Pax6 Modulates the Dorsoventral Patterning of the Mammalian Telencephalon

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The *Pax6* gene encodes a transcription factor with a restricted expression in the ventricular zone of the pallium and subpallium. We tested whether the function of *Pax6* is necessary for the correct patterning and morphogenesis of the vertebrate telencephalon. Homozygous embryos of the *Pax6*/*Small eye* mutant lack functional PAX6 protein because of a point mutation of the gene. In the mutant *Small eye* embryos we detected a ventralization of the molecular patterning of the telencephalon at two borders, the pallium/subpallium and the lateral/medial ganglionic eminence. The results indicate that *Pax6* controls the lateral limit of the expression of *Nkx2.*1, *Shh,* and *Lhx6* in the prechordal neural tube, the telencephalon. This finding is in agreement with previous studies and supports a model for a common genetic mechanism for modulation of the dorsoventral patterning of the prechordal and epichordal CNS. The pattern defects caused by the loss of Pax6 function result in multiple morphological abnormalities in the *Small eye* brain: dysgenesis of the piriform, insular, and lateral cortices, the claustrum–endopiriform nucleus, and a failure in the differentiation of a subpopulation of the cortical precursors. Together the results demonstrate that Pax6 has an essential role for the modulation of the dorsoventral patterning of the embryonic telencephalon, influencing thereby the forebrain morphogenesis.

Key words: Pax6; Small eye; *dorsoventral patterning*; *telencephalon*; *borders*; *pallium/subpallium*; *MGE/LGE*

The two main subdivisions of the embryonic telencephalon, pallium (cortex) and subpallium (basal ganglia), have a distinct molecular patterning and strikingly different developmental potentials. During development, the initial sheet of uniform pseudostratified neuroepithelium generates dorsally the six-layered cortex and ventrally the three eminences, the medial ganglionic eminence (MGE), lateral ganglionic eminence (LGE), and caudal ganglionic eminence (CGE), which later differentiate into the nuclei of the basal ganglia.

The *Pax6* gene plays a crucial role in the development of the vertebrate CNS. The mouse *Small eye* (allele *Sey)* mutation is caused by a point mutation in the *Pax6* gene, resulting in the production of a nonfunctional protein (Hill et al., 1991). The homozygous *Small eye* animals die at birth with multiple CNS defects in the eye, forebrain, cerebellum, and spinal cord (Schmahl et al., 1993; Stoykova et al., 1996; Burrill et al., 1997; Caric et al., 1997; Ericson et al., 1997; Grindley et al., 1997; Mastick et al., 1997; Osumi et al., 1997; Engelkamp et al., 1999; Warren et al., 1999). We have previously found that Pax6 mediates the establishment of distinct adhesive properties between the dorsal and ventral compartments of the embryonic telencephalon (Stoykova et al., 1997) and that Pax6 controls the differentiation of the cortical radial glia cells (Götz et al., 1998). Here we explore the role of *Pax6* in the control of the dorsoventral (DV) regionalization of the telencephalon and the consequences for the brain morphogenesis in loss of *Pax6* function.

In the embryonic telencephalon, the expression of *Pax6* is confined to the mitotically active ventricular neuroepithelium (Ne) of the pallium (Walther and Gruss, 1991). The pallium is classically subdivided into the medial pallium (MP), dorsal pallium (DP), and lateral pallium (LP), giving rise to the archicortex (hippocampus), neocortex, and paleocortex, respectively. In addition, *Pax6* exhibits a particularly strong expression in a small lateralmost region of the ventricular zone of the LGE at the level of the pallial/subpallial border (Stoykova et al., 1996, 1997). This domain is intercalated between the neuroepithelium of the striatum and the lateral pallium (Fig. 1*A*) and was recently designated as "ventral pallium" (VP) (Puelles et al., 1999, 2000) or "intermediate zone" (Smith-Fernandez et al., 1998). The *Pax6* mRNA level shows a lateral-to-medial gradient, being highest in the region of the VP (Walther and Gruss, 1991; Stoykova et al., 1997; Puelles et al., 1999). *Pax6* is also expressed in the ventricular zone (VZ) of LGE, although at a very low level (Hallonet et al., 1998; Puelles et al., 1999). A number of transcription factors and regulatory molecules with a restricted expression in the embryonic telencephalon are respecting the pallial/subpallial and MGE/LGE border (for review, see Rubenstein and Shimamura, 1997; Rubenstein et al., 1998). We examined therefore whether the strikingly different *Pax6* expression levels at these two boundaries might have a biological function for the regionalization of the telencephalon. We show in this work that a similar constellation of genes, including *Pax6, Nkx2.1*/*2.2,* and *Shh* appears to modulate the DV patterning not only in the epichordal part of the neural tube (Ericson et al., 1997; Briscoe et al., 1999), but also in the prechordal part of the CNS, the telencephalon. Furthermore, we found that the disruption of the normal DV patterning in the *Sey*/*Sey* brain leads to a hypoplasia of the basolateral cortex, affecting the structures that derive from the region of the ventral pallium. Our results further suggest that *Pax6* possibly controls the activity of the neural determination gene *Ngn2* in a subpopulation of the cortical precursors.

MATERIALS AND METHODS

Animals. Embryos were derived from crosses of heterozygous *Small eye* mice, *Sey* allele (Roberts, 1967; Hogan et al., 1986) on a C57BL/6Jx-DBA/2J background. The point mutation in the *Pax6* gene results in the generation of truncated nonfunctional protein (Hill et al., 1991), whereas the transcription is not affected, thus allowing us to study the activity of the gene in the affected brain regions. The day of the vaginal plug was considered as embryonic day 0.5 (E0.5). The brains of matched homozygous and wild-type littermates were used for the expression analysis.

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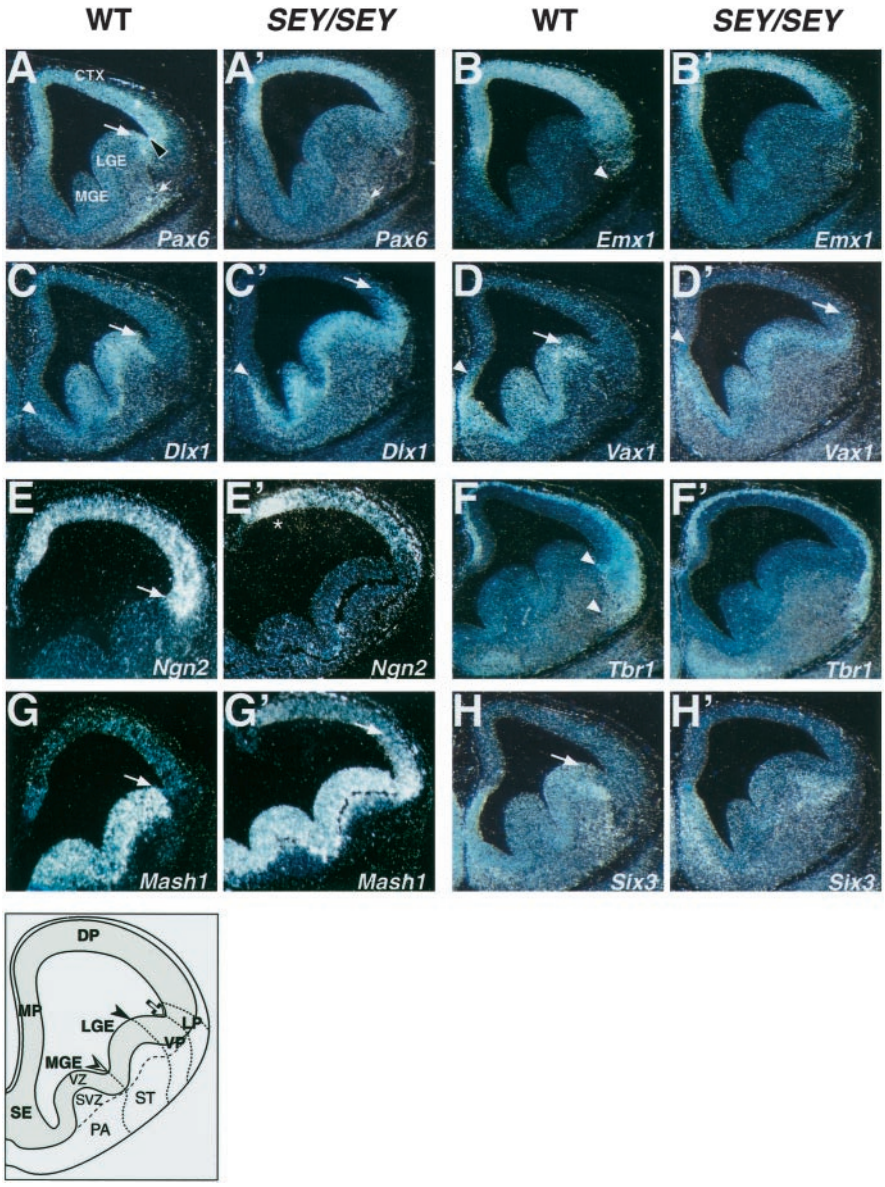

