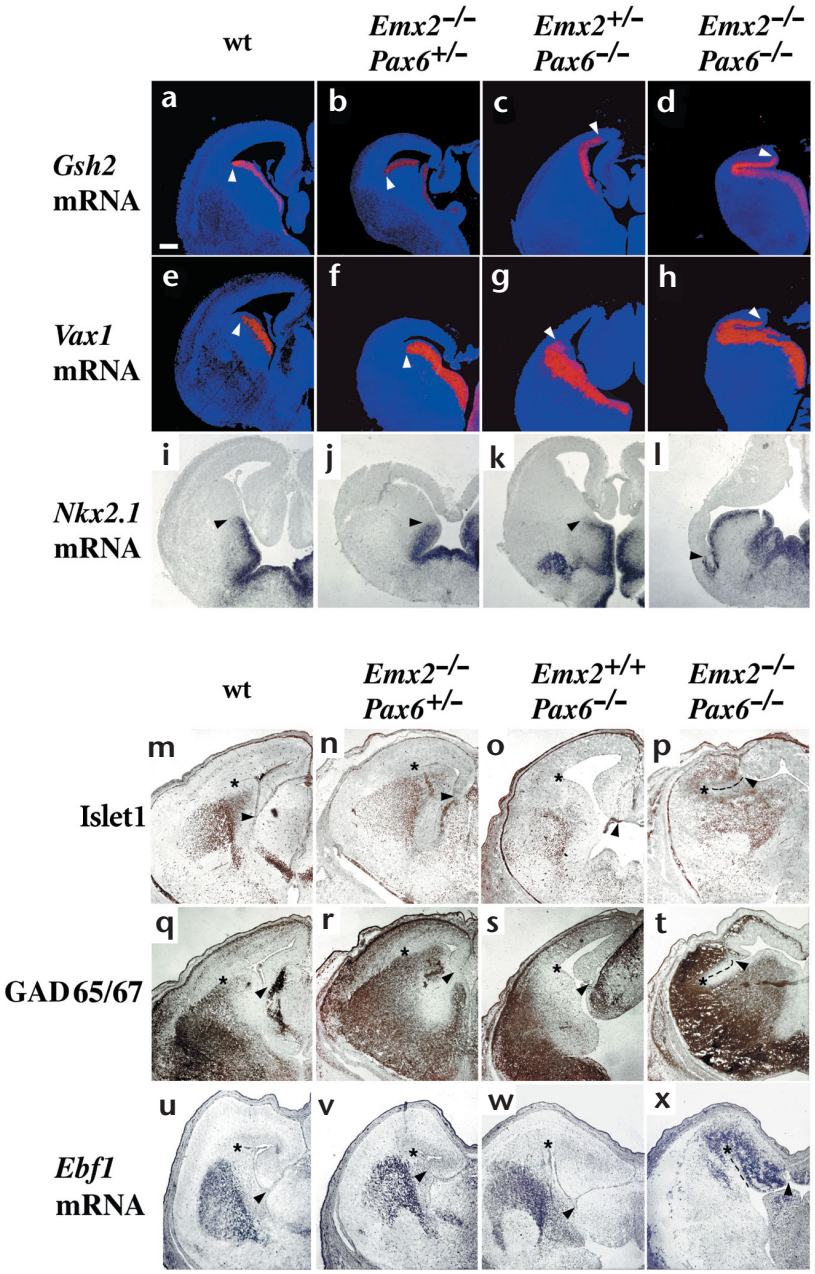

Fig. 3. Ectopic expression of basal ganglia markers in the cortex of late gestation *Emx2 Pax6* mutants. *Gsh2* (**a**–**d**), *Vax1* (**e**–**h**) and *Nkx2.1* (**i**–**l**) mRNAs, Islet1 (**m**–**p**) and GAD65/67 (**q**–**t**) proteins and *Ebf1* mRNA (**u**–**x**) in telencephalons of wild-type (**a**, **e**, **i**, **m**, **q**, **u**), $Emx2^{-/-}$ $Pax6^{+/-}$ (**b**, **f**, **j**, **n**, **r**, **v**), $Emx2^{+/-}$ $Pax6^{-/-}$ (**c**, **g**, **k**), $Emx2^{+/+}$ $Pax6^{-/-}$ (**o**, **s**, **w**), and $Emx2^{-/-}$ $Pax6^{-/-}$ (**d**, **h**, **l**, **p**, **t**, **x**) E14.5 (**a**–**t**) and E16.5 (**u**–**x**) embryos. Frontal sections, medial to the right. In (**a**–**l**), arrowheads point to the dorsal boundary of the expression domains; in (**m**–**x**), arrowheads point to the dorsomedial edge of the dorsal telencephalic wall, asterisks demarcate presumptive corticostriatal notch. Scalebar, 200 μm.

