# Compromised generation of GABAergic interneurons in the brains of *Vax1*$^{-/-}$ mice

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## Summary

**The subcortical telencephalon is the major source of GABAergic interneurons that, during development, tangentially migrate to the cerebral cortex, where they modulate the glutamatergic excitatory action of pyramidal cells. The transcription factor Vax1, an intracellular mediator of both Shh and Fgf signaling, is expressed at high levels in the medial and lateral ganglionic eminences (MGE and LGE, respectively), in the septal area (SA), in the anterior entopeduncular area (AEP) and in the preoptic area (POA). We show that *Vax1* expression in the neuroepithelium is graded: low in the ventricular zone (VZ) and high in the subventricular zone (SVZ), in a pattern that closely reproduces that of several members of the Dlx and Gsh family of homeobox transcription factors. We provide evidence that *Vax1* plays an important role in proliferation and differentiation of MGE, POA/AEP and septum, and that the last structure is completely absent in *Vax1*$^{-/-}$ mice. We show that the absence of Vax1 causes a severe depletion of GABAergic neurons in the neocortex, ranging from 30% to 44%, depending on the cortical areas considered. Taken together, our data indicate that a loss of function mutation in the *Vax1* gene generates abnormalities in basal ganglia subventricular zone development and that it prevents the formation of the septum, impairing GABAergic interneuron generation.**

Key words: Telencephalon, Septum, MGE, Mouse

## Introduction

During development, the forebrain acquires precise subdivisions both along the dorsoventral (DV) and the anteroposterior (AP) axis (Puelles et al., 1987; Puelles and Rubenstein, 1993; Puelles and Rubenstein, 2003; Rubenstein et al., 1994; Shimamura et al., 1995). Several morphogens act early and are required for this mosaic organization and for the specification of different cell types within these territories. Multipotent neural progenitors are first maintained in a proliferative state, in order to ensure that the correct number of cells will be generated. Transition from precursor cell proliferation to neural differentiation requires the coordinated action of several basic helix-loop-helix (bHLH) and homeobox transcription factors, which – directly or indirectly – ultimately act on cell cycle molecules to instruct cells to leave their mitotic state, differentiate and migrate to their final location (Schuurmans and Guillemot, 2002; Yun et al., 2002). Often a combinatorial code of several genes is necessary to start a specific program of differentiation. By mechanisms of activation and mutual repression, transcription factors establish boundaries within precursor cells of the forebrain yielding the characteristic regionalization.

Along the DV axis, the telencephalon becomes subdivided into two large and profoundly different territories: pallium and subpallium (Puelles et al., 2000). The pallium is the major source of glutamatergic neurons and it comprises the cerebral cortex, the hippocampus and part of the septum. The subpallium is instead the major source of GABAergic neurons and it includes the basal ganglia, the rostral telencephalic stalk and the ventral part of the septum. Within the basal ganglia, the lateral ganglionic eminence (LGE) will generate the striatum; the medial ganglionic eminence (MGE) the globus pallidum, and the preoptic area (POA) will generate oligodendrocytes and cholinergic neurons of the basal forebrain. Originally, it was thought that cell mixing was not possible between pallium and subpallium; however, recently, it has been demonstrated that GABAergic interneurons are generated in the basal ganglia and migrate across the pallium/subpallium border to populate the cerebral cortex (Anderson et al., 1997a; Wichterle et al., 2001) (for reviews, see Marin and Rubenstein, 2001; Marin and Rubenstein, 2003).

In the subcortical telencephalon, several genes have been found responsible for regionalization, cell type determination or precursor cell migration. Among the genes important for specification, is the homeobox *Nkx2.1*. Mice that lack *Nkx2.1* display a ventral-to-dorsal change of fate, with precursor cells that should generate MGE converted into LGE precursors (Sussel et al., 1999). In the absence of *Gsh2*, instead, it has been observed a loss of DV territorialization, shown by the expansion of MGE markers, such as *Gsh1*, into the LGE, and ectopic expression of pallial markers in the dorsal LGE (Corbin et al., 2000; Toresson and Campbell, 2001; Yun et al., 2001). Dlx genes also play a crucial role in basal telencephalon development. These genes do not seem to be related to forebrain regionalization, but instead are responsible for differentiation and migration of subpallial projection neurons

and interneurons that tangentially migrate to the cerebral cortex, hippocampus and olfactory bulb (Anderson et al., 1997a; Bulfone et al., 1998; Pleasure et al., 2000; Yun et al., 2002). Moreover, ectopic expression of Dlx genes is sufficient to induce a GABAergic phenotype (Stuhmer et al., 2002).

In the context of basal forebrain genetic determination, we studied *Vax1*, a homeobox transcription factor expressed in the subpallium in a pattern highly similar to that of Dlx and Gsh genes (Anderson et al., 1999). Its function has been studied mainly in the developing visual system (Bertuzzi et al., 1999a; Hallonet et al., 1998; Hallonet et al., 1999; Ohsaki et al., 1999; Schulte et al., 1999), where it is responsible for conferring ventral identity to the optic stalk and neural retina (S. H. Mui, J. W. Kim, G. Lemke and S.B., unpublished). Its expression has been reported in the forebrain (Bertuzzi et al., 1999a; Hallonet et al., 1998; Stoykova et al., 2000), but its function has not been studied in great detail in this territory, especially with respect to cell migration from the ganglionic eminences.

In this paper, we study the function of Vax1 in the subcortical telencephalon. In *Vax1* mutant brains, despite dorsoventral patterning occurring correctly, we have found that neurons originating from MGE and POA/AEP do not differentiate properly and accumulate in the subventricular zone (SVZ), failing to migrate into the mantle zone (MZ) efficiently. Moreover, *Vax1* mutant embryos display a severe ventral midline defect, with an apparent complete morphological absence of the septal formation. As a consequence, the impaired differentiation of MGE, POA/AEP and septum leads to a loss of GABAergic neurons in the neocortex, revealing a role for Vax1 in precursor cell determination.

## Materials and methods

### Genotyping of *Vax1* mutant mice

*Vax1*$^{-/-}$ embryos die at birth because of a severe cleft palate (Bertuzzi et al., 1999a; Hallonet et al., 1998). Few pups (about 6%) survive up to postnatal day (P) 20. *Vax1*$^{-/-}$ mice were generated by crossing heterozygous animals on a 129SV×C57Bl/6 background. Genotyping was performed by PCR.

### Tissue preparation and histology

Noon of the day when the vaginal plug of the pregnant dam was identified was considered to be embryonic day (E) 0.5 (Nagy et al., 2003). Embryos younger than E15.5 were immersion fixed in 4% paraformaldehyde (PFA). E16.5 embryos and older were anesthetized and perfused transcardially with 4% PFA in 0.1 M PBS.

### RNA in situ hybridization

In situ hybridization on frozen sections was carried out using digoxigenin-labeled probes as already described (Tuttle et al., 1999; Zhadanov et al., 1995).