Figure 1. Ventralization of the neuroepithelium at the pallio/subpallial border at stage E12.5 in the *Sey*/*Sey* telencephalon. Adjacent coronal sections from the brain of wild-type (*WT*; *A–D*; *F*, *H*; *E*, *G*) and homozygous (*Sey*/*Sey;* *A′–D′*; *F′*, *H′*; *E′*, *G′*) littermates at E12.5 were hybridized with RNA probes for region-specific markers as indicated. The *empty arrowhead* in *A* points to the morphological corticostriatal sulcus, whereas the *arrows* in *A*, *C*, *D*, and *E*, *H* point to the pallial/subpallial border. *A,* The region of the ventral pallium is located between the *arrowhead* and the *arrow* (also in Figs. 5, 7). The *thin arrows* in *A* and *A′*) point to early-born *Pax6*+ cells possibly generated from the VP and migrating toward the presumptive anlage of the piriform cortex and anterior amygdala. *B*, *B′*, *Emx1* is dorsally retracted from the LP in the mutant as compared with the WT brain. The subpallial markers for the VZ–SVZ are ectopically expressed in the Ne of the VP, LP, and DP of the *Sey*/*Sey* as illustrated for: *Dlx1* (*C′*), *Vax1* (*D′*), and *Mash1* (*G′*). The *arrowheads* in *C′* and *D′* point to the extension of the *Dlx1* and *Vax1* expression into a more dorsal domain within the septum in the mutant brain. In *H′*, note that the normal expression of *Six3* in the striatal mantle extends laterally into the mantle of the VP. In *E* and *E′*, note that *Ngn2* expression is abolished in the VZ of the VP, strongly suppressed in the LP and in a part of the DP, but appeared unaffected in the MP (designated by the *asterisk* below the MP). The *two arrowheads* in *F* point to the medial limit of *Tbr1* expression along the pallial/subpallial border, thus including in its expression domain the mantle zone of the VP (the anlage of the ventromedial claustrum; Puelles et al., 1999). The expression of *Tbr1* in this domain is abolished in the *Sey*/*Sey* brain (*F′*).

In situ *hybridization and immunohistochemistry.* Sectioning, *in situ* hybridization, and emulsion autoradiography were performed as previously described (Stoykova and Gruss, 1994). ^{35}S-labeled sense and antisense RNA probes were synthesized in the presence of two radioactive nucleotides from linearized plasmid templates according to the supplier's instructions (Promega, Madison, WI). Two independent *in situ* analyses for each stage were performed. For the colocalization of the *Ngn2* mRNA and the Pax6 antigen first a nonradioactive *in situ* hybridization with the *Ngn2 in situ* probe was performed on 12 μm cryostat sections from E13.5 wild-type brain as described by Gradwohl et al. (1996). For the antibody staining the sections were further proceeded for immunohistochemistry according to Götz et al. (1998) using the anti-mouse Pax6 antibody (Development Studies Hybridoma Bank, Iowa City, IA), 1:200 and "Alexa" 568 goat anti-mouse conjugate (MoBiTec), 1:500. The terminology is in accordance with the rat brain atlases of Paxinos et al. (1994), Altman and Bayer (1995) and Foster (1998).

RESULTS

Ventralization of the molecular patterning of the pallial neuroepithelium in the *Pax6*/*Small eye* mutant telencephalon

To examine whether *Pax6* plays a role in the dorsoventral regionalization at the pallial/subpallial border, we studied the molecular patterning by *in situ* hybridization in sections of E12.5 wild-type (WT) and homozygous *Small eye* (*Sey*/*Sey)* brains using the following markers: *Emx1* (Simeone et al., 1992) as a dorsal telencephalic marker, which is expressed in the whole pallium except for the VP (Puelles et al., 1999, 2000); *Pax6 (*Walther and Gruss, 1991*), Ngn2 (*Gradwohl et al., 1996), and *Tbr1* (Bulfone et al., 1995; Puelles et al., 1999, 2000) as pallial markers that include the VP in their expression domains and *Dlx1* (Bulfone et al., 1993*), Vax1* (Hallonet et al., 1998*), Mash 1* (Guillemot and Joyner, 1993), and *Six3* (Oliver et al., 1995) as ventral telencephalic markers. The comparative analysis was performed at three rostrocaudal levels of sectioning, and the detected patterns are illustrated in Figure 1.

At E12.5, *Emx1* is expressed in mitotic and postmitotic cells in the anlage of the medial, dorsal, and lateral pallium (Fig. 1*B*). In the mutant brain, *Emx1* expression was retracted from the depth of the basolateral wall, except for some *Emx1*+ cells, located very superficially (Fig. 1*B′*). The expression of *Tbr1* is restricted to early postmitotic cells in the pallium (Bulfone et al., 1995). In the basolateral telencephalon, the *Tbr1* expression extends more medially than *Emx1* so that the subventricular zone (SVZ), submantle, and mantle zone of the VP expresses the *Tbr1*, but not the *Emx1* gene (Fig. 1*F, arrowheads*). Thus, the medialmost expression domain of *Tbr1* in the basolateral telencephalic wall seems to consist of postmitotic cells that are generated predominantly from the neuroepithelium of the VP (Puelles et al., 2000). In *Sey*/*Sey*, the expression of *Tbr1* was abolished in the SVZ, submantle, and mantle zone of the VP and appeared less affected in the postmitotic neurons of the preplate in the DP and LP (Fig. 1*F′*).

Pax6 is expressed in the VZ of the entire pallium, showing a particularly strong signal within the region of the VP (Figs. 1*A*, 2*A*). In *Sey*/*Sey* the mutant transcripts were much less abundant in the VP, and the pallial/subpallial border was not well delineated (Figs. 1*A′*, 2*A′*). The *Pax6* and *Ngn2* expression domains overlap in the pallial VZ (Fig. 1*E*; see Fig. 5*A*,*B*). Interestingly, the expression of *Ngn2* in *Sey*/*Sey* was completely abolished in the region of the VP, substantially reduced in the LP and DP, but appeared at a normal level in the MP (Fig. 1*E′*).