hem marker *Wnt8b*[32] was also not affected (**Fig. 4q** and **t**). This molecular profile was specific to knockout mice for both genes; anomalies detectable in mice knockout for either *Emx2* or *Pax6* were much less pronounced. Cortical markers *Tbr2*, *Ngn2* and *Ngn1* were downregulated medially in $Emx2^{-/-}$ $Pax6^{+/-}$ mutants (**Fig. 2b**, **f** and **j**, arrowheads) and rostro-laterally in $Emx2^{+/-}$ $Pax6^{-/-}$ mutants (**Fig. 2c** and **g**, arrowheads, and data not shown); *Pax6* was also rostro-laterally downregulated in the latter mutants (**Fig. 2o**, arrowhead). Basal markers were unaffected in the presence of at least one functional *Pax6* allele (Fig. **3b**, **f**, **j**, **n**, **r** and **v**); *Gsh2* and *Vax1* domains were only moderately enlarged in $Emx2^{+/-}$ $Pax6^{-/-}$ embryos (**Fig. 3c** and **g**). Notably, this pattern of cortex-to-basal ganglia and cortex-to-cortical hem conversion was specific to mice mutant for *Emx2* and *Pax6*. In double-knockout embryos for *Pax6* and the *Emx2* paralog, *Emx1*[19], *Tbr2* and *Ngn2* were expressed throughout the archicortical anlage and *Otx2* was tightly confined to cortical hem and choroid plexus; moreover, *Gsh2* was not differentially expressed in their brains as compared with those of $Pax6^{-/-}$ embryos (data not shown).

Respecification of $Emx2^{-/-}$ $Pax6^{-/-}$ cortical neuroblasts

To cast light on mechanisms leading to the large-scale regionalization defects described above, we re-analyzed the molecular profile of $Emx2^{-/-}$ $Pax6^{-/-}$ mutants at earlier stages. Defects in cortical specification were evident from the onset of cortical neuronogenesis (E11). *Pax6*, normally expressed in the dorsal telencephalon in a high-lateral to low-medial gradient (**Fig. 5a**), was dramatically downregulated, being detectable in a few cells in the medial cortical field (**Fig. 5b**, solid arrowheads); *Emx1* was also downregulated (**Fig. 5d**, solid arrowheads); *Tbr2*, normally expressed at this age in postmitotic neurons in the marginal zone (MZ) and in proliferating cells clustered in the lateral ventral zone (VZ; **Fig. 5e**), was almost undetectable, with its expression confined to rare marginal cells in the medial cortical field (**Fig. 5f**, solid arrowheads). This mis-specified cortical primordium was instead ventralized and acquired features of LGE. Laterally, it expressed the LGE-specific homeogene *Gsh2* (**Fig. 8j**); pan-basal markers *Mash1*, *Dlx1*, *Dlx2*, *Islet1* and *Gad65/67* (but not MGE-specific markers *Shh*[28] and *Lhx6*[33]) were activated throughout its medial-lateral extent (**Fig. 6d**, **h** and **l** and data not shown). Finally, we detected no spreading of cortical-hem fates into the cortical field at this stage, as suggested by *Wnt3a* and *Wnt8b* expression profiles[32] (data not shown). These findings are in agreement with the more pronounced phenotype that characterizes mid-neuronogenetic stages, when all ventricular cortical markers are switched off and cortical neuroblasts exhibit molecular properties highly similar to those of striatal neuroblasts, suggesting that late morphological abnormalities may arise largely from early patterning defects.