### Immunohistochemistry

Sections were postfixed in 4% PFA for 5 minutes, rinsed in PBS and blocked in 10% normal goat serum/0.1% Triton X-100 at room temperature for 1 hour. The following antibodies were incubated overnight in 0.1% normal goat serum and 0.1% Triton X-100 at 4°C: monoclonal rat anti-mouse Ki67 (Dako number M7249, 1:100); rabbit anti-Dlx (generous gift of Dr Panganiban, 1:300); monoclonal anti-neural class IIIB-tubulin (Babco, number MM-S405-P, 1:500); rabbit anti-calbindin D-28K (Swant number CB-38, 1:10,000). Anti-Ki67 staining was performed after microwave epitope retrieval in 10 mmol/L citrate buffer (pH 6). Sections were incubated for 1 hour with biotinylated secondary antibodies: goat anti-rabbit (Jackson Immunoresearch, 1:100), goat anti-mouse (Jackson Immunoresearch, 1:100) or donkey anti-rat (Jackson Immunoresearch 1:500). Subsequently, tissues were incubated for 30 minutes with an avidin-biotin-peroxidase complex (Elite PK-6100, Vector Laboratories) at room temperature, and revealed with DAB peroxidase substrate (SK-4100, Vector Laboratories). Sections were finally dehydrated, mounted with Permount (Fisher, number SP15-100) and photographed with an Olympus bright field microscope (Olympus Provis AX 70) equipped with a digital camera (Nikon DXM 1200).

### Birthdating analysis

Pregnant mice were injected intraperitoneally at E13.5 with a sterile solution of bromodeoxyuridine (BrdU, Sigma) at a dose of 100 μg BrdU/g of body weight. Newborn pups were anesthetized and perfused transcardially with 4% PFA in 0.1 M PBS. Immunohistochemistry was performed as described using a mouse monoclonal anti-BrdU antibody (Sigma, number B2531, 1:100).

### GABAergic cell counts and statistics

To obtain accurate and statistically significant counts of GABAergic cells, we performed fluorescent immunohistochemistry using a rabbit anti-GABA antibody (SIGMA, n° A2052, 1:1000) in an overnight incubation at 4°C, followed by a 1 hour room temperature incubation with an anti-rabbit FITC-conjugated secondary antibody (Jackson Immunoresearch, 1:100). Nuclei were counterstained with DAPI (Sigma, 1:10000). Sections were subsequently mounted on slides and photographed with a Nikon ES600 microscope equipped with digital camera (Nikon, Coolpix 990). Three pairs of matching P0 wild type and mutants were examined. GABA-positive cells were counted in rostral, medial and caudal cortex within a standardized rectangular area of 200×150 $\mu m^2$. Counts were performed in at least 10 slices selected at the same level for each couple of animals. The ratio of the total number of GABA-positive cells between mutant and wild-type cortical areas was calculated. Ratio values were compared and represented in graphics by Excel 2000. A ratio of 1 (100%) indicates the number of GABAergic cells in the wild type.

### Organotypic slice culture experiments

Organotypic slice cultures were performed as described (Lavdas et al., 1999; Tobet et al., 1994). To examine the migratory pathways of neurons generated in septum or MGE, we placed small crystals of 1-1′-dyoctodecyl-3-3-3′-3′ tetramethylindocarbocyanine (DiI, Molecular Probes, Eugene OR) in the region of interest. Slices were examined under a fluorescent microscope (Nikon ES600) after 48 hours in culture.

## Results

### *Vax1* is expressed in the neuroepithelium of the developing ventral forebrain

To understand *Vax1* function in basal forebrain development, we have first studied its expression profile in this area at embryonic day (E) 12.5, when *Vax1* is strongly expressed in LGE, MGE, AEP, POA, hypothalamus (HT) and in the septum (Fig. 1A-D). *Vax1* expression is sharply downregulated at the corticostriatal border (arrowhead in Fig. 1A), with the consequent reduction of *Vax1* presence in the pallium. As it is evident from Fig. 1, in the subpallium, *Vax1* is expressed in the neuroepithelium and is excluded from the differentiated mantle zone. Interestingly, *Vax1* message is not uniformly detected throughout the neuroepithelium of the subpallium, but its expression is graded: low on the ventricular side and high at the border with the mantle zone. To determine if *Vax1*-

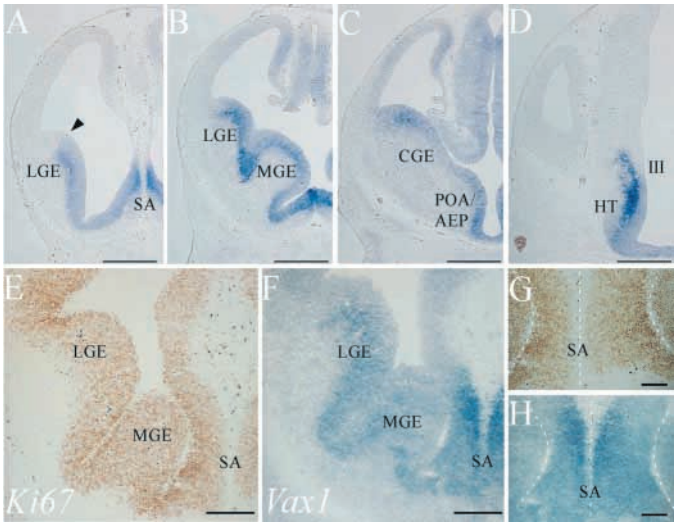



**Fig. 1.** *Vax1* expression in the ventral telencephalon. (A-D,F) In situ hybridization revealing *Vax1* expression in telencephalic coronal sections in E12.5 wild-type mice. H is a magnification of F. (A-D) Different rostrocaudal levels of the same brain (A is the most rostral and D is the most caudal). *Vax1* is strongly expressed in the SA, LGE, MGE and post-optic area. There is sharp downregulation of *Vax1* expression at the corticostriatal boundary (arrowhead in A). (E) Immunohystochemical localization of the cell cycle marker Ki67 on an adjacent section to that shown in F. G is a magnification of E. (E-H) In the ganglionic eminences (F) and in the septum (H), *Vax1* expression overlaps with Ki67 (E,G) with an inverse gradient. III, third ventricle; AEP, anterior entopeduncular area; CGE, caudal ganglionic eminence; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; POA, pre-optic area; HT, hypothalamus; SA, septal area. Scale bars: 500 μm in A-D; 200 μm in E,F; 100 μm in G,H.

expressing cells are proliferating, in Fig. 1E-H we compare *Vax1* expression with that of the Ki67 protein (Mki67 – Mouse Genome Informatics). Ki67 is a protein that interacts with members of the heterochromatin protein 1 family and is strictly associated with cellular proliferation, present in all of the active phases of the cell cycle ($G_1$, S, $G_2$ and mitosis), but not in resting cells in $G_0$ (Scholzen et al., 2002; Scholzen and Gerdes, 2000). As it can be observed, there is a wide overlap of expression between *Vax1* and Ki67, indicating that the vast majority of *Vax1*-expressing cells are mitotically active. However, Ki67 presents an opposing gradient to that of *Vax1*, which is particularly evident in the septal area (Fig. 1G,H). A graded expression pattern similar to that of *Vax1* has been reported for several members of the Dlx homeobox family (Eisenstat et al., 1999), which are essential genes for correct basal forebrain differentiation. We will discuss the relationship between *Vax1* and Dlx genes below. In this paper, we focus our analysis on the *Vax1* knockout defects in the MGE and septum, where we have observed specific defects that are also described below.