In the WT brain, the transcripts of the subpallial markers *Dlx1, Vax1*, and *Mash1* in the VZ–SVZ and *Six3* in the mantle zone were clearly not detectable in the VP (Fig. 1*C,D,G,H*). In the *Pax6*-deficient brain, the markers for the ventral telencephalic VZ–SVZ were ectopically expressed within the Ne of the VP and LP (Fig. 1*C′*,*D′*,*G′*). Similarly, the expression of *Six3* expanded into the mantle zone of the VP (Fig. 1*H′*). In addition, the limit of the *Dlx1* and *Vax1* expression extended more dorsally in the mutant septum (Fig. 1*C*,*C′*,*D*,*D′*, *arrowheads*), which appeared enlarged as compared with the septum of the wild-type brain.

Together these data indicate that in the *Sey*/*Sey* telencephalon, the domain of the VP and LP is ventralized so that the limit of the *Emx1* expression is retracted to a more dorsal position within the pallium, the expression of *Ngn2* and *Tbr1* is abolished in the VZ and SVZ-mantle of the VP, respectively, and the subpallial markers *Dlx1, Vax1, Mash,* and *Six3* are ectopically expressed into more dorsal pallial domains.

Ventralization of the subpallial patterning in *Sey/Sey*

To test whether the extremely low level of the expression of *Pax6* in the VZ of the entire LGE may have a biological significance for the patterning of the basal telencephalon, we studied the expression of several markers for the MGE in sections of WT and *Sey*/*Sey* brains at stages E11.5–E14.5. From E10.5 onward, the proliferative and later on the postmitotic Ne of the MGE begins to express *Shh* (Sussel et al., 1999) and *Nkx2.1* (Shimamura et al., 1997). The gene *Shh* encodes a powerful morphogen with ventralizing activity that can induce the expression of *Nkx2.1* (Ericson et al., 1995; Shimamura et al., 1997) and *Dlx* (Kohtz et al., 1998). Furthermore, *Shh* can inhibit the activity of the dorsal pattern genes such as *Pax6* (Ericson et al., 1997), *Emx1,* and *Tbr1* (Kohtz et al., 1998).

As illustrated in Figure 2, *B*, *B′*, the expression of *Shh* at E13.5, whereas normally confined to the submantle and mantle of the MGE, was expanded into the adjacent territory of the LGE in *Sey*/*Sey*. A similar pattern was seen at stage E12.0 as well (data not shown). Likewise, the *Nkx2.1* expression, while normally restricted to the germinative Ne and mantle of MGE, expanded beyond the MGE/LGE border in the mutant brain (Fig. 2*C′*, *arrow*). At stage E12.0 the ectopic expression of *Nkx2.1* was spread over the VZ of the adjacent LGE domain (Fig. 2*D′*, *arrows*), which normally expresses *Pax6* at a very low level. Thus, it is likely that an interaction between *Pax6* and *Nkx2.1* genes might contribute for the maintenance of the MGE/LGE border. At stage E14.5, the *Nkx2.1* expression outlined an enlarged MGE in the mutant brain (Fig. 2*E,E′*; also *F,F′*).

Next we examined the expression of the LIM-homeobox containing gene *Lhx6,* which is assumed to play a specific role in defining the MGE territory (Grigoriou et al., 1998). The expression of *Lhx6* at E12.5 was restricted to a subpopulation of cells in the SVZ and submantle of the MGE with only a few *Lhx6*+ cells in the LGE in a proximal vicinity to the sulcus between MGE and LGE (Fig. 3*A*; Wanaka et al., 1997; Grigoriou et al., 1998). After E13.0, an increasing number of *Lhx6*+ cells were observed in the mantle of the wild-type LGE, accompanied by the appearance of a *Lhx6*+ layer of cells in the intermediate zone (IZ) and marginal zone (MZ) of the cortex (Fig. 3*B–D*; see also Lavdas et al., 1999; Parnavelas, 2000). In *Sey*/*Sey*, already at E12.5 the expression of *Lhx6* in the LGE was much more widespread, strongly suggesting that the mutant LGE contains a higher number of *Lhx6*+ cells (Fig. 3*A′*). To further characterize this pattern defect, *in situ* hybridization analysis was performed on sections from E14.0 wild-type and

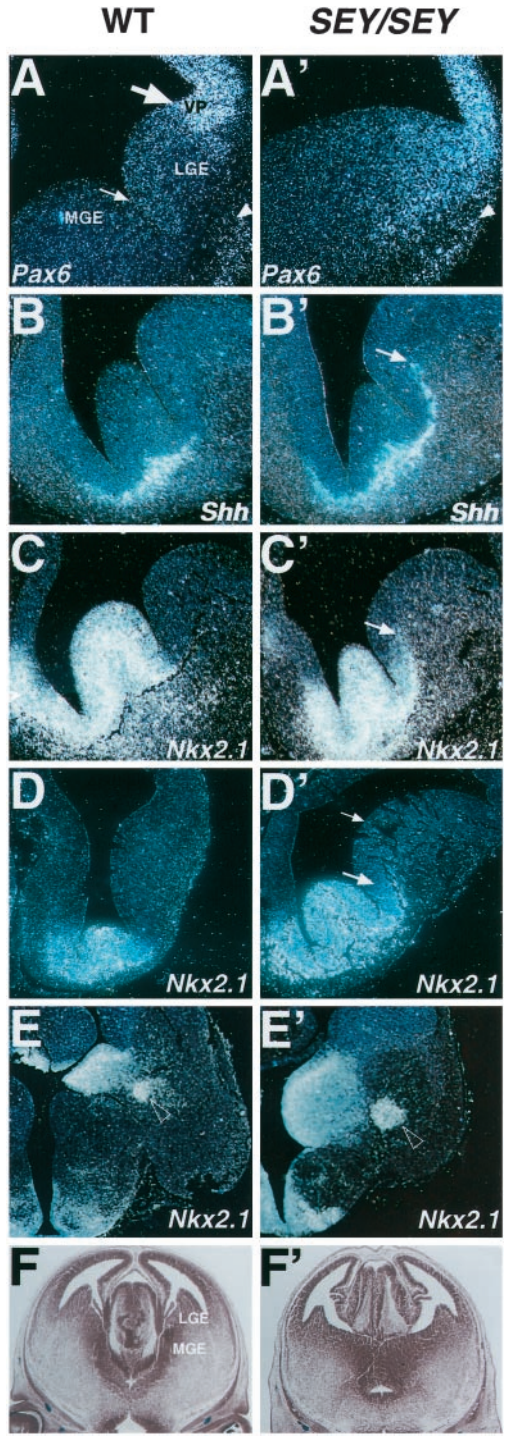

Figure 2. Ventralization of the neuroepithelium of the LGE in *Sey*/*Sey*. *In situ* hybridization on coronal (*A–D′*) and cross (*E*, *E′*) sections from the WT and *Sey*/*Sey* brains. Different markers for the MGE were tested at stages: E13.5 (*A–C′*), E12.0 (*D,D′*), and E14.5 (*E,E′*). In *A,* note the strikingly different level of *Pax6* expression in the VZ of the VP and LGE. The *arrowheads* in *A* and *A′* point to *Pax6*+ cells that appear to stream out from the Ne of the VP toward the basolateral telencephalon. *B–C′*, In the mutant telencephalon, the lateral limit of the expression of *Shh* (*B*,*B′*) and *Nkx2.1* (*C*,*C′*) extends from the MGE into the adjacent territory of the LGE. In *D′*, the *large* and *small arrows* point to the lateral limit of the strong and the faint ectopic expression of *Nkx2.1* within the VZ of the mutant LGE, respectively. In *E* and *E′*, note the enlarged MGE (which includes at this late stage the adjacent LGE domain with a ventralized identity) and the differentiating globus pallidus, labeled by the *Nkx2.1* probe (*open arrowhead*). *F*, *F′*, Coronal sections from E15.5 WT (*F*) and *Sey*/*Sey* (*F′*) brain at the level of the preoptic area stained with neutral *red*, illustrating the enlarged MGE in the *Sey*/*Sey* telencephalon.