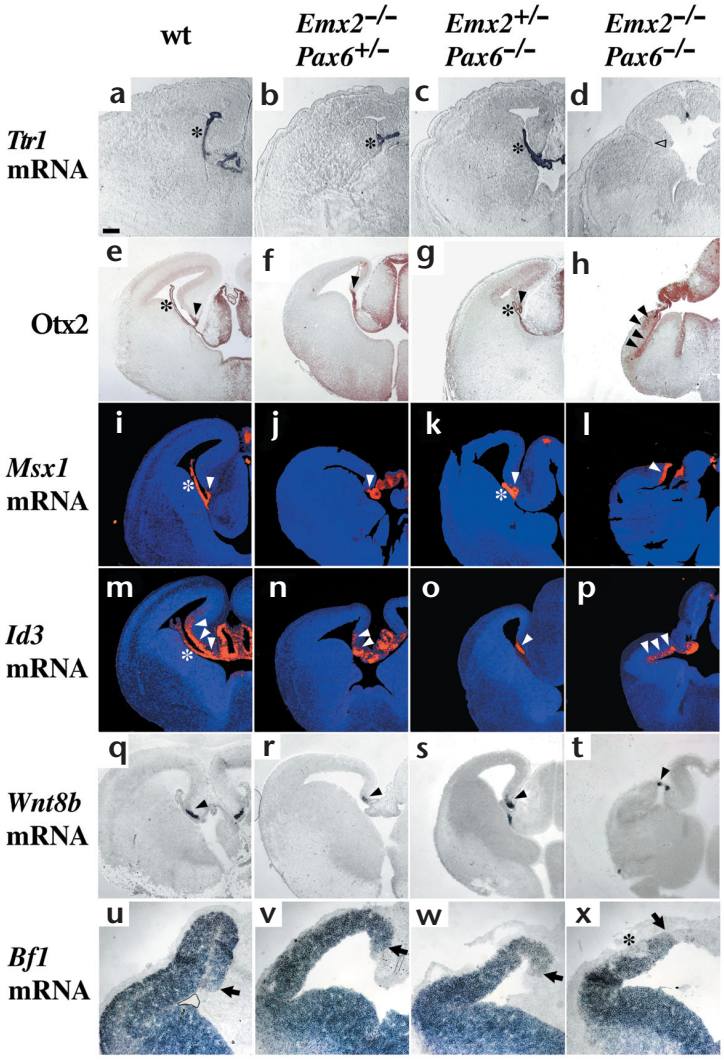

Fig. 4. Spreading of cortical hem identities in the cortex of late gestation *Emx2 Pax6* mutants. *Ttr1* mRNA (**a**–**d**), Otx2 protein (**e**–**h**), *Msx1* (**i**–**l**), *Id3* (**m**–**p**), *Wnt8b* (**q**–**t**) and *Bf1* (**u**–**x**) mRNAs in telencephalons of wild-type (wt; **a**, **e**, **i**, **m**, **q**, **u**), $Emx2^{-/-}$ $Pax6^{+/-}$ (**b**, **f**, **j**, **n**, **r**, **v**), $Emx2^{+/-}$ $Pax6^{-/-}$ (**c**, **g**, **k**, **o**, **s**, **w**) and $Emx2^{-/-}$ $Pax6^{-/-}$ (**d**, **h**, **l**, **p**, **t**, **x**) E14.5 (**a**–**t**) and E12.5 (**u**–**x**) embryos. Frontal sections, medial to the right. In (**e**–**t**), solid arrowheads point to expression domains in the hem and in the pallial field, asterisks mark choroid plexus domains; in (**d**) an empty arrowhead indicates presumptive dorsal tele-diencephalic border; in (**u**–**x**), arrows demarcate the dorsomedial boundary of the *Bf1* expression domain; in (**x**), asterisk marks a large patch of cortical tissue not expressing *Bf1*. Scalebar, 200 μm.

Remarkably, large numbers of neurons expressing Islet1, GAD65/67, Calbindin and *Ebf1* accumulated in the marginal zone of the cortical field between E11 and E16, giving it a striatum-like molecular profile. To explain this accumulation, we considered three hypotheses. First, interneurons born in the basal telencephalon and migrating to more dorsal locations may have massively penetrated abventricular layers of the dorsal telencephalon, thus diluting the less numerous, locally born neurons of cortical phenotype. Second, after a proliferative collapse and/or a burst of apoptosis in cortical germinative layers, basal neuroblasts may have massively infiltrated these layers and replaced local proliferating populations, subsequently generating neurons of basal phenotype. Third, cortical neuroblasts may have been respecified, that is, homeotically transformed into basal neuroblasts able to generate $Islet1^+$ GAD^+ neurons.

To assay the first hypothesis, we scored the distribution of three markers expressed by migrating neurons born in the MGE: the LIM homeobox gene *Lhx6*, expressed by GABAergic neurons, which spread throughout the telencephalon[33]; the LIM homeobox gene *Lhx7*, possibly restricted to acetylcholinergic neurons that diffuse into the adjacent striatum[34]; and the semaphorin receptor *Nrp1*, expressed by a very large set of interneurons that escape the striatum and migrate through the cerebral cortex[35]. Almost no $Lhx6^+$, $Lhx7^+$ or $Nrp1^+$ cells could be detected in the dorsal telencephalic wall of *Emx2 Pax6* double knockouts (data not shown), suggesting that ventral interneurons did not flow in an uncontrolled manner into the cortex of these mutants. This interpretation was also confirmed by *in vivo* short-term 5-bromodeoxyuridine (BrdU) pulse-chase experiments, in which E11.5 wild-type and *Emx2 Pax6* double-mutant embryos were pulsed by BrdU and fixed after 8 h, followed by analysis of the distribution of cells immunoreactive for BrdU and the basal ganglia postmitotic marker Islet1. We reasoned that this delay should be long enough to allow newborn neurons to reach the marginal edge of proliferative layers[36] and to switch *Islet1* on[28], and too short for them to subsequently move far in the tangential plane from their place of birth[37,38]. As expected, numerous $BrdU^+$ $Islet1^+$ cells were found in the basal telencephalon of both genotypes (**Fig. 7a**, **e**–**g** and data not shown), but a substantial number of $BrdU^+$ $Islet1^+$ cells were also found throughout the medial-lateral extent of the marginal cortical field of $Emx2^{-/-}$ $Pax6^{-/-}$ mutants (**Fig. 7a**, **b**–**d**). We obtained similar results by comparing distributions of BrdU and GAD on the same embryos (not shown). These findings suggest that a large fraction of $Islet1^+$ and GAD^+ cells detectable in the pallial telencephalon of $Emx2^{-/-}$ $Pax6^{-/-}$ mutants was locally generated.

To determine whether progenitors of these $Islet^+$ and GAD^+ cells were autochthonous or came from basal forebrain, we analyzed the growth kinetics of cortical population using a variety of approaches. In both wild-type and $Emx2^{-/-}$ $Pax6^{-/-}$ telencephalons, almost all cells in the ventricular zone expressed the proliferating cell nuclear antigen (PCNA)[39] independently on their dorsoventral location (**Fig. 8b** and **f**). Mitotic cells immunopositive for phosphohistone H3 (ref. 40), were found at similar spatial frequencies along the entire ventricular edge of the telencephalic wall, in both wild type and double knockouts (**Fig. 8c** and **g**). BrdU-uptake profiles, as detected in pulse-chase experiments previously described (**Fig. 7a** and **b** and data not shown), did not suggest any marked reduction of proliferation rates in the dorsomedial pallium of double mutants, where a residual cortical specification is still retained at E11 (**Fig. 5**). Finally, cells immunopositive for the apoptotic marker activated caspase3 (ref. 41) were very rare in the cortical fields of both

