Gsh2 and Pax6 play complementary roles in dorsoventral patterning of the mammalian telencephalon

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SUMMARY

The telencephalon has two major subdivisions, the pallium and subpallium. The pallium, which primarily consists of glutamatergic cortical structures, expresses dorsal molecular markers, whereas the subpallium, which primarily consists of the GABAergic basal ganglia, expresses ventral molecular markers. Here, we present evidence that the progenitor and postmitotic cells flanking the pallial/subpallial boundary (PSB) in the embryonic mouse can be subdivided into multiple regions that express unique combinations of transcription factors. The domains that immediately flank the PSB are the ventral pallium (VP) and the dorsal lateral ganglionic eminence (dLGE). The early expression of the Pax6 and Gsh2 homeobox transcription factors overlaps in the region of the dLGE. Analyses of mice that lack functional alleles of either Gsh2 or Pax6 demonstrate that these genes have complementary roles in patterning the primordia flanking the PSB. In the Gsh2 mutants, the dLGE is respecified into a VP-like structure, whereas in the Pax6 mutants the VP is respecified into a dLGE-like structure. The role of Pax6 in dorsalizing the telencephalon is similar to its role in the spinal cord, supporting the hypothesis that some dorsoventral patterning mechanisms are used at all axial levels of the central nervous system.

Key words: Gsh2, Pax6, Telencephalon, Dorsoventral patterning, LGE, Ventral pallium, Striatum, Pallial/subpallial boundary, CNS development, Mouse

INTRODUCTION

In the last decade, significant inroads have been made into elucidating mechanisms that regulate dorsoventral (D/V) patterning in the mammalian spinal cord (Ericson et al., 1997a; Lee and Jessell, 1999). Several lines of evidence suggest that D/V patterning of the telencephalon involve similar mechanisms (Wilson and Rubenstein, 2001). For instance, sonic hedgehog has a role in ventral patterning of the spinal cord and telencephalon through the induction of the Nkx family of homeobox genes (Chiang et al., 1996; Sussel et al., 1999; Briscoe et al., 1999; Sander et al., 2000). Likewise, WNT and TGFβ signaling is implicated in regulating the development of dorsal structures in the spinal cord and telencephalon (Lee and Jessell, 1999; Solloway and Robertson, 1999; Galceran et al., 2000; Lee et al., 2000). While some progress has been made in understanding the mechanisms that pattern spinal cord subdivisions located between the most dorsal and ventral tissues (Pierani et al., 1999), little is known about the patterning of intermediate regions of the telencephalon.

The telencephalic vesicles are bilateral evaginations from the rostral forebrain that contain two principal subdivisions: pallium and subpallium. The pallium, or telencephalic roof, primarily consists of laminar structures that contain glutamatergic projection neurons, whereas the subpallium primarily consists of non-laminar aggregates of GABAergic projection neurons. The discovery of regulatory genes that are expressed in regionally restricted patterns within the developing telencephalon has renewed efforts to elucidate the organization of telencephalic subdivisions. Two groups have demonstrated conserved telencephalic expression patterns of homeobox and other transcription factors across vertebrate species, suggesting that the embryonic telencephalon has a common basic organization (Smith-Fernandez et al., 1998; Puelles et al., 2000). Puelles and co-workers have proposed that the pallium consists of four major subdivisions that extend from dorsocaudal to ventrorostral. They are the medial, dorsal, lateral and ventral pallium (MP, DP, LP and VP, respectively; see Figs 1C, 2Ad). The medial pallium (MP) abuts the choroidplexus and consists of the hippocampal complex; the dorsal pallium (DP) consists of the isocortex; the lateral pallium (LP) consists principally of the olfactory cortex and parts of the amygdala; and the ventral pallium (VP) consists of the caudal and amygdalar components. This model proposes that the subpallium is made up of three primary regions: the striatum, pallidum and rostral telencephalic stalk. Most of the striatum and pallidum develop from transient embryonic structures called the lateral and medial ganglionic eminences (LGE and MGE) (reviewed by Puelles et al., 2000; Marín and
Accordingly, the pallial-subpallial boundary (PSB) corresponds to the boundary between the VP and LGE progenitor zones. The PSB also marks the limit where there are profound changes in molecular, physiological and hodological properties (Fishell et al., 1993; Stoykova et al., 1996; Stoykova et al., 1997; Anderson et al., 1997a; Anderson et al., 1997b; Smith-Fernandez et al., 1998; Puelles et al., 2000).