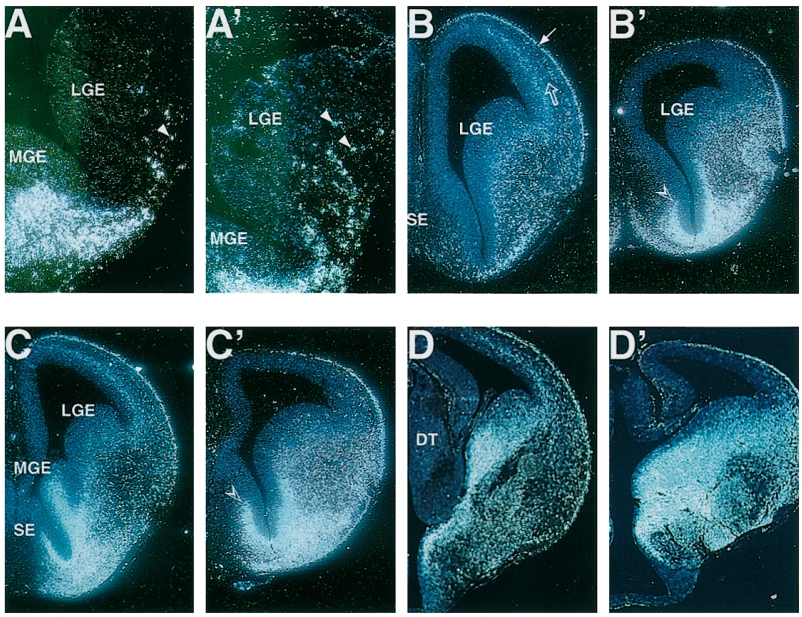

Figure 3. Expression of *Lhx6* in the basolateral telencephalon in wild-type and *Sey*/*Sey* brain. *A*, *A′*, Coronal sections from E12.5 wild-type (*A*) and mutant (*A′*) brain. Note the enhanced number of *Lhx6*+ cells in the mutant LGE. *B–D′*, Coronal sections from E14.0 wild-type (*B–D*) and mutant (*B′–D′*) brain at different rostrocaudal levels. The *open* and the *thin arrows* in *B* point to a *Lhx6*+ layer of cells in the intermediate and marginal zones of the CP, respectively. In all levels note the enhanced expression of *Lhx6* in the LGE in the mutant as compared with the WT brain and the lack of *Lhx6*+ cells in the lower part of the *Sey*/*Sey* CP. *B, B′*, Note the increased expression of *Lhx6* in the rostral septum in *Sey*/*Sey* (*open arrowhead*) from where more *Lhx6*+ cells seem to populate directly the LGE.

Sey/*Sey* brains at different rostrocaudal levels. The expression of *Lhx6* along the entire rostrocaudal axis was much more abundant in the mutant as compared with the wild-type LGE (Fig. 3*B–D′*). Furthermore, while present in the MZ of the *Sey*/*Sey* cortex, *Lhx6*+ cells were not detectable in the lower part of the mutant cortical plate (Fig. 3*B′–D′*). In accordance with previous data showing that the *Lhx6*+ cells originate mainly from the Ne of the MGE (Grigoriou et al., 1998) at a very rostral level the WT septum contained only a few *Lhx6*+ cells (Fig. 3*B*). In contrast, the SVZ–submantle of the septum in *Sey*/*Sey* was abundantly populated with *Lhx6*+cells that seem to migrate directly into the mutant LGE (Fig. 3*B′*). Thus, in Pax6 loss of function the Ne of the rostral septum and MGE appears to produce a higher number of *Lhx6*+ cells that migrate into the territory LGE, but these cells fail to populate the lower part of the mutant cortical plate. Different possibilities may account for the observed wider expression of *Lhx6* in the basolateral telencephalon in *Sey*/*Sey*: (1) enhancement of the rate of the *Lhx6 mRNA* synthesis implicating a transcriptional regulation between *Pax6* and *Lhx6*; (2) increase of the number of the generated *Lhx6*+ cells and/or enhanced ventrodorsal cell migration between the MGE and LGE as a result of the ventralization of a part of the Ne of LGE, as noticed above; and (3) accumulation of *Lhx6*+ cells within the mutant LGE because of a malformation of the corticopetal axons (Kawano et al., 1999) that normally help the subpallial cells in their tangential migration toward the cortex. Further experimentation will be required to definitively distinguish between these possibilities.

Taken together, the results from the performed analysis of the patterning of the basal telencephalon indicate that in the lack of a functional Pax6 protein a more dorsal domain (LGE) of the basal telencephalon achieves characteristics of a more ventral domain (MGE).

Defects in the rostral basolateral telencephalon of the *Sey*/*Sey* brain

The origin of the telencephalic basolateral structures is still under debate. Morphological studies suggested that whereas the claustrum has a neocortical origin, the endopiriform nucleus and piriform cortex originate from the Ne of the corticostriatal wedge (Bayer and Altman, 1991a) and/or from the Ne of the LGE (Valverde and Santacana, 1994; De Carlos et al., 1996).

At stage E12.5, cells expressing *Pax6* or *Tbr1* appear to extend out from the Ne of the VP toward the basolateral telencephalon (Fig. 1*A,F*; see Fig. 7). To study which basolateral structures were specifically patterned by either of the two pallial markers, we examined the patterning at stage E18.5. In agreement with Bulfone et al. (1995), *Tbr1* transcripts were detected in postmitotic cells of the neocortex and paleocortex, classical claustrum (Cl) (Fig. 4*A,C,F*), and the dense layer II of the piriform cortex (Fig. 4*B, open arrowhead*). Recent results indicate that the classical claustrum is a derivative of the "dorsolateral claustrum" whose precursors are possibly generated from the Ne of the *Tbr1*+/*Emx1*+ lateral pallium (Puelles et al., 1999, 2000). Rostrally, *Pax6* was not expressed in the differentiating classical claustrum (Fig. 4*D*). However, *Pax6* transcripts were detected in the presumptive domain of the olfactory tubercle (Tu) and in the ventral part (presumptive layer I) of the piriform cortex (Fig. 4*E, filled arrowhead*). In addition, *Pax6* was expressed in a region, which has been designated by different authors as the endopiriform nucleus, anterior amygdalar area, ventral pallidum, and/or lateral striatal area (Fig. 4*E,F, arrow*). At early developmental stages this domain was referred to as the "ventromedial claustrum", a derivative of the VP (Fig. 1*F, arrowheads*) (for discussion, see Puelles et al., 1999, 2000). Results from autoradiographic studies indicated that early-born cells from the Ne of the corticostriatal wedge (included in the territory of VP) are divided by the growing tip of the cortical plate at late developmental stages into a superficial part corresponding to layer I and a deep part, corresponding to cells located in the adult layer III of the piriform cortex (Valverde and Santacana, 1994). Thus, our results suggest that the early- and the late-born constituents of the piriform cortex (the primary olfactory cortex) are differentially patterned by *Pax6* and *Tbr1*.