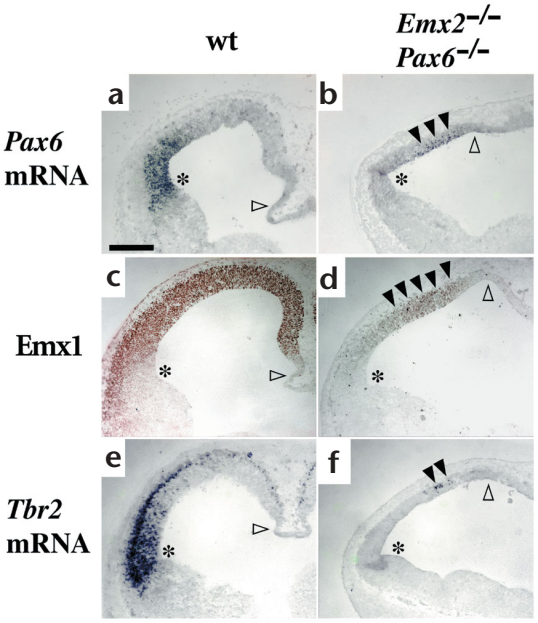

Fig. 5. Downregulation of cortical markers in mid-gestation *Emx2 Pax6* mutants. *Pax6* mRNA (**a**, **b**), Emx1 protein (**c**, **d**) and *Tbr2* mRNA (**e**, **f**) in telencephalons of E11.5 wild-type (**a**, **c**, **e**) and $Emx2^{-/-}$ $Pax6^{-/-}$ (**b**, **d**, **f**) embryos. Frontal sections, medial to the right. Asterisks demarcate the corticostriatal notch, empty arrowheads point to presumptive dorsal tele-diencephalic boundary. In (**b**, **f**), solid arrowheads point to very few cells expressing *Pax6* and *Tbr2*; in (**d**), they demarcate cells in the middle cortical field, more intensely immunopositive for Emx1. Scalebar, 200 μm.

genotypes (**Fig. 8d** and **h**). Thus, no growth collapse occurred in the $Emx2^{-/-}$ $Pax6^{-/-}$ cortical VZ, suggesting that cortical neuroblasts of double mutants were not fully replaced by basal neuroblasts and could contribute, upon striatal respecification, to generation of the local neuronal complement. To verify this inference, we scored early dorsal telencephalon for cells co-immunopositive for pallial and subpallial markers, reasoning that these cells would serve as an index of ongoing homeotic respecification. Emx1 and Gsh2, normally restricted to distinct telencephalic progenitors, respectively committed to cortical and ganglionic morphogenetic programs[3], were co-expressed in the majority of neuroblasts the lateral cortical field of E11.5 $Emx2^{-/-}$ $Pax6^{-/-}$ mutants (**Fig. 8i–j**). This phenomenon did not occur in either *Emx2* or *Pax6* simple-knockout mutants (data not shown). At the same stage, *Mash1* and *Dlx1* mRNAs, normally confined to basal telencephalon, were also activated in a substantial fraction of cells in the dorsal telencephalon, where almost all neuroblasts still expressed low levels of Emx1 (data not shown). Thus, at E11, cortical neuroblasts of $Emx2^{-/-}$ $Pax6^{-/-}$ mutants proliferate and seem to undergo extensive pallial-to-subpallial respecification, paving the way to massive ectopic generation of striatal-like neurons in the abortive cortical primordium.

Discussion

Striatogenesis in the $Emx2^{-/-}$ $Pax6^{-/-}$ cortical field

Telencephalic patterning is a multistep process that largely relies on a dynamic interplay between signals from patterning centers at the borders of the telencephalic field and response to these signals by cells composing the field itself[2,28,31,42]. Of crucial importance in this process is differential activation of transcription factor genes, able—in different combinations—to promote morphogenetic programs specific to various structures arising from the primitive telencephalic wall. We show here that if the two homeobox genes *Emx2* and *Pax6*, normally expressed at high levels in the developing cortical primordium, are both inactivated, the telencephalic pallial neuroblasts are re-specified as subpallial neuroblasts, and this is followed by formation of an additional striatum-like structure in place of the cerebral cortex.

The analysis of mid-neuronogenetic molecular profiles of $Emx2^{-/-}$ $Pax6^{-/-}$ telencephalons suggests that both *Emx2* and *Pax6* are involved in more than one primary developmental choice. On the dorsomedial side of the dorsal telencephalic wall, it is determined at an early stage whether neuroblasts will give rise to cortical hem or to cortex. The BMP-dependent restriction of *Lhx2* to the cortical field is essential for this process[1,2], molecular correlates of which are the confinement of *Bf1* to the cortical field[43] and the activation of *Msx1* and *Otx2* in the cortical hem[29,30,43]. The upregulation of *Msx1* and *Otx2* and the presence of patches of tissue free of *Bf1* expression in the $Emx2^{-/-}$ $Pax6^{-/-}$ cortical primordium suggest that *Emx2* and *Pax6* normally cooperate with *Lhx2* in repressing hem-specific programs in the cortical field. At more ventral locations, neuroblasts will generate either cerebral cortex or basal ganglia. *Shh*-dependent activation of *Nkx2.1* and *Gsh2* in the anlagen of MGE and LGE promotes pallidal and striatal morphogenetic programs, respectively, and downregulates cortical markers[27,28,44,45]. Low levels of *Pax6* mRNA, normally available in the LGE, are sufficient to confine *Nkx2.1* to the MGE; in the absence of *Pax6*, *Nkx2.1* is activated in the medial LGE, which acquires features of the MGE[21]. In a similar way, high levels of *Pax6* product, normally avail-