## Abnormal proliferation of progenitor cells in the subcortical telencephalon

Given the expression pattern of *Vax1* in the ventricular zone of the subcortical telencephalon, we first wanted to understand if in the absence of *Vax1* the ganglionic eminences develop correctly. In Fig. 2 we have analyzed three issues related to *Vax1* function: (1) the anatomical and molecular identity of MGE and LGE; (2) the precursor cell transition from ventricular to mantle zone; and (3) the dorsoventral forebrain patterning.

### Anatomical and molecular identity of MGE and LGE

Nissl stained sections at E13.5 display a lack of separation of the basal ganglionic eminences with the consequent loss of the interganglionic sulcus, which is reduced to a small dent between LGE and MGE (Fig. 2A,B). Owing to this, the MGE region of the eminences assumes an oblong appearance instead of the distinct bulb-shaped structure protruding into the lateral ventricles, as shown in Fig. 2A. LGE and MGE normally fuse in the posterior part of the forebrain to form the caudal ganglionic eminence (CGE) (Jimenez et al., 2002; Nery et al., 2002), while in *Vax1* mutants the fusion of the two eminences is present throughout the telencephalon. Given the loss of separation between LGE and MGE, we considered the hypothesis that the anterior lack of separation of the eminences could be due to identity loss of MGE or LGE, which in normal development give rise to two different structures: the globus pallidum and the striatum, respectively. We examined the expression of the zinc-finger transcription factor *Gli1*, which marks the border between MGE and LGE (Tole et al., 2000). As it can be seen in Fig. 2D, in the absence of *Vax1* the expression of *Gli1* is modified, loosing its characteristic staining at the MGE-LGE border (Fig. 2C), presenting instead a diffuse signal extending widely into the presumptive MGE. We then considered the homeobox gene *Nkx2.1*. Loss of *Nkx2.1* causes a ventral-to-dorsal respecification within the subcortical telencephalon, with the main derivative of the MGE, the globus pallidum, transformed into striatum (Sussel et al., 1999). As shown in Fig. 2F, in *Vax1*$^{-/-}$ mutants *Nkx2.1* expression is correctly restricted to the MGE territory, indicating that the identity of the MGE neuroepithelium has not changed in the absence of a functional Vax1 gene product. To assess LGE identity, we checked the expression of *Ebf1*, a transcription factor normally expressed in this area. As shown in Fig. 2G,H, *Ebf1* is correctly restricted to LGE without invading the MGE (compare Fig. 2F with 2H), despite some minor abnormalities (see text below). Therefore, the loss of correct *Gli1* expression seems to be related to the MGE-LGE border, rather than to a loss of specification of these two structures. At E16.5, markers of the globus pallidum and striatal formation appear to correctly label the mature structures, although defects seem to be apparent in the organization of these nuclei. We will address these issues in detail elsewhere.

### Precursor cell transition from ventricular to mantle zone

*Nkx2.1* in situ hybridization and Nissl stain clearly highlight an aberrant shape of the MGE ventricular zone. In fact, *Nkx2.1* does not show the typical contoured pattern of expression (Fig. 2E), following the ventricles abutting the MGE, but instead labels a large, compact mass of darkly stained cells (Fig. 2B). This mass of cells is located in the medial region of the basal ganglia and has the appearance of VZ. To determine if this was correct, we examined the expression of the LIM-homeobox gene *Lhx8*, which is mostly expressed in the mantle zone of MGE and POA (Zhao et al., 2003). As shown in Fig. 2J,

although *Lhx8* expression is maintained in *Vax1* mutants, it is reduced if compared with wild-type sections (Fig. 2I). *Lhx8*-positive cells accumulate in the SVZ instead of spreading into the MZ, possibly indicating a differentiation defect. *Ebf1* is a transcription factor required for coupling neuronal differentiation with cell cycle exit and is therefore essential for the transition of precursor cells from the VZ to the striatal mantle (Garcia-Dominguez et al., 2003; Garel et al., 1999). As shown in Fig. 2G,H, in *Vax1*$^{-/-}$ brains the *Ebf1* expression domain is greatly reduced, with most of the cells expressing *Ebf1* confined to a small area of the LGE mantle zone, at the border with the VZ. Although located in roughly the correct position with respect to the lateromedial axis (suggesting again that the MGE-LGE identity has not changed), *Ebf1*-positive cells seem to have failed to penetrate into the mantle zone in an efficient way. This result, together with that obtained with *Lhx8*, suggests that *Vax1* mice present a defect in the differentiation of mature neurons, from both MGE and LGE. In agreement with this, the analysis of Ki67 at a later stage of development, E16.5 (Fig. 3D), reveals that in Vax mutants the VZ is significantly expanded. As expected, this region is negative for Tuj1 immunostaining, showing a labeling pattern that is complementary to that of Ki67 (Fig. 3E,F). Neuronal birthdating obtained by injection of BrdU at E13.5 and immunohistochemistry at P0, reveals that neurons born at E13.5 do not disperse into the MZ, but accumulate at the border of the VZ (arrowheads in Fig. 3H). This area corresponds to the developing nucleus accumbens, where *Lhx8* is expressed (Zhao et al., 2003).

### Dorsoventral forebrain patterning

*Vax1* presents a clear border of expression at the corticostriatal corner (Fig. 1A,B). To control the possibility that the identity of pallium and subpallium was changed in *Vax1* mutants, we checked for the expression of two established dorsal regulators, neurogenin 2 and *Pax6* (Fig. 2K-N), which are ectopically expanded into the subpallium in the absence of the ventral regulator *Gsh2* (Toresson et al., 2000; Yun et al., 2001). As it can be observed, the boundary of expression at the corticostriatal corner in *Vax1*$^{-/-}$ brains is precisely maintained (broken black line in Fig. 2K-N), indicating the absence of subcortical telencephalon dorsalization. In fact, in *Vax1* mutants *Gsh2* expression is correctly maintained along the dorsoventral axis (Fig. 2O,P).