As illustrated in Figure 4, the piriform cortex, the claustrum (assumed to represent the deep layers of the insular cortex), the endopiriform nucleus, and the reservoirs cells (r) (Bayer and Altman 1991a,b) were not detectable in the rostral *Sey*/*Sey* telencephalon. Likewise, the lateral cortex including the prospective insular cortex was severely disorganized without a recognizable cortical plate at a very rostral level (Fig. 4*C,H*). Cells expressing defective *Pax6* transcripts were detectable in the stream that extends from the Ne of the VP along the pallial/subpallial border (Fig. 1*A′*), implicating that *Pax6* would not have a cell autonomous function for the generation of the early-born cells of the piriform cortex. We assume rather that the dysgenesis of the piriform, lateral cortex and claustrum in the *Sey*/*Sey* telencephalon is a consequence of the prominent ventralization of the molecular patterning of the VP in *Sey*/*Sey* as demonstrated in this work.

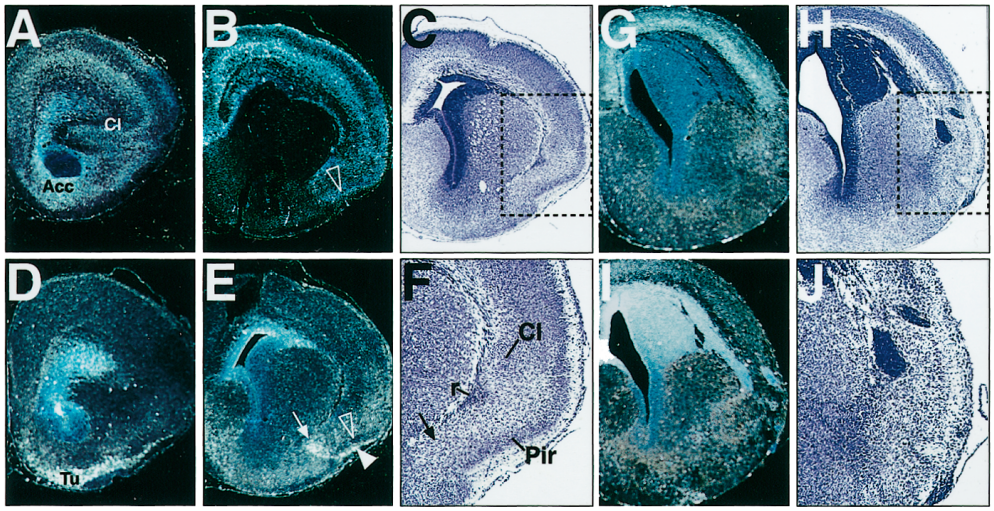

Figure 4. Differently patterned structures by *Pax6* and *Tbr1* are distorted in the *Sey*/*Sey* basolateral telencephalon. *A, B, D, E*, Adjacent coronal sections from the E18.5 WT brain were hybridized with probes for *Tbr1* (*A, B*) and *Pax6* (*D, E*). *C* is a bright-field picture of an adjacent section to the section (*B*) after hematoxylin–eosin (HE) staining. *F* is a close-up of *C* for the indicated field. *A, B, Tbr1* expression is detected in the differentiating claustrum proper (*Cl*) and in the dense layer II (*B, open arrowhead*) of the piriform cortex. *D, E, Pax6* is expressed in the olfactory tuberculum (*Tu*), in the ventral part (presumptive layer I) of the piriform cortex (*E, arrowhead*), and in the presumptive anlage of the anterior amygdala–endopiriform nucleus, a *thin arrow* in *E* and *F*. In *E*, note that the dense layer of the piriform cortex is *Pax6*-negative (*open arrowhead*). *G–J* are adjacent coronal sections from the E18.5 *Sey*/*Sey* brain, hybridized with *Tbr1* (*G*) and *Pax6* (I) probes or stained with HE (*H, J*). In *C*, *F*, *H*, and *J* note that in the mutant brain, the piriform cortex, the claustrum proper, the endopiriform nucleus–anterior amygdala and the reservoir (*r*) are not distinguishable. The dark-stained structures in *H* and *J* are cells from the pallial germinative neuroepithelium that form clumps (or a thick band at other levels) located all along the pathway of the lateral migratory stream (Fig. 5) in the *Sey*/*Sey* pallium.

Defects in the differentiation of the cortical plate in *Sey/Sey*

The intriguing finding that at E12.5 *Pax6* and *Ngn2* have overlapping expression domains and that the activity of *Ngn2* was downregulated within the *Sey*/*Sey* pallium was confirmed also later in development (Fig. 5). Recent data (Hartfuss et al., 2000) indicated that a subpopulation of acutely dissociated cortical progenitors colocalize Ngn2 and Pax6, implicating that a direct regulation between the two genes might cause the lack of the Ngn2 expression in the VP–LP. The performed double *in situ* and immunolabeling for *Ngn2* and Pax6 revealed at E13.5 a nuclear signal for Pax6 only in a limited number of the *Ngn2*+ pallial cells, including the region of VP and LP (Fig. 5*D*). These data suggest that the observed lack of *Ngn2* expression might be a consequence of both, the ventralization of the neuroepithelium as described above and possibly involving also a direct gene regulation in a subset of the cortical progenitors. We assume therefore that a region-specific downregulation of the activity of the proneural gene *Ngn2* in the ventrolateral pallium might contribute to the complex cortical phenotype in *Sey*/*Sey*.

A prominent feature of the *Sey*/*Sey* pallium is the thin cortical plate (CP) and the enlarged germinative neuroepithelium (VZ–SVZ) that occupies the IZ domain (Fig. 6*A,A′*; for discussion, see also Warren et al., 1999). Highly accumulated cells appear adherent to each other all along the lateral migratory stream (Fig. 6*B′*), a pathway that normally carries postmitotic cells populating the basolateral cortex. At stage E14.5, the early differentiation markers *Tbr1* and *SorLA* showed only a faint expression in the superficial zone of the forming CP, but were not detected in the mutant VZ–SVZ (data not shown). We tested the differentiation of the mutant cortex further at stage E18.5 using the available layer-specific markers: *Emx1* and *Tbr1* for all layers of the cortex (Bulfone et al., 1995), *Otx1* for layer V–VI (Frantz et al., 1994), *mSorLA* for layers V-II (Hermans-BorgMeyer et al., 1998; our unpublished observations) and *reelin* for the MZ (D'Arcangelo et al., 1995; Ogawa et al., 1995). In the abortive CP, the *Emx1, mSorLa,* and *Otx1* showed a diffuse expression at a similar strength