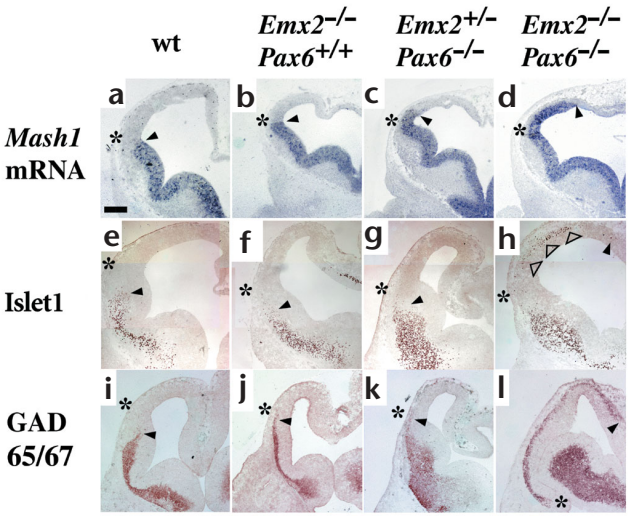

Fig. 6. Ectopic expression of ventral markers in the cortical anlage of mid gestation *Emx2 Pax6* mutants. *Mash1* mRNA (**a**–**d**), Islet1 (**e**–**h**) and GAD (GAD65 plus GAD67) proteins (**i**–**l**), in telencephalons of wild-type (**a**, **e**, **i**), $Emx2^{-/-}$ $Pax6^{+/+}$ (**b**, **f**, **j**), $Emx2^{+/-}$ $Pax6^{-/-}$ (**c**, **g**, **k**) and $Emx2^{-/-}$ $Pax6^{-/-}$ (**d**, **h**, **l**) E11.5 embryos. Frontal sections, medial to the right. Asterisks demarcate the corticostriatal notch; solid arrowheads point to the dorsal boundary of the expression domains; in (**h**), empty arrowheads indicate Islet1-expressing cells in the marginal cortical field of *Emx2 Pax6* mutants. Scalebar, 200 μm.

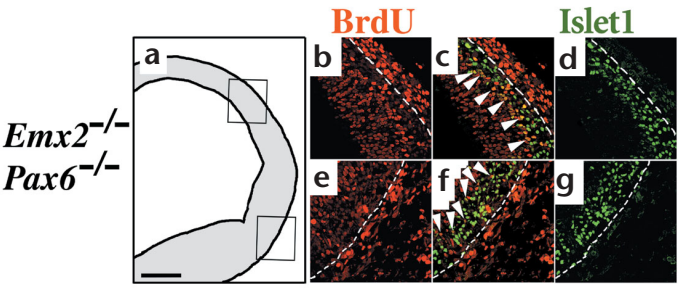

Fig. 7. Colocalization of BrdU and Islet1 in the telencephalon of an E11.5 *Emx2*$^{-/-}$ *Pax6*$^{-/-}$ mutant embryo, administered with BrdU and killed after 8 h. (**a**) Silhouette of a midfrontal telencephalic section; (**b**–**d**) and (**e**–**g**) are magnifications of the two areas boxed in (**a**). Several BrdU^{+} Islet1^{+} cells can be detected in the submarginal basal telencephalon (**f**, arrowheads) as well as in the dorsal telencephalon, at the border between the ventricular zone and marginal layer (**c**, arrowheads). Scalebar, 200 μm.

able in ventral and lateral pallial field, restrict *Gsh2* to the LGE; in the absence of *Pax6*, *Gsh2* spreads into the ventral pallium and cortical markers such as *Ngn2*, *Ngn1*, *Tbr2*, *Pax6* are downregulated in this area[3,4,17], which acquires molecular features of striatum. However, the dorsal shifts of striato–pallidal and cortico–striatal boundaries detectable in *Pax6*$^{-/-}$ mice are limited, and the lateral LGE and the dorsomedial pallium retain striatal and cortical specifications, respectively[3,4,17,21]. In this study we found that a more severe phenotype results if *Emx2* is knocked out in addition to *Pax6*. *Nkx2.1* reaches the corticostriatal notch, conferring MGE properties to the entire ganglionic eminence (compare **Fig. 3u** and **x**); this implies that EMX2, expressed at low levels in baso-lateral telencephalon (unpub. data), normally cooperates with PAX6 in patterning this structure. Moreover, the entire dorsal telencephalon, including dorsal and medial pallium, loses the expression of a large set of transcription factor genes normally restricted to the developing cortex (*Emx1*, *Ngn2*, *Ngn1*, *Tbr2* and *Pax6*) and conversely expresses a set of transcription factor genes peculiar to baso-lateral telencephalon (*Gsh2*, *Vax1*, *Mash1*, *Dlx1*, *Dlx2* and *Six3*). This is associated with ectopic activation of striatal abventricular markers (*Islet1, Calbindin*, *Gad65/67* and *Ebf1*) in the cortical intermediate-marginal zone, suggesting that, in the absence of *Emx2* and *Pax6*, the entire cortical primordium converts into striatum. This phenotype was highly specific to *Emx2*$^{-/-}$ *Pax6*$^{-/-}$ mutants; nothing similar was found in *Emx1*$^{-/-}$ *Pax6*$^{-/-}$ mutants, in spite of structure and expression similarities between the two *Emx* paralogs. Moreover, dorsal markers were also downregulated in partial *Emx2 Pax6* null mutants, but this was limited to restricted cortical regions. Therefore, high-level expression of either *Pax6* or *Emx2* in the telencephalic wall is specifically necessary and sufficient to promote corticogenesis and repress morphogenesis of basal ganglia. Indeed, selective downregulation of *Tbr2*, *Ngn1* and *Ngn2* in pallial regions of *Emx2*$^{-/-}$ *Pax6*$^{+/-}$ and *Emx2*$^{+/-}$ *Pax6*$^{-/-}$ mutants where the fully knocked-out gene normally would be more intensely expressed[9], strongly supports the hypothesis that *Emx2* and *Pax6* promote corticogenesis by acting along parallel pathways.