## Dlx expression is affected in the LGE, MGE and septal area

Four Dlx genes (*Dlx1*, *Dlx2*, *Dlx5* and *Dlx6*) are expressed at different stages of differentiation in the subcortical telencephalon (Anderson et al., 1997b; Liu et al., 1997; Porteus et al., 1991; Price et al., 1991; Robinson et al., 1991; Simeone et al., 1994). In particular, at E13.5 *Dlx1* and *Dlx2* are expressed in a subset of VZ and SVZ precursors, with a dominance of *Dlx2* in the VZ. *Dlx5* is largely absent from the VZ and is almost

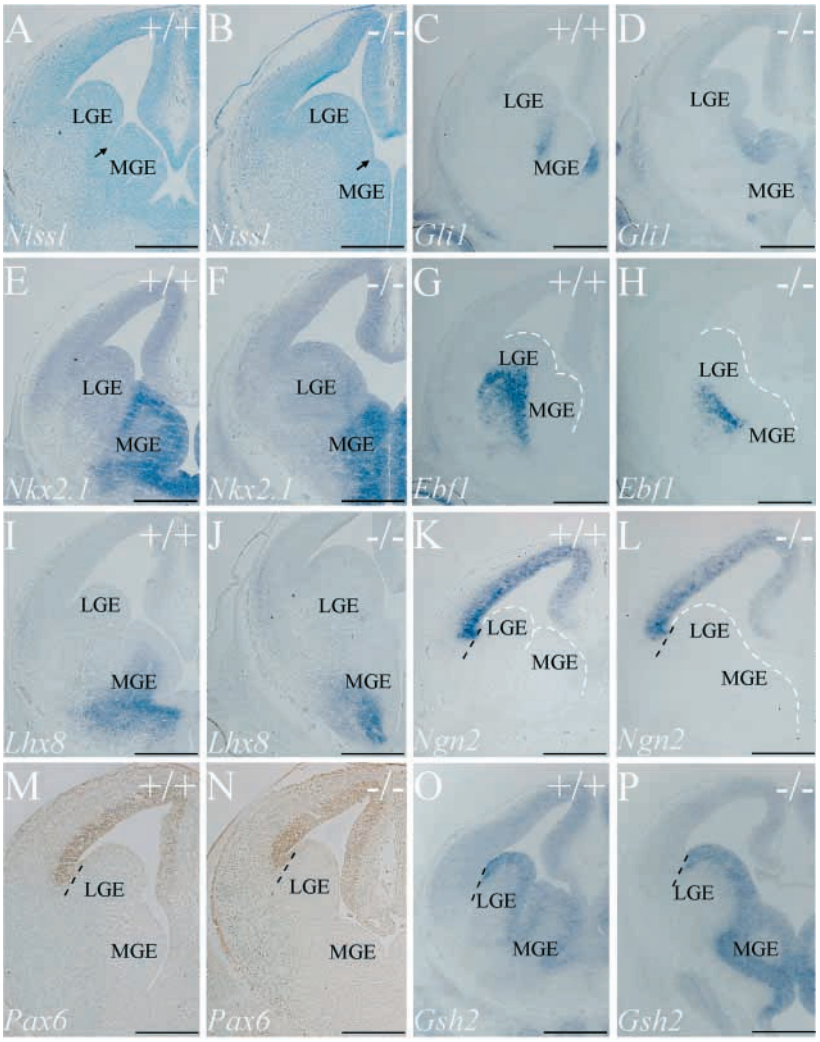



**Fig. 2.** Ventral telencephalic defects in *Vax1* mutant brains. Coronal sections from wild-type (A,C,E,G,I,K,M,O) and *Vax1* knockout (B,D,F,H,J,L,N,P) E13.5 embryos. The expression of several pallial and subpallial markers has been analyzed by RNA in situ hybridization (C-L,O,P) or by immunohistochemistry (M,N). Nissl analysis of comparable sections from wild-type and mutant telencephalon (A,B) reveals that in the absence of *Vax1* the interganglionic sulcus is missing (black arrows). This defect is accompanied by the modification of the expression pattern of *Gli1* (C,D), which shows a diffuse labeling pattern in the territory surrounding the mutant interganglionic sulcus. (E,F) In wild-type brains, the homeobox gene *Nkx 2.1* is expressed in the proliferative zone of the MGE (E); in *Vax1* $^{-/-}$ embryos, its expression is expanded (F). (G,H) *Ebf1* is a marker of the mantle zone of the LGE (G). In the mutant brain, its expression is strongly reduced but correctly restricted to this region (H). (I-J) The MGE mantle marker *Lhx8* shows a significant reduction in mutants (J) compared with wild types (I). (K-N) The boundary between ventral pallium and subpallium (broken black line) is maintained in the absence of *Vax1*: expression domains of the pallial markers *Ngn2* (K,L) and *Pax6* (M,N) are unaltered. (O,P) The subpallial marker *Gsh2* does not show a dorsal shift. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence. Scale bars: 500 μm.

exclusively expressed in the SVZ and in the postmitotic mantle zone. *Dlx6* instead is mainly present in the mantle zone (Eisenstat et al., 1999; Liu et al., 1997; Perera et al., 2004). Therefore, if we consider the combined expression of all of the relevant Dlx genes we should observe the highest expression in the SVZ, a situation closely resembling that of *Vax1* expression. We analyzed the expression of Dlx genes in *Vax1* mutant mice using a pan-Dlx antibody (Panganiban et al., 1995). As expected, cumulative Dlx expression is strongest in the SVZ and spans the LGE, MGE and SA (Fig. 4A). When we analyzed the expression of Dlx in the *Vax1* mutants (Fig. 4B), we noticed two major defects: (1) the peaking expression in the SVZ is largely reduced throughout the basal ganglia generating a uniform staining pattern without a defined gradient; and (2) a significant reduction of Dlx expression in the MGE (Fig. 4A,D). Based on the Dlx analysis, it seems that in the absence of Vax1 the separation of VZ, SVZ and MZ is partly lost, generating a rather flat gradient of Dlx expression, compared with the sharp one present in the wild type. Interestingly, although *Vax1* and Dlx present a wide overlapping expression throughout the subcortical telencephalon, Dlx expression is mostly affected in the MGE (Fig. 4D). We hypothesized that this could be due to a specific