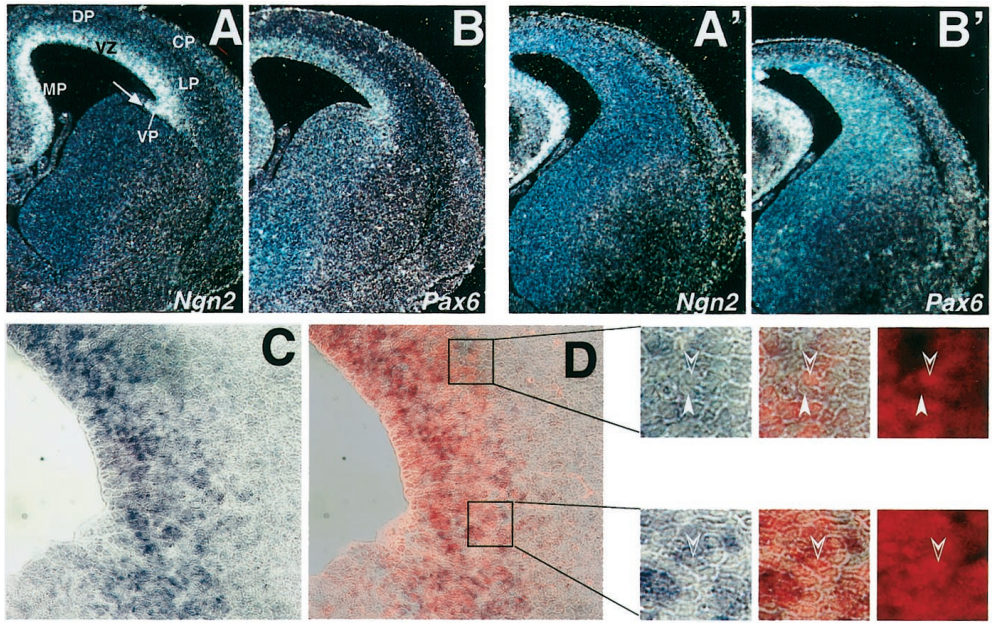

Figure 5. Inhibition of *Ngn2* activity in a subpopulation of the cortical progenitors in *Sey*/*Sey.* Coronal sections from E16.5 wild-type (*A, B*) and *Sey*/*Sey* (*A′*, *B′*) brain hybridized with *Ngn2* (*A*, *A′*) and *Pax6* (*B*, *B′*) probes. In *A* note that the region of the VP in the VZ of the LGE is still distinguishable at this late developmental stage. In the mutant, the expression of *Ngn2* is completely abolished from the region of the VP and LP, severely repressed in the DP, but appears unaffected in the MP. In *B′* note that the enlarged VZ–SVZ in the *Sey*/*Sey* pallium is expressing abundantly the mutant *Pax6* mRNA (*B′*). *C, D,* Coronal sections from E13.5 WT brain were double-labeled by *in situ* histochemistry with the *Ngn2* antisense RNA probe (*blue* cytoplasmic staining) and by immunohistochemistry with the Pax6 antibody (*red* nuclear stain). The enlarged *inserts* are higher magnifications showing that some Pax6-immunoreactive cortical progenitors express *Ngn2* mRNA (*open arrowheads*). The *filled arrowheads* point to progenitors that are only *Ngn2*+. *D* is a composite picture of *C* and the Pax6 immunostaining; the overlay has been done in Adobe Photoshop.

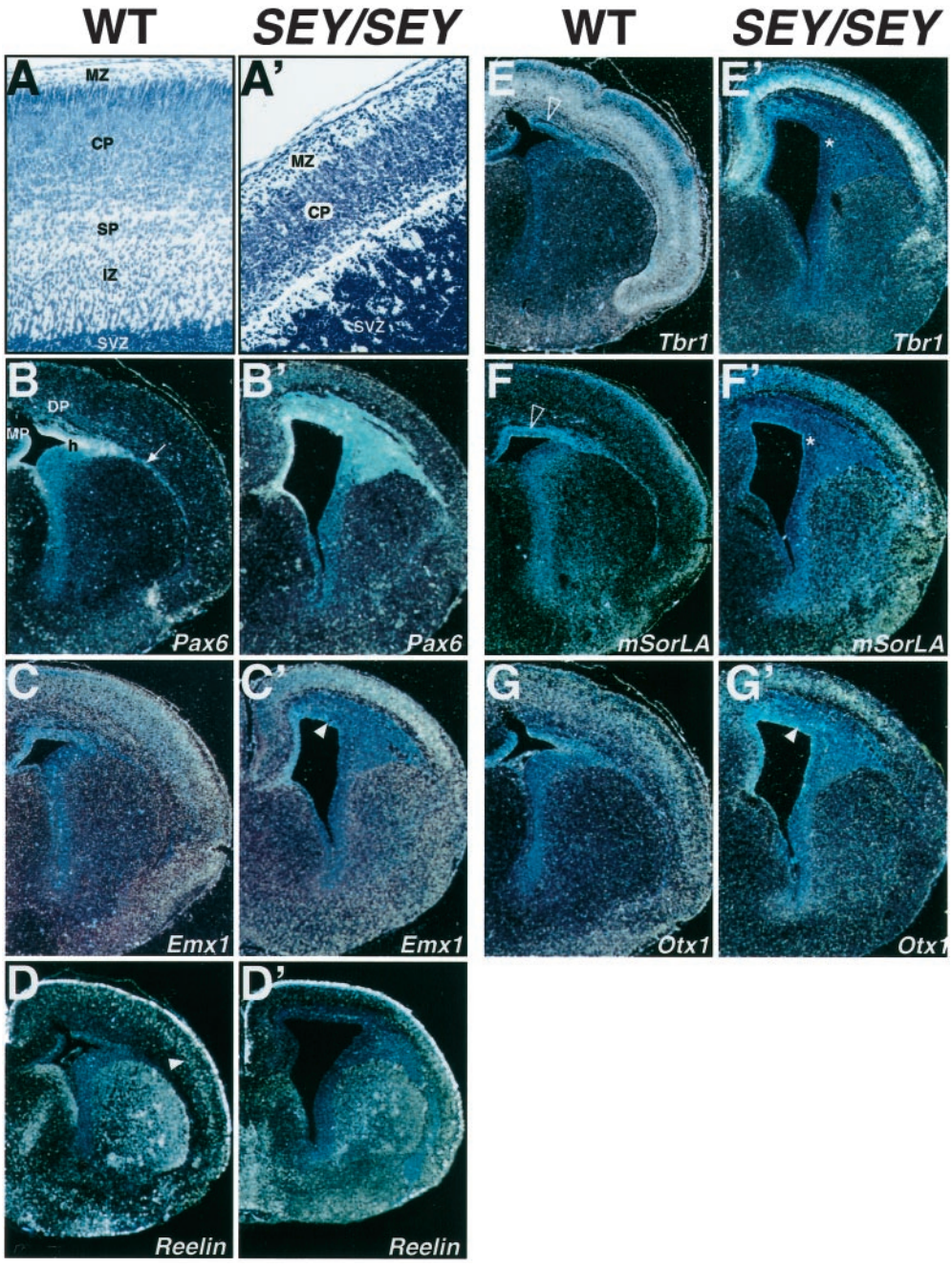

Figure 6. Failure in the differentiation of the cortical plate in *Sey*/*Sey.* Coronal sections at a rostral level from E18.5 WT (*B–D*) and *Sey*/*Sey* (*B′–D′*) brain were hybridized with different cortical markers, as indicated. *A* and *A′* are Nissl-stained sections from a WT and a mutant brain illustrating the severe abnormalities of the pallium in the mutant: an enlarged germinative zone (*VZ–SVZ*), a lack of a delineated intermediate zone (*IZ*) and subplate (*SP*), a thin cortical plate (*CP*) without radial alignment of the cells, and a wide and hypercellular marginal zone (*MZ*). The *asterisks* in *E′* and *F′* point to the germinative Ne of the VP + LP that are compressed by the growing striatum and therefore not easily distinguishable; the *thin arrow* in *B* points to the lateral migratory stream (LMS). In *B′* note the *Pax6*-positive VZ–SVZ in the mutant pallium, which expands within the domain of the VP, LP, DP, in the hilus (*h*) of the LMS, but not in the MP. In *C′* and *G′* note that the expression of *Emx1* and *Otx1* in the pallial VZ–SVZ (*arrowheads*) of *Sey*/*Sey* is detectable. *E, F,* In the WT brain, *Tbr1* and *mSorLa* are expressed in the SVZ (*empty arrowhead*) of the entire pallium and their expression outline the piriform cortex as well. *E′*, *F′*, In *Sey*/*Sey,* the expression of *Tbr1* and *SorLA* is abolished in the enlarged SVZ of the VP–LP (*asterisks*), except for the region of the MP. In the basolateral telencephalon note the disorganization of the lateral insular and piriform cortex. *C′*, *F′*, *G′*, *Emx1, Otx1,* and *mSorLa* show diffuse expression in the abortive mutant CP, whereas the *Tbr1* transcripts are accumulated in the lower part of the CP (*E′*). In *D′* note the stronger expression of *reelin* in the mutant MZ. The *arrowhead* in *D* points to a layer of *reelin*+ cells in the CP that is not detectable in the *Sey*/*Sey* cortex (*D′*).