The present study aims to elucidate some of the genetic and molecular mechanisms that regulate a major choice in telencephalic regional specification: whether to form pallial or subpallial neuroepithelial progenitors. We have identified molecular markers that distinguish the telencephalic primordia flanking the PSB, including the VP and subdivisions within the LGE (dLGE and vLGE). Using these markers, we have studied the phenotypes of mice that have mutations in Pax6 and Gsh2, homeobox transcription factor genes that are strongly expressed in progenitors around the PSB.

Pax6 is most strongly expressed in the pallium, and Pax6 mutants express markers of the subpallium (Dlx1) in cortical regions (Stoykova et al., 1996; Stoykova et al., 1997). It is postulated that the primary telencephalic defect in Pax6 mutants lies in the failure to establish/maintain the ‘cortico-striatal boundary’ (Stoykova et al., 1996; Stoykova et al., 1997; Chapouton et al., 1999). Recently, Chapouton and colleagues reported evidence for increased tangential migration of subpallial cells into the cortex. This result was used to argue that the increased migration in Pax6 mutants is due to altered properties of the ‘cortico-striatal boundary’ (Chapouton et al., 1999). The results described herein lead us to propose an additional mechanism that leads to the dorsal expansion of subpallial gene expression. We suggest that Pax6 is required for dorsoventral regional specification of the pallium (particularly of the VP), and in its absence, pallial progenitors take on subpallial properties (i.e. are ventralized).

Gsh1 and Gsh2 are homeobox genes that are expressed in subpallial progenitors (Valerius et al., 1995; Hsieh-Li et al., 1995). Their expression patterns largely overlap, which suggests functional redundancy. However, Gsh2 expression extends beyond the dorsal limit of Gsh1 in the LGE, implying that Gsh2 has unique functions in the dorsal LGE. Gsh1 mutants do not show an obvious telencephalic phenotype (Li et al., 1996), while Gsh2 mutants have a hypoplastic LGE (Szusick et al., 1997). The report describing the Gsh2 mutant did not explore the mechanism(s) underlying this defect. Here, we present evidence that in the Gsh2 mutant the dLGE is mis-specified to develop into a VP-like tissue. Therefore, these studies provide evidence that Pax6 and Gsh2 have complementary functions in patterning the progenitors that flank the PSB by regulating opposing genetic programs.

MATERIALS AND METHODS

Pax6 and Gsh2 mutants

Mice carrying the Seyneo mutant allele of Pax6 (Hill et al., 1991) were mated to CD1 wild type animals to maintain the strain. Genotyping of the Pax6 mutants was performed by PCR as described by Hill et al. (Hill et al., 1991). The Gsh2 mutant was also previously generated (Szusick et al., 1997). Gsh2 heterozygous animals were genotyped by PCR for the neomycin gene (Qiu et al., 1995) and the homozygous embryos were identified by the lack of wild-type Gsh2 PCR product using the following primers: 5′-ATG GAT GTG TTG GTG TAG ACT GGG TTC TGG-3′; 5′-TGC TTC ACG CGG TTC TGA AAC CAT ATT-3′.

The morning the vaginal plugs were found was considered embryonic day (E) 0.5. Embryos from both mutant backgrounds were collected at E10.5, E11.5, E12.5, E14.5 and E18.5. The embryos were fixed overnight in 4% paraformaldehyde, cryoprotected in sucrose, embedded in OCT (Tissue-Tek) and sectioned using a cryostat. 10 µm sections were collected on separate slides.

In situ RNA hybridization

cDNA plasmids used for in situ hybridization analysis were gifts from the following people: Brian Condie (GAD67), Chris Evans (μ-opioid receptor), Charles Gerfen (dopamine receptor 2 (Drd2) and substance P (Tac1)), Peter Gruss (Six3, Pax6), Francois Guillemot (Mash1, Ngn1, Ngn2), Thomas Jessell (Er81), Vassilis Pachnis (Lhx6); Steve Poter (Gsh1 and Gsh2), Frank Ruddle (Dbx1) and Heiner Westphal (Lhx). Photographs were taken using darkfield optics on an Olympus SZH10 microscope. The pseudo-colored pictures were generated by importing digital images into Photoshop 5.5 through either the green or red channels; the images were then overlaid. For this purpose, only adjacent sections were used.