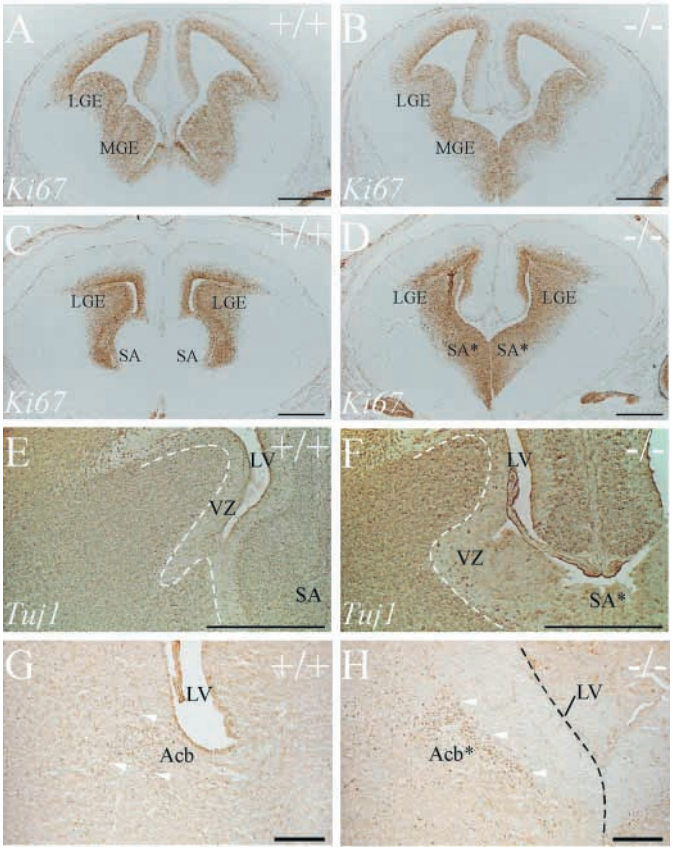



**Fig. 3.** Abnormal differentiation in *Vax1*$^{-/-}$ brains. Proliferating cells at E13.5 (A,B) and E16.5 (C,D) were revealed with anti-Ki67 antibody. Ki67 shows strong labeling in the VZ and SVZ of the wild-type LGE and MGE (A,C). Labeling in the *Vax1* mutant is significantly expanded in the MGE and presumptive septal area (D). The expansion of the VZ in the mutant MGE is confirmed by the complementary Tuj1 staining at E15.5 (E,F). There is strong expansion of the Tuj1-negative domain in the mutant brain (F). (G,H) Birthdating analysis. Brains were injected with BrdU at E13.5 and analyzed at P0. Cells labeled with BrdU at E13.5 are dispersed in the wild-type P0 brain (G, arrowheads); by contrast, BrdU-positive cells linger at the border between proliferating and differentiated regions in the mutant MGE (H, arrowheads). Acb, nucleus accumbens; Acb*, presumptive nucleus accumbens; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; SA, septal area; SA*, presumptive septal area; VZ, ventricular zone. Scale bars: 500 μm in A-F; 100 μm in G,H.

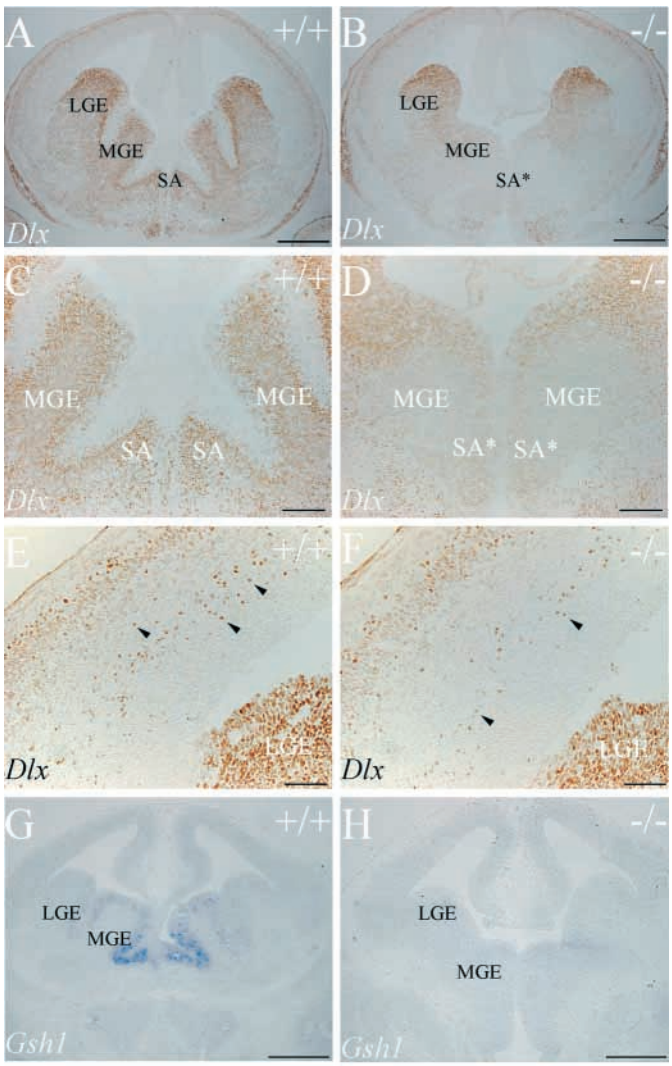
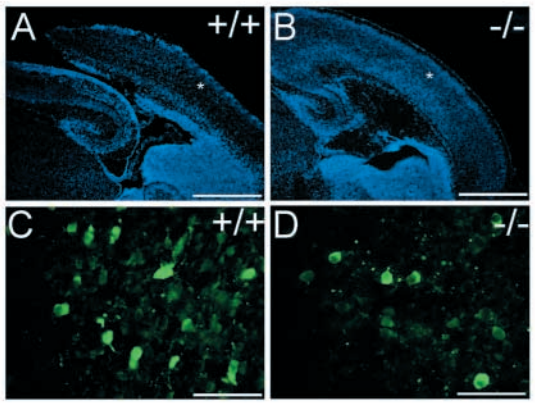



**Fig. 4** *Vax1* mutant MGE shows reduced levels of Dlx proteins and loss of Gsh1 expression. Coronal sections from control (A,C,E,G) and *Vax1* mutant (B,D,F,H) telencephalons at E13.5 analyzed by immunohistochemistry (A-F) and in situ RNA hybridization (G,H). Dlx proteins are expressed in the VZ and SVZ of wild-type SA, LGE and MGE (A,C) in a gradient similar to that of *Vax1* mRNA. In *Vax1*$^{-/-}$ ganglionic eminences, Dlx expression is reduced in the MGE and presents a shallower graded pattern in the LGE (B,D). Dlx-positive cells are reduced in the cortex of mutant mice (arrowheads in E and F). In the MGE of mutant brains, *Gsh1* expression is almost completely lost (compare G with H). LGE, lateral ganglionic eminence; LV, lateral ventricle; MGE, medial ganglionic eminence; SA, septal area; SA*, presumptive aplastic septal area; SVZ, subventricular zone; VZ, ventricular zone. Scale bars: 500 μm in A,B,G,H; 100 μm in C-F.

interaction occurring between a Dlx gene and *Vax1* in the MGE via a MGE-specific regulator. For this reason, we checked the *Gsh1* transcription factor, which is mainly expressed in the MGE and only minimally in the LGE (Corbin et al., 2003; Toresson and Campbell, 2001; Valerius et al., 1995; Yun et al., 2003). In Fig. 4G,H we show that in *Vax1*$^{-/-}$ mutants *Gsh1* expression is almost completely absent (at E13.5 its signal is minimal, slightly above noise levels, while at E16.5 is completely absent, data not shown), consistent with the possibility of a genetic cascade controlled by *Vax1*, regulating both Dlx and *Gsh1*.