(Fig. 6*C′*,*F′*,*G′*), and the *Tbr1* transcripts accumulated in the lower part of the CP (Fig. 6*E′*; Warren et al., 1999). The VZ and SVZ of the mutant pallium was labeled by the *Emx1* and *Otx1* probes (Fig. 6*C′*,*G′*, *arrowheads*). In contrast, the expression of *Tbr1* (Fig. 6*E′*) and *SorLA* (Fig. 6*F′*) within the enlarged VZ–SVZ was abolished, most strongly within the region of VP and LP (Fig. 6*E′*,*F′*, *asterisks*) as compared with the dorsomedial SVZ. This finding is consistent with the noticed above region specific inhibition of the *Ngn2* activity in *Sey*/*Sey.* Together, these data suggest that the inhibition of the *Ngn2* activity in the *Sey*/*Sey* pallium might prevent/or causes a delay in the differentiation of a subpopulation of the cortical progenitors (mostly within the ventrolateral pallial domain) that fail to migrate and accumulate within the pathological germinative Ne.

Given the key role of reelin in the laminar cortical development (Lambert de Rouvroit and Goffinet, 1998), it is of special interest to note that whereas the *reelin* expression in the *Sey*/*Sey* MZ was at a higher level as compared with the wild-type brain, the *reelin* transcripts were lacking from the IZ of the pallium in the mutant brain (Fig. 6*D′*,*D*, *arrowhead*).

DISCUSSION

Pax6 modulates the DV regionalization of the neuroepithelium along the entire anteroposterior axis of the developing CNS

Accumulating evidence indicates that the expression of *Shh* in the axial mesendoderm is essential for the ventral specification of the developing CNS, including the forebrain (Ericson et al., 1995; Chiang et al., 1996; Rubenstein and Shimamura, 1997). In the ventral neural tube the Shh signal secreted from the floor plate mediates a long-range repression of the *Pax6* level, forming thereby four zones of distinct progenitors, a most ventral *Pax6*−/*Nkx2.2*+ domain and progressively more dorsally located domains with low, moderate, and high levels of *Pax6* expression (Ericson et al., 1995, 1997). The progenitors of these domains generate distinct neuronal

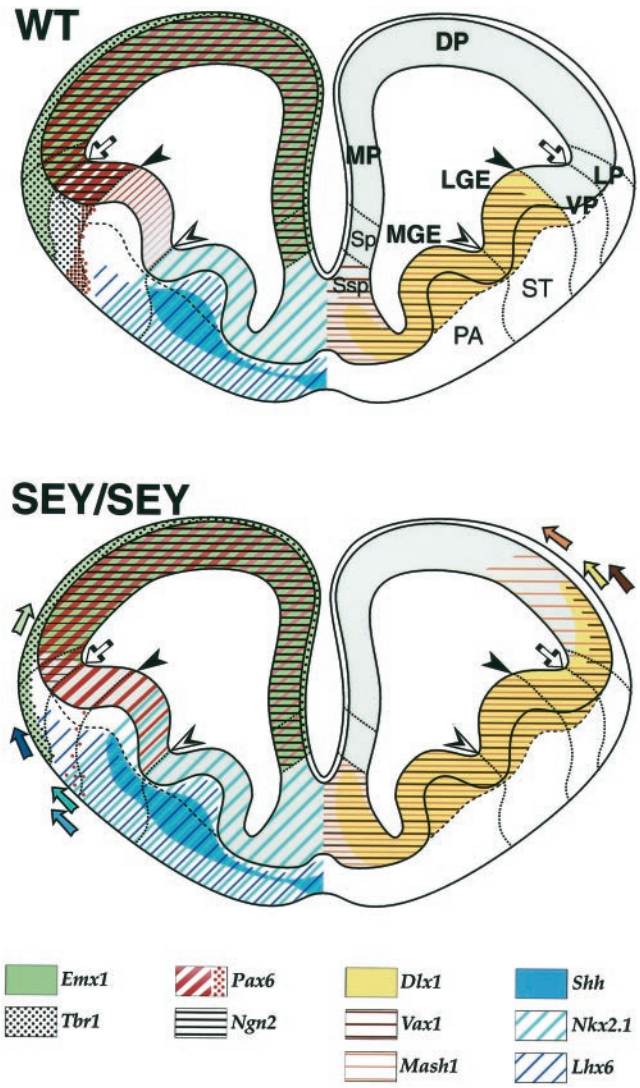

Figure 7. Schematic representation of the DV pattern defects in the telencephalon of the *Pax6*/*Small eye* mutant. The scheme illustrates that in the absence of functional Pax6 protein, the molecular patterning of the embryonic telencephalon is ventralized at the level of the pallial/subpallial and MGE/LGE borders. The drawing is based on the proposed subdivision of the telencephalic Ne (Puelles et al., 1999, 2000) and the results obtained from the expression analysis performed on coronal sections at a rostral level of the E12.5 wild-type (*WT*) and homozygous *Small eye* (*Sey*/*Sey*) brain. The pallial and subpallial markers have been color-coded as indicated. The *arrow* points to the morphological corticostriatal sulcus. The *filled arrowhead* points to the pallial/subpallial border, from where a of *Pax6*+ stream of cells (*red dots*) and *Tbr*1+ (*black dots*) cells migrate toward the basolateral telencephalon as discussed in the text. Noteworthy, results from a very recent homology study in chick and mouse suggest that the *Pax6*+ cells migrate within the striatal territory (Puelles et al., 2000). The *open arrowhead* points to the boundary between the MGE (pallidum) and the LGE (striatum).

populations of the motor neurons and the three columns of interneurons. Our analysis revealed a similar characteristic in three ventrodorsal domains of the telencephalic neuroepithelium for the expression of *Pax6* and *Nkx2.1* which is another member of the Nkx gene family with a restricted expression in the anlage of the MGE (Price et al., 1992; Shimamura et al., 1995; Sussel et al., 1999). It should be noted however that the "dorsoventral" terminology used to describe our observations is preliminary because the topological relationship of the telencephalic subdivisions is still an open question (for discussion, see Rubenstein et al., 1998).

We found that the most ventrally located domain, the VZ of the MGE, that generates the cells of the pallidum is a *Pax6*−, but *Nkx2.1*+/*Dlx1,2*+/*Vax1*+/*Mash1*+ region. In the MGE, *Shh* is initially expressed in the VZ (Sussel et al., 1999), and later it is expressed in the SVZ and mantle zone. The next domain is the VZ of the LGE, which produces the striatum. It expresses *Pax6* at a very low level and is *Nkx2.1*−/*Dlx1,2*+/*Vax1*+/*Mash1*+. The domain of the VP is the third zone, located further dorsally. It contributes to the generation of the claustrum–endopiriform nucleus, the piriform cortex, and a part of the amygdala (Bayer and Altman, 1991; for further discussion see Puelles at al., 2000). Here the expression of *Pax6* (and *Ngn2* as well) is very high, whereas the transcripts of *Nkx2.1, Dlx1, Vax1,* and *Mash1* are absent.