By BrdU administration and subsequent BrdU/Islet and BrdU/GAD colocalization, we have shown that neurons accumulating in marginal layers of the *Emx2*$^{-/-}$ *Pax6*$^{-/-}$ cortical primordium were presumably locally born. Pallial absence of interneuron markers, such as *Lhx6*, *Lhx7* and *Nrp1*, and pallial activation of genes normally restricted to resident populations of basal ganglia, such as *Islet1* and *Ebf1*, further support this interpretation. However, to prove that *Emx2* and *Pax6* act as proper selector genes for corticogenesis, it is crucial to assess that progenitors generating subpallial neurons in the 'cortex' of *Emx2*$^{-/-}$ *Pax6*$^{-/-}$ embryos were, at least partially, respecified pallial neuroblasts. First, in the absence of *Emx2* and *Pax6*, cortical proliferating populations could collapse; second, *Emx2*$^{-/-}$ *Pax6*$^{-/-}$ mutants, like simple *Pax6*$^{-/-}$ knockouts,

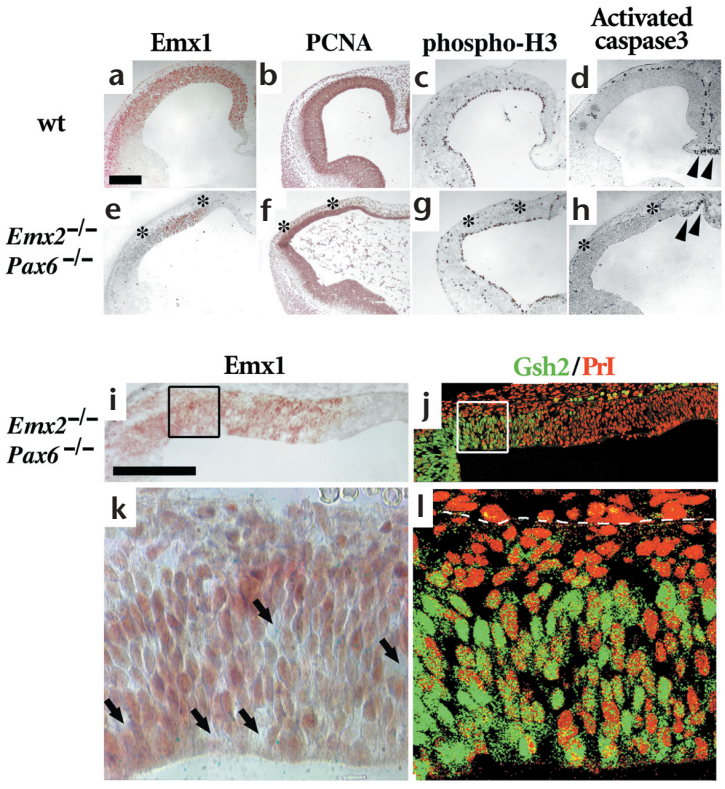

Fig. 8. Vitality and cortical-ganglionic specification of neuroblasts, in the dorsal telencephalon of *Emx2 Pax6* midgestation mutants. (**a**–**h**) Emx1 (**a**, **e**), PCNA (**b**, **f**), phosphohistone H3 (**c**, **g**) and activated caspase3 (**d**, **h**) on midfrontal sections of E11.0 wild-type (**a**–**d**) and *Emx2 Pax6* double knockout (**e**–**h**) brains. All of these markers display similar expression patterns in wild-type and *Emx2 Pax6* double knockouts, including the sector of the double mutant cortical neuroepithelium expressing Emx1 more intensely (**e**–**h**, asterisks); activated caspase3 is prominent in the choroid area (**d**, **h**, arrowheads). (**i**–**l**) Emx1 (**i**, immuno-histochemistry/bright field microscopy) and Gsh2 (**j**, immuno-fluorescence/confocal microscopy with nuclei counterstained by propidium iodide) on adjacent intermediate frontal sections of E11.75 *Emx2 Pax6* double knockouts. (**k**, **l**) Magnifications of areas boxed in (**i**) and (**j**). Emx1 and Gsh2 overlap in the lateral half of the cortical field: here all neuroblasts express Emx1 (**k**) and the majority of them also express Gsh2 (**l**). Rare Emx1^{-} cells are indicated with arrows (**k**). Scalebar, 200 μm.

could lack the functional barrier which normally inhibits migration of basal blasts into the cortical VZ[46–48]. These events would result in rapid and full replacement of cortical neuroblasts by dorsally migrating basal neuroblasts, and no proper respecification would occur. However, distributions of PCNA, phosphohistone H3, BrdU and activated caspase3 indicate that dorsal neuroblasts retaining residual cortical specification at E11.5 were somehow healthy and actively proliferating. Moreover, many of these cells abnormally co-expressed *Emx1* and *Gsh2*, or *Emx1*, *Dlx1* and *Mash1;* this suggests they were undergoing cortical-to-basal respecification, making them able to contribute to generation of the striatum-like structure, detectable in brains of late gestation *Emx2*$^{-/-}$ *Pax6*$^{-/-}$ embryos, in place of cerebral cortex (**Figs. 1e** and **3x**).