### The cerebral cortex of *Vax1*$^{-/-}$ mice is severely depleted of GABAergic neurons

As mentioned in the introduction, GABAergic neurons are born in the subcortical telencephalon. Although late tangentially migrating interneurons are contributed by the LGE (Anderson et al., 1997a; Anderson et al., 2001; Jimenez et al., 2002), considering the analysis of *Nxk2.1* (Sussel et al., 1999), *Dlx1/2* (Anderson et al., 2001) and *Pax6* (Chapouton et al., 1999) mutants, it seems that most of the tangentially migrating interneurons originate from the MGE and POA/AEP. In *Vax1* mutant MGE, POA/AEP differentiation is abnormal (Figs 2, 6), with Dlx expression greatly reduced (Fig. 4A-D). Moreover, the number of Dlx-positive cells in the mutant cortex appears diminished compared with the wild-type controls (Fig. 4E,F). It is therefore logical to assume a consequent loss of GABAergic neurons in cortex. We stained P0 cerebral cortices with an anti-GABA antibody, which showed a significant reduction, but not a complete loss, in the number of GABA-positive cells (Fig. 5C,D). We accurately quantified the number of GABAergic cells in neocortices of wild-type and knockout mice, counting GABA-positive cells in three standardized rectangles selected at comparable levels along the rostrocaudal axis in three wild-type and three knockout mice. We counted at least 10 different slices for each genotype at each level. The ratios of average values (1=100%, no defect) for GABA-positive cells indicate that *Vax1* mutant mice show a general reduction of these cells (Fig. 5E), with a more pronounced loss of GABAergic neurons in the caudal cortical areas (44% reduction), an intermediate reduction in the medial cortex (38% reduction) and a less severe one in the frontal areas (30% reduction). This result is in keeping with the reduced differentiation of the MGE and the partial loss of Dlx expression, which is known to severely affect the differentiation of GABAergic interneurons (Anderson et al., 1997a). Although also not complete, in *Dlx1/2* mutants the reduction of GABAergic cells is ~75% in the neocortex and greater than 95% in the hippocampus (Anderson et al., 1997a; Marin and Rubenstein, 2003; Pleasure et al., 2000). This is a bigger reduction than that observed in *Vax1* mutants. One possible reason for this discrepancy in number is that the residual expression of Dlx still allows some interneurons to differentiate and to migrate to the pallium. To determine if this was the case, we analyzed cell migration in organotypic slice cultures (Tobet et al., 1994), placing DiI crystals in the MGE at E13.5 and culturing the slices for 2 days. As shown in Fig. 6A-D we were able to detect a significant number of cells labeled with DiI that had reached the neocortex, indicating the presence of migration from the MGE to the pallium (migratory cells have also been identified from the mutant POA in matrigel




E

| | Rostral | Medial | Caudal |
|---|---|---|---|
| wt 1 | 72 | 54 | 62 |
| ko 1 | 54 | 31 | 34 |
| | | | |
| wt 2 | 60 | 55 | 72 |
| ko 2 | 48 | 42 | 36 |
| | | | |
| wt 3 | 141 | 132 | 147 |
| ko 3 | 81 | 72 | 93 |
| | | | |
| Ratio ko/wt (1) | 75% | 57% | 55% |
| Ratio ko/wt (2) | 80% | 76% | 50% |
| Ratio ko/wt (3) | 57% | 54% | 63% |
| | | | |
| Average | 71% | 63% | 56% |
| SD | ±12 | ±12 | ±7 |

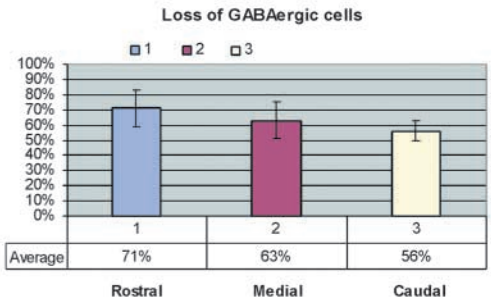



**Fig. 5.** Decreased number of GABAergic interneurons in the cortex of *Vax1* mutant mice. GABA-positive cells were counted in sagittal sections from wild-type (A,C) and mutant (B,D) P0 mice. (A,B) DAPI staining of two comparable sections from wild-type (A) and mutant (B) brains. (C,D) Immunohistochemistry with FITC-labeled anti-GABA antibody on the same sections; images are high magnifications of the regions indicated with asterisks in A and B, respectively. (E) Summary of cell counts (see text for details). Graphical representation of the results, indicating the amount of GABAergic cell decrease in *Vax1*-knockout brains (wild type is 100%). Scale bars: 500 μm in A,B; 50 μm in C,D.

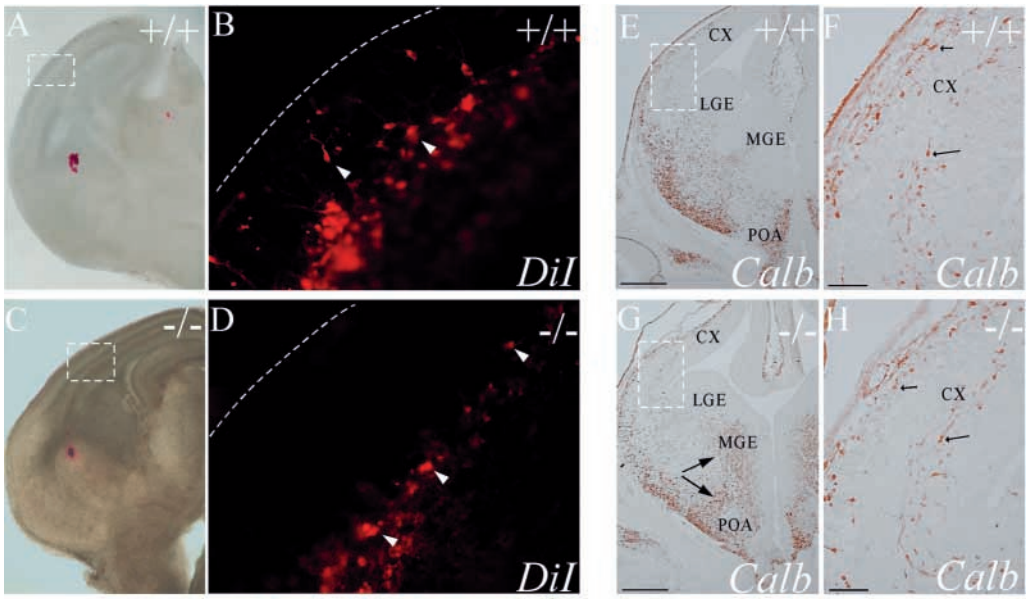



**Fig. 6.** Tangential migration from MGE and POA. (A-D) Cell tracing by DiI injection. Labeled cells from E13.5 MGE after 48 hours in culture can migrate to the cortex. (A,C) Bright-field images of a wild-type (A) and a mutant (C) slices with DiI crystals placed in the MGE. (B,D) Fluorescent images of the region boxed in A and C, respectively; white arrowheads show migrating cells in the cortex of wild-type and mutant embryos. (E-H) Immunohistochemistry for calbindin on coronal sections at E13.5 wild-type (E,F) and *Vax1*$^{-/-}$ embryos (G,H). (F,H) High-magnification images of the region boxed in E and G, respectively; black arrows indicate labeled cells in a tangential orientation. Clear leading processes are present. Arrows in G indicate an aberrant accumulation of calbindin-positive cells in the area of the MGE and POA. CX, cortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; POA, preoptic area. Scale bars: 100 μm.

cell cultures). Because the number of cells labeled by DiI is highly dependent on the quantity of dye released, it is difficult to standardize this type of experiment. To better understand this issue, we checked the expression of calbindin-positive cells at E 13.5. Calbindin is a calcium-binding protein; at this stage, it is present in all tangentially migrating GABAergic interneurons (Liu and Graybiel, 1992; Van Eden et al., 1989). In *Vax1* mutant brains, a significant number of calbindin-positive cells was readily detectable at the expected location, showing a typical migratory cell morphology (Fig. 6H). However, a large population of calbindin-positive cells was also evident, lightly stained and lingering in the area of the MGE and POA subventricular zone (Fig. 6G, arrows). It seems, therefore, that cells accumulate and differentiate abnormally, without migrating efficiently to the MZ. This defect is also observed, in a much more severe form, in the *Dlx1/2* mutants (Anderson et al., 1997b).