In the caudal neural tube, the loss of Pax6 function leads to a dorsal expansion of ventral markers and to a change of the cell fate (Ericson et al., 1997). Likewise we found that the Pax6 mutation leads to an expansion of the expression of the MGE marker genes *Shh, Nkx2.1,* and *Lhx6* into the territory of the more dorsally located LGE. This pattern defect appears to result in the alteration of the regional identity of the adjacent LGE area reflected in an enlargement of the MGE territory at midgestation and underdevelopment of the striatum later on—a puzzling morphological phenotype for the Pax6 mutant brain for a long time (Glaser et al., 1994). We found further that the ventralization of the Ne of the VP, where *Pax6* is expressed at a very high level, causes defects in the generation of the piriform, rostral lateral (insular) cortex, and the claustrum–endopiriform nucleus. These defects are reminiscent to observations in the *Sey*/*Sey* hindbrain and spinal cord where the columns of the dorsally and more ventrally located neurons, produced by domains with a very high and low level of *Pax6* expression, are either missing or show an altered identity, respectively (Burrill et al., 1997; Ericson et al., 1997). Thus, in Pax6 loss of function in appears that domains that normally have a comparable level of *Pax6* expression, show similar morphological disturbances in the epichordal and prechordal part of the CNS as a result of the ventralization of the molecular identity of adjacent regions. These results indicate that the level of Pax6 expression is an essential determinant of the DV regionalization of the Ne along the entire anteroposterior axis of the developing CNS.

In the spinal cord of *Nkx2.2*−/− mice the fate of the most ventral column of neurons is dorsalized into the fate of the somatic motoneurons, but without a change in the *Pax6* expression—a fact implicating that *Nkx2.2* has a decisive role for interpreting the ventralizing activity of the Shh protein produced by the notochord and floor plate (Briscoe et al., 1999). Although Shh, which is produced by the rostral mesendoderm, is an essential factor for establishing the ventral identity in the forebrain (Ericson et al., 1995; Shimamura and Rubenstein, 1997), the final specification of the DV domains in the telencephalon seems to include additional mechanisms. The expression of *Nkx2.1* and *Shh* in the MGE and *Pax6* in the pallium appears almost simultaneously at ~E10.5 (Hentges et al., 1999). In Pax6 loss of function we observed ectopic expression of both *Shh* and *Nkx2.1* into more dorsal telencephalic domains. A recent analysis of *Nkx2.1*−/− mice revealed opposite pattern defects as compared with the *Small eye* telencephalon (Sussel et al., 1999). In these mice, the lateral domain of the MGE is dorsalized showing ectopic expression of *Pax6*, whereas the expression of *Shh* in the MGE is suppressed. The alteration of the patterning leads to a lack of the globus pallidus and an enlargement of the striatum. Thus, in the *Sey*/*Sey* and *Nkx2.1*−/− mutants, although the anlage of the MGE and LGE are specified presumably by the ventralizing activity of the mesendodermal Shh, these structures show a complementary DV pattern and morphological defects in the adjacent domains of the MGE or LGE. It is worthy to note that in the absence of the low level expression of *Pax6* in the VZ of the LGE, the dorsal ectopic expression of *Nkx2.1* includes the VZ of a part of the LGE. Together, these data suggest that either a direct regulation of the activity of these genes or protein–protein interactions between their products might contribute for the maintenance of the MGE/LGE border in the telencephalon.

Pax6 and the patterning of the cortex

The development of the cortex is severely affected in the *Sey*/*Sey* mutant: the CP is hypocellular without radial alignment of the cells, whereas the germinative neuroepithelium (VZ–SVZ) is enlarged and consists of accumulated precursors in large clumps that occupy the area of the IZ (Schmahl et al., 1993; Warren and Price, 1999). These cells show a high level of expression of the mutant *Pax6* message (Stoykova et al., 1997; this study) and active incorporation of BrdU after pulse labeling at early (E10–E12.5) (Warren et al., 1999) and later (E12.5-E18.5) stages (Brunjes et al., 1998; Götz et al., 1998).

We show in this work a severe defect of the DV patterning in the *Sey*/*Sey* telencephalon. As a result of the early developmental ventralization of the NE at the pallial/subpallial border, the morphogenesis of the basolateral cortex appears to be strongly affected, as shown by the malformation of the claustrum, endopiriform nucleus, piriform, and lateral cortex.

From E14.5 onward, the pallium of the *Sey*/*Sey* mutant fails to properly differentiate. The accumulated cells in the mutant VZ–SVZ express the neuron-specific marker TuJ1 (Caric et al., 1997). However, the expression of the differentiation markers *Tbr1*, *mSorLa,* and *Emx1* were not detected in the SVZ of the ventrolateral and dorsal pallium, but being preserved in the MP and in the abortive cortical plate. This suggests that only a portion of the later cortical progenitors are either not generated or they are unable to properly differentiate in the *Sey*/*Sey* cortex. A similar regional inhibition of the activity of the *Ngn2* gene was detected in the VZ of the mutant pallium. *Ngns* are vertebrate neuronal determination genes encoding for basic helix-loop-helix transcription factors, essential for the neurogenesis, including the cortex (Ma et al., 1996; 1999; Cai et al., 2000). Our previous results indicated that the expression of *Pax6* is a characteristic trait of the cortical RC2+ radial glial cells with an essential role for their differentiation (Götz et al., 1998). The cortical radial glial cells might have a neurogenic potential (Alvarez-Buylla et al., 1990; Lendahl et al., 1990; Gray and Sanes, 1992). In accordance with recent results indicating that Ngn2 is detected only in those Pax6+/RC2+ radial glial cells, that contain neither the astrocyte-specific glutamate transporter (GLAST) nor the brain- lipid-binding protein (BLBP) (Götz, 2000; Hartfuss et al., 2000) we show here that at E13.5 the expression of *Ngn2* and Pax6 normally colocalizes only in some cortical progenitors. Most intriguingly, the misexpression of *Ngn2* in the cortical progenitor cells results in the production of neurons (Cai et al., 2000). Furthermore, isolated radial glial cells from *Sey*/*Sey* cortex generated *in vitro* only 44% of the neuronal clones produced by the WT radial glial cells (Malatesta and Götz, 2000). Thus, our results and the literature data support the possibility that the differentiation of not all cortical precursors in the *Sey*/*Sey* pallium is affected; indeed the mutant CP shows expression of all tested cortical markers. We favor rather the idea that only a portion, mainly the Ngn2+/Pax6+ progenitors of the ventrolateral pallium are hampered to differentiate in Pax6 loss of function.

Accumulating evidence indicates that some postmitotic cells born in the subpallium invade the pallium. Thus, a part of the cortical interneurons are produced in the subpallial Ne and populate through a tangential migration the CP as postmitotic Dlx-, GABA-, GAD67-, Lhx6-, calbindin-, calretinin-, or reelin-positive cells (for review, see Parnavelas, 2000). The absence of *Dlx1/2* (Anderson et al., 1997a,b) and *Mash1* (Casarosa et al., 1999) in the MGE/LGE leads to an almost complete loss of the GAD67+ cells in the CP or in the MZ, respectively, whereas the loss of *Nkx2.1* in the MGE (Sussel et al., 1999) is associated with absence of calbindin+ cells. We show in this work that early in development the proliferative Ne of the VP and LP in *Sey*/*Sey* expresses ectopically *Dlx1, Mash1*, *Vax1,* and *Six3,* whereas the restricted expression of *Nkx2.1* and *Lhx6* to the MGE expands into the adjacent LGE territory. Therefore it is likely that the ventralized Ne in the basal telencephalon of *Sey*/*Sey* produces progenitors with altered identity, increasing thereby the portion of the subpallial cells that migrate into the cortex. This is in line with data showing that the lateral telencephalon of *Sey*/*Sey* contains twice as much postmitotic GABA+, calbindin+ and calretinin+ cells as compared with the wild-type littermates (Chapouton et al., 1999). Thus, the defects of the dorsoventral patterning of the telencephalic neuroepithelium in Pax6 loss of function are part of the complex cortical phenotype of the *Small eye* mutant.

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