Emx2, Pax6 and dorsoventral telencephalic patterning

Activation of basal ganglia markers in the cortical anlage is not peculiar to *Emx2 Pax6* mutants, but takes place also in other mutants.

Cortical upregulation of *Gad67* and *Dlx1* occurs upon cortical overexpression of the subcortical proneural gene *Mash1* and/or inactivation of cortical proneural genes *Ngn1* and *Ngn2*. However, other basal ganglia markers, such as *Islet1,* are not ectopically induced by *Mash1*; moreover, whereas *Mash1* and *Ngn2/1* expression patterns are deeply perturbed in *Emx2 Pax6* double knockouts (**Figs. 2** and **6**), neither overexpression of *Mash1* nor ablation of *Ngn2* impair expression of *Emx1, Emx2* and *Pax6* in cortical neuroblasts; more generally, neither of these genetic manipulations lead to dramatic changes in gross morphology and cytoarchitecture, characterizing the cortex of *Emx2*$^{-/-}$ *Pax6*$^{-/-}$ mutants[5]. This suggests that *Emx2* and *Pax6*, as general activators of corticogenesis, act at a higher hierarchical level as compared to *Ngn1* and *Ngn2*, which, moreover, would master execution of more limited aspects of cortical morphogenetic programs. Similar considerations might apply to *Nkx2.1*, *Gsh2* and *Mash1,* respectively, in development of basal ganglia.

Cortical upregulation of *Dlx2* and downregulation of *Emx1* also occur in mice naturally mutant for the zinc finger gene *Gli3*. Here, however, ventral master genes, such as *Nkx2.1,* do not spread to more lateral locations; moreover *Emx2*, *Pax6* and *Ngn2* are still expressed in the dorsal telencephalon, even if at lower levels[6,7]. This suggests that *Gli3* does not properly act as selector of cortical fates, but rather promotes cortical morphogenetic pathways, by positively modulating expression of *Emx2* and *Pax6*.

Finally, even in the *Emx2*$^{-/-}$ *Pax6*$^{-/-}$ cortical primordium, corticostriatal respecification is not complete around E11, when substantial amounts of Emx1 can still be found in the dorsomedial cortical field. The presence of rare *Pax6*$^{+}$ neuroblasts and rare *Tbr2*$^{+}$ presumptive neurons as well as the almost complete absence of *Gsh2* in this area (**Figs. 5** and **8**) led us to suggest that, in the absence of *Emx2* and *Pax6, Emx1* could initially promote corticogenesis versus morphogenesis of basal ganglia, subsequently failing to sustain the former process because of the requirement of either *Emx2* or *Pax6* for late maintenance of its expression in the cortical VZ. However, simultaneous inactivation of all three genes, *Emx1, Emx2* and *Pax6*, neither abolished early expression of *Tbr2* nor led to early activation of *Gsh2* in this area (data not shown). This did not allow us to verify the former hypothesis and conversely suggested that at least another gene, different from *Emx1, Emx2* and *Pax6*, should be able to transiently and independently activate corticogenesis. *Lhx2*, expressed at high levels in dorsal telencephalon, even in the absence of *Emx2 or Pax6*, seems to be a reasonable candidate. Further stringent genetic analysis will be needed to test this prediction.

Methods

Animal husbandry, recovery of embryos and tissue sampling. *Pax6 Emx2.* mutant embryos were obtained by intercrossing heterozygous *Small eye* mutants, *Sey* allele[22], hereafter called *Pax6*$^{+/-}$ mutants, heterozygous *Emx2*$^{+/-}$ mutants[18] and heterozygous *Emx1*$^{+/-}$ mutants[19], the last ones provided by S. Aizawa. *Pax6*$^{-/-}$ embryos were recognized by the absence of eyes, *Pax6*$^{+/-}$ embryos by ocular abnormalities; *Pax6* genotypes were confirmed by direct sequencing of genomic DNA[22]. *Emx2* mutant embryos were genotyped by PCR [oligos: *E2F*, 5′ CAC AAG TCC CGA GAG TTT CCT TTT GCA CAA CG 3', *E2R/WT*, 5′ ACC TGA GTT TCC GTA AGA CTG AGA CTG TGA GC 3', *E2R/KO*, 5′ ACT TCC TGA CTA GGG GAG GAG TAG AAG GTG G 3'; program: 98°C/5' (1X), 98°C/1' – 72°C/2' (5X), 94°C/1' – 72°C/2' (30X), 72°C/10' (1X); PCR products: 180bp (wt allele), 340bp (null allele)] and genotypes were confirmed by Southern when appropriate[18]. *Emx1* mutant embryos were genotyped by PCR [oligos: *E1F*, 5′ CGA CGT TCC CCA GGA CGG GCT GCT TTT GC 3', *E1R/WT*, 5′ GTC TCG GAG AGG CTG AGG CTG CCT GCC AGC 3', *NR/KO*, 5′ ACT TCC TGA CTA GGG GAG GAG TAG AAG GTG G 3'; program: 98°C/5' (1X), 98°C/1' – 72°C/2' (5X), 94°C/1' – 72°C/2' (30X), 72°C/10' (1X); PCR products: 180bp (wt allele), 230bp (null allele)] and genotypes were confirmed by Southern when appropriate[19].