The spatial and temporal pattern of expression of the adhesion molecule TAG-1 (Cntn2 – Mouse Genome Informatics) in corticofugal fibers coincides with the appearance of GABAergic cells in the developing cortex; moreover, TAG-1 is also recognized to play an active role in neuronal migration (Denaxa et al., 2001; Morante-Oria et al., 2003). To determine whether the defect found in our mice was due to an impairment of the corticofugal projection system, we examined the expression of TAG-1. However, we were not able to detect any abnormality in the mutant brain.

One alternative hypothesis that could be considered is that *Vax1* mutants present a delay in GABAergic cell migration from the subcortical telencephalon to the cortex. Unfortunately, 94% of *Vax1* mutants die at birth, and only 6% survive to P20. Given the small number of viable P20 *Vax1* mutants, it was impossible to perform thorough statistics, as we did at P0. However, we performed GABA cell counts at P20 in a single mutant brain, confirming the P0 defect, a finding that probably rules out the possibility of a delayed migration of GABAergic precursor cells.

Earlier studies provided evidence in support of the hypothesis that loss of inhibitory GABAergic neurons determines neuronal hyperexcitability in cortex, eventually leading to seizures (Ribak et al., 1982; Ribak et al., 1979). More recent investigations using animal models have confirmed that impairment of GABAergic neurotransmission is implicated in epilepsy (DeLorey et al., 1998; Powell et al., 2003; Treiman, 2001), and provided genetic evidence that the $GABA_A$ receptor is directly involved in human idiopathic epilepsy (Baulac et al., 2001; Treiman, 2001). Indeed, during routine handling of *Vax1*-mutant mice we have observed spontaneous convulsions, resembling clonic seizures, possibly indicating that the loss of GABAergic neurons in these mice has severe consequences on cortical physiology.

In summary, we have shown that P0 mutants have a decreased number of cortical GABAergic interneurons; however, the MGE defective differentiation does not seem to be severe enough to explain the 30% to 44% reduction in GABAergic interneurons. One reasonable possibility to explain this discrepancy would be cell loss by apoptosis, but we were unable to detect significantly altered levels of cell death by TUNEL staining (data not shown). Other work (J. M. Soria and A. Fairén, personal communication) has found that the septum significantly contributes to cortical GABAergic interneurons. For this reason, we turned our attention to the septum of *Vax1*$^{-/-}$ mutants.

### The septal area is absent in *Vax1*$^{-/-}$ mice

The septum is part of the limbic system and is a telencephalic structure located under the corpus callosum, above the anterior commissure and between the medial walls of the lateral ventricles. Soria and Fairén describe a migratory stream of cells moving subpially from the septum to the neocortex and including both presumptive Cajal-Retzius cells and also interneurons (personal communication). *Vax1* mutants do not form the septum, presenting a midline fusion of the ventral forebrain (Bertuzzi et al., 1999b; Hallonet et al., 1999),

therefore this structure is completely absent at E 16.5 (Fig. 7A,B; Fig. 4C,D) (Hallonet et al., 1999). Owing to the lack of septum formation, (broken line in Fig. 7A,B) instead of showing the normal contoured appearance the lateral ventricles are fused together forming a single U-shaped holoprosencephalic ventricle. We will describe the generation of the septal defect in detail in the future; here, we address the consequences of the fact that the septal midline region is occupied by the large, compact mass of undifferentiated cells, which cannot differentiate and migrate correctly (Fig. 7). We wanted to verify that the GABAergic cell migration from the septum was indeed missing in the *Vax1*$^{-/-}$ brains. We cultured E13.5 brains from wild-type and *Vax1* mutants in an organotypic culture assay (Tobet et al., 1994), implanting in the developing septal area a small piece of bamboo soaked in the fluorescent tracer DiI. Fig. 7C-H shows that whereas an abundant stream of migratory cells has taken the lateral pathway of migration toward the pallium in wild-type brains, there is no sign of migration from the *Vax1* mutants. This result helps significantly in explaining the loss of cortical GABAergic cells, which could not be taken into account only by the partial loss of differentiation of MGE precursor cells.

In conclusion, *Vax1* mutants exhibit differentiation defects that reduce the production and the migration of subpallial neurons, particularly from rostroventral regions of the telencephalon.

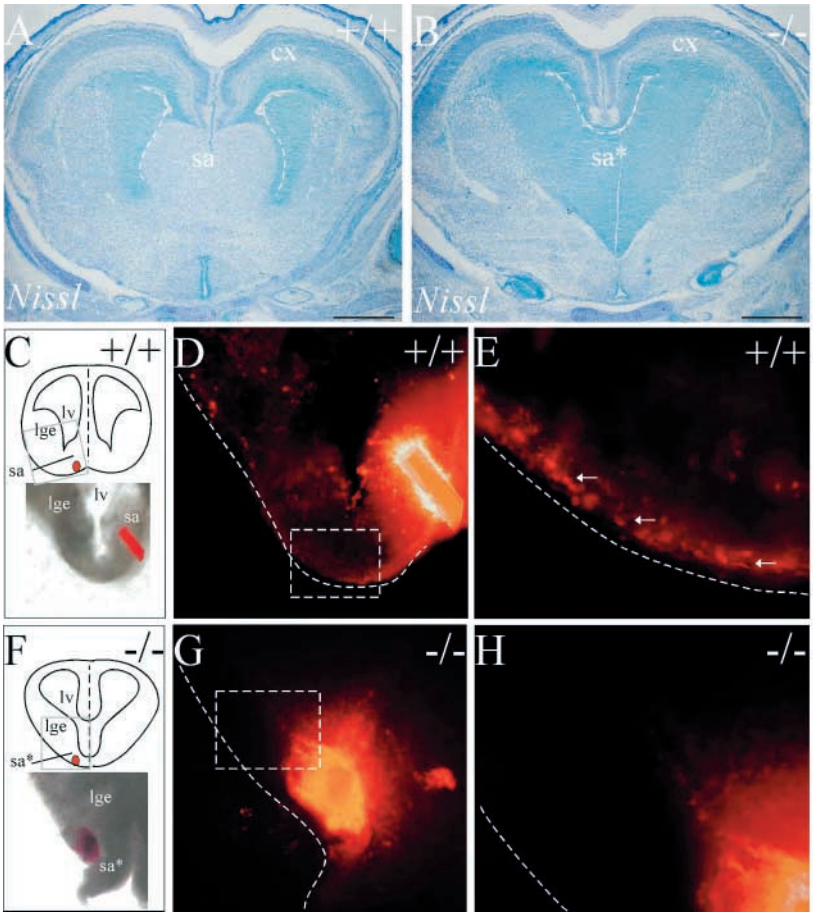



**Fig. 7.** Tangential migration from the septum is severely impaired in *Vax1*$^{-/-}$ embryos. (A,B) Nissl staining of coronal sections from wild-type and mutant brains at E16.5. There is complete loss of the septum in *Vax1* $^{-/-}$ telencephalon. (C,F) (Top) The experimental procedure. The red spots represent DiI crystals placed at E13.5 in an organotypic slice culture assay. (Bottom) Bright-field images of wild-type (C) and mutant (F) slices corresponding to those shown in the scheme. Sections are taken at comparable rostrocaudal levels. (D,E,G,H) Fluorescent images of the same slices from wild-type (D,E) and *Vax1*$^{-/-}$ (G,H) embryos; (E,H) high-magnification photographs of the region boxed in D and G, respectively. Cells that migrate along a lateral-tangential pathway are present in the wild-type slice (E, white arrows) but there is complete loss of this stream in the mutant brain (H). lv, lateral ventricle; lge, lateral ganglionic eminence; sa, septal area; sa*, presumptive septal area.