Pregnant females were anasthetized by CO_2 and killed by cervical dislocation, in compliance with European laws (European Communities Council Directive of November 24, 1986 (86/609/EEC)) and according to guidelines of H San Raffaele Institutional Animal Care and Use Committee (HSR-IACUC). Embryos were fixed in 4% paraformaldehyde-PBS overnight at 4°C and then washed, dehydrated, and embedded in wax according to standard protocols. Embryonic brains were cut at 10 mm and sections were mounted on Fischer SuperFrost Plus slides. Samples were subsequently dewaxed by xylene, rehydrated in descending ethanol series, and processed for *in situ* hybridization or immunohistochemistry. Alternatively, embryonic brains, upon 4% paraformaldehyde-PBS fixation, were cryoprotected by 30% sucrose-1X PBS, included in OCT and cut at 10 μm by cryostat. Cryosections, mounted on Fischer SuperFrost Plus slides, were used as such.

Immunohistochemistry-immunofluorescence. Antigens were generally unmasked by boiling samples in 10 mM sodium citrate, pH 6.0, for 5 min and allowing them to cool down slowly, prior to applying conventional immunohistochemistry-immunofluorescence protocols. In the case of BrdU detection, to depurinate genomic DNA and make the epitopes accessible, slides were kept in 2 M HCl for 30 min at 60°C and then neutralized in 0.1 M borate buffer, pH 8.5, for 15 min at RT. Immunohistochemistries were performed according to ref. 9; for immunofluoresences, Alexa secondary antibodies (Molecular Probes, Eugene, Oregon) were used, according to Manifacturer's instructions. When double immunos were performed with two primary antibodies raised in the same species, the two tests were performed sequentially and, just before the second immuno, the first primary antibody was masked by a large excess of unconjugated secondary antibody; in this case, specific negative controls were run as appropriate. The following primary antibodies were used: anti-BrdU, mouse monoclonal (Becton-Dickinson, San Josè, California), 1:75; anti-(activated) caspase3, rabbit monoclonal (BC PharMingen, San Diego, California), 1:300; anti-Emx1, rabbit polyclonal (Briata *et al.*, 1996, gift of G.Corte) 1:500; anti-GABA, rabbit polyclonal (Sigma, Saint Louis, Missouri), 1:300; anti-GAD65/67, rabbit polyclonal (Chemicon, Temecula, California), 1:250; anti-GAD65/67, rabbit polyclonal (Sigma), 1:1600; anti-Gsh2, rabbit polyclonal (gift of K. Campbell), 1:2000; anti- phosphohistone H3, rabbit polyclonal (Upstate Biotechnology, Lake Placid, New York), 1:600; anti-Islet1, mouse monoclonal (gift of P. Bovolenta), 1:500; anti-MAP2, mouse monoclonal (Roche, Indianapolis, Indiana,), 1:100; anti-neurospecific classIII β-tubulin, mouse monoclonal (BabCo, Richmond, California), 1:500; anti-Otx2, rabbit polyclonal[49], 1:500; anti-PCNA, mouse monoclonal (Dako, Glostrup, Denmark), 1:50.

***In situ* hybridization.** Radioactive and non-radioactive *in situ* hybridizations were performed according to ref. 11 and 17, respec-

tively. The following probes were used: *Bf1* (PCR-amplified, Genbank NM_008241, nt1626–2900); *Dlx1* (plasmid MOD6.14, ref. 50); *Dlx2* (plasmid M524, gift of V. Broccoli); *Ebf1* (plasmid Ebf1, gift of G.G.Consalez); *Gsh2* (PCR-amplified, Genbank S79041, nt812–1611); *Id3* (PCR-amplified, Genbank M60523, nt90–905); *Lhx2* (PCR-amplified, Genbank NM_010710.1, nt1128–1748); *Lhx6* (plasmid Lhx6, gift of J. Parnavelas); *Lhx7* (plasmid Lhx7, gift of J. Parnavelas); *Mash1* (PCR-amplified, Genbank NM_008553 and MMU68534. 796 bp fragment encompassing 582 bp of 5'UTR plus 214 bp of cds); *Msx1* (PCR-amplified, Genbank NM_010835, nt849–1707); *Ngn1* (plasmid NGN1, gift of K.A. Nave); *Ngn2* (plasmid NGN2/2, gift of K.A. Nave); *Nkx2.1* (plasmid Nkx2.1, gift of G. Fishell); *Nrp1* (PCR-amplified, Genbank D0086.1, nt1202–3258); *Shh* (plasmid mShh, gift of G. Fishell); *Pax6* (PCR-amplified, Genbank NM_013627, nt1485–2450); *Tbr2* (plasmid D12, gift of A. Bulfone); *Ttr* (PCR-amplified, Genbank D00071, nt13–1484); *Vax1* (PCR-amplified, Genbank AF064554, nt13–214); *Wnt3a* (PCR-amplified, Genbank NM_009522, nt29–1451); *Wnt8b* (PCR-amplified, Genbank NM_01172, AW488375 and AA874401, 1370 bp fragment encompassing the last 488 bp of cds plus the first 882 bp of the 3'UTR).

Photography and editing. Micrographs of immunohistochemistries and *in situs* were take using an SV Micro CV3000 digital microscope camera (Taunton, Massachusetts). Immunofluorences were analyzed on a Zeiss Axiophot microscope, equipped with a Biorad confocal detection apparatus. Electronic files were processed on a MacIntoshG3 computer by Adobe Photoshop 5.0 software (San Jose, California).

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Competing interests statement

The authors declare that they have no competing financial interests.

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