## Discussion

### *Vax1* is required for correct neuronal differentiation in the MGE, but not for dorsoventral patterning of the forebrain

*Vax1* mutants do not show apparent abnormalities in dorsoventral patterning in the forebrain (Fig. 2K-P). This was somewhat surprising to us, as we are used to thinking of Vax genes as determinants for ventral identity in the budding optic vesicle. In fact, several authors (Barbieri et al., 2002; Mui et al., 2002) have shown that *Vax2* is a fundamental regulator of ventral identity for retinal ganglion cells. Moreover, Schulte and colleagues (Schulte et al., 1999) have shown that misexpression of Vax genes in the dorsal retina leads to its ventralization. The findings presented in this work show that this is not the case in the telencephalon, which, in the absence of *Vax1*, develops the correct pallial-subpallial identity. In the case of MGE and LGE, in *Vax1*$^{-/-}$ mice we observe a lack of separation between these two structures; however, their identities seem to be correctly maintained (Fig. 2). Over the past few years, a large body of work has dealt with the role of several transcriptional regulators in the basal forebrain, identifying genes that are responsible for setting the boundaries between pallium and subpallium (Campbell, 2003; Marin and Rubenstein, 2002; Wilson and Rubenstein, 2000). Surprisingly, the Vax and Dlx gene families do not seem to fall into this category. Rather, they appear to be crucially involved in commitment decisions of neural progenitor cells generated in the basal forebrain. Loss of function of the homeobox gene *Gsh2* is characterized by an early expansion of pallial markers across the corticostriatal border (Corbin et al., 2000). It seems that the establishment of the corticostriatal border is dependent on the cross-repression of the subpallial *Gsh2* and the pallial *Pax6* genes (Toresson et al., 2000; Yun et al., 2001). In *Vax1* mutants, we detect a normal expression of both genes. Although *Pax6* is largely the complement of *Vax1*, *Gsh2* in the LGE neuroepithelium shows a widely overlapping expression pattern with that of *Vax1* (Fig. 2) (Toresson et al., 2000; Yun et al., 2001). We hypothesize that it is the maintained *Gsh2* expression in the LGE of *Vax1*$^{-/-}$ brains that contributes to the correct regionalization of pallium

and subpallium, and that *Gsh2* regulation is independent of *Vax1*. The dorsalization of the subpallium in *Gsh2* mutants is not complete. In fact, at later stages of development there is a significant self-rescue of the phenotype, indicating that other regulators are compensating for *Gsh2* loss. It is possible that *Vax1* could be one of these compensatory genes; however, we also have to take into consideration the expression of *Gsh1*, a transcription factor closely related to *Gsh2*, expressed mainly in the MGE neuroepithelium. It has been described that in *Gsh2* mutants, *Gsh1* expression is expanded into the LGE (Toresson and Campbell, 2001; Yun et al., 2003); however, the MGE in *Gsh2*$^{-/-}$, *Gsh1*$^{-/-}$ or *Gsh1/2* double mutants does not present severe alterations (Li et al., 1996; Toresson and Campbell, 2001; Toresson et al., 2000; Yun et al., 2003). Interestingly, we have discovered that in *Vax1* mutants, *Gsh1* expression in the MGE is barely detectable as early as E13.5, but differently from the *Gsh1*$^{-/-}$ mice, *Vax1* mutants present a more severe phenotype in this area, with cellular differentiation compromised and loss of the interganglionic sulcus, indicating that an interaction between *Vax1* and *Gsh1* has a synergistic effect on MGE development. Further experiments will be directed towards understanding this relationship.

### Evidence that in *Vax1*$^{-/-}$ mice reduced migration from the basal ganglia and absent migration from the septum cause a severe GABAergic cell depletion in cortex

An important feature of CNS development is that pools of multipotent precursor cells proliferate in the neuroepithelium, close to the ventricles, and migrate to their final location becoming part of highly organized circuits. Within the telencephalon, the key process of radial migration provides neurons to the cortex (Marin-Padilla, 1971; Rakic, 1974). The existence of tangentially oriented cells in the cerebral cortex has been recognized for a long time, but only recently it has been proven that the process of tangential migration from the subcortical telencephalon contributes to GABAergic interneurons of the dorsal pallium (for reviews, see Marin and Rubenstein, 2001; Marin and Rubenstein, 2003). Information obtained from several mouse mutants (see Results section) has demonstrated that the vast majority of GABAergic interneurons that modulate the function of glutamatergic pyramidal cells originate from precursor cells located in the MGE and POA/AEP. In this work, we wanted to study whether the loss of expression of *Vax1* in the VZ and SVZ of the subcortical telencephalon impaired tangential migration. We have found that migrating cells from the MGE, labeled with calbindin and *Lhx8*, in part tend to accumulate at the border of the SVZ without reaching their final destination. We propose that this defect could be mediated by Dlx genes. In support of this hypothesis, we have observed a partial loss of Dlx proteins in the MGE. Moreover, *Dlx1/2* mutants show a similar defect, in that cells differentiate abnormally and do not leave the SVZ efficiently (Anderson et al., 1997b). We have carefully estimated the GABAergic cell loss in *Vax1* mutants in the range between 30% and 44%, depending on the rostrocaudal levels considered. As the reduction of migration from the MGE was rather mild, and migratory cells have also been identified from the mutant POA in matrigel cell cultures, it was not possible to explain such a severe loss of GABAergic cells in cortex. For this reason, we turned our attention to the septum, which we knew was completely missing in *Vax1* mutants. Soria and Fairén (personal communication) have found that the septal area is a source of GABAergic interneurons that are Arx, PSA-NCAM and calbindin positive, which migrate subpially to the neocortex. The stream of cells labeled by DiI placed in the septal area at E13.5 is conspicuous in the wild type and completely absent in *Vax1* mutants (Fig. 7G,H). Indeed, the complete loss of septal GABAergic interneurons and the partial reduction of the MGE and POA/AEP pools, can account for the numbers of total GABAergic cell loss. Unfortunately, the number of cells labeled with DiI is highly dependent on the quantity and precise location of dye application, making quantitative comparisons between different samples difficult using this method. However, a rough estimate suggests that the loss of cortical GABAergic cells can be explained by the basal ganglia and septum defects combined.

We find it interesting that the conspicuous cortical GABAergic cell loss in *Vax1*$^{-/-}$ mutants could be the cause, at a physiological level, of the spontaneous and induced convulsions that resemble clonic seizures, which we have observed in the few surviving P20 mutant mice.

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