Immunohistochemistry

Anti-BrdU antibody was purchased from Harlan and used at 1:10 dilution. Before the anti-BrdU primary antibody was added to sections, the antigen was unmasked by 2N HCl treatment for 20 minutes at room temperature. Production and characterization of the DLX2 antibody is described elsewhere (Porteus et al., 1994). For DLX2/PCNA double labeling (DLX2, rabbit polyclonal at 1:200 dilution; PCNA (Novocastra), mouse monoclonal at 1:2000 dilution), the sections were first treated in boiling 10 mM sodium citrate for approx. 5 seconds. Phosphorylated Histone-III antibody was purchased from Upstate Biochemicals and used at 1:2000 dilution. The sections were blocked in 5% NGS/PBS/0.1%TritonX-100 for 1 hour at room temperature. Primary antibodies were diluted in the blocking solution and incubated overnight at 4°C. The sections were rinsed in PBS, then incubated with secondary antibodies at 1:200 dilution for 1 hour at room temperature, then rinsed in PBS and finally mounted with Vectashield (Vector Labs). The secondary antibodies were conjugated with either Alexa-488 or Alexa-596 (Molecular Probes). The fluorescent images were photographed on a Nikon Optiphot 2 microscope. TUNEL assay was performed according to the manufacturer’s instruction (Intergen, #S7100).

RESULTS

Unique combinations of transcription factor expression define subdivisions around the pallial/subpallial boundary

The pallial/subpallial boundary (PSB) is characterized by an abrupt change in histological and molecular properties (reviewed by Puelles et al., 2000). As early as E10.5 in the mouse, this boundary can be defined by the restricted expression of transcription factors in progenitor cells in the pallium (e.g. Tbr2 (Eomes – Mouse Genome Informatics) Ngn2 (Atoh4 – Mouse Genome Informatics)) and in the subpallium (e.g. Gsh2, Mash1 (Ascl1 – Mouse Genome Informatics) and Dlx2) (Fig. 1A and data not shown; Belfone et al., 1999; Eisenstat et al., 1999; Fode
subdivision that we designate as the dorsal LGE (dLGE). In contrast, the ventral LGE (vLGE) expresses Gsh1 and has lower expression of Pax6, Gsh2, Dbx2 and Mash1 at this age (Fig. 1Aa-e,e and data not shown). By E11.5, expression of Er81 (Ersp81 – Mouse Genome Informatics) in the VZ of the dLGE also distinguished this progenitor zone from the vLGE (see Figs 1Bd; 6Bc). These results suggest that the dLGE and vLGE are distinct progenitor subdivisions within the LGE that may give rise to distinct populations of postmitotic neurons (see below).

Neurons in the mantle zone that are derived from pallial and subpallial progenitors express distinct sets of molecules. For instance, Tbr1 was expressed in most postmitotic pallial neurons (Fig. 2Ae,Be) whereas glutamic acid decarboxylase 67 (Gad67; Gad1 – Mouse Genome Informatics) was expressed in most postmitotic subpallial cells (Fig. 2Aa,Ba). Within the pallium, postmitotic cells derived from pallial subdivisions close to the PSB, the LP and VP, can be distinguished from the DP-derived cells by the combinations of genes that they express. We propose that the mantle cells in the VP express Lhx2 (Porter et al., 1997; Fig. 2Ag,Bg,h), whereas mantle cells in the LP express Emx1 (Figs 1Ah; 2Af,Bf,h; Puuelles et al., 2000). Note that Lhx2 and Emx1 were also expressed in subpial cells in other pallial regions where their expression

**Fig. 1.** Expression of transcription factors in neuroepithelial progenitors. (A) In situ RNA hybridization of telencephalic coronal sections (only the right side is shown) from E10.5 wild-type mice. The top row shows expression of subpallial markers: Gsh2 (a), Gsh1 (b) and Mash1 (c). The bottom row shows expression of genes that are primarily pallial markers: Pax6 (e), Tbr2 (f), Ngn2 (g) and Emx1 (h). The location of the pallial-subpallial boundary (PSB) is indicated with arrowheads. Strong Pax6 expression is also observed in a small region of the subpallium where it overlaps with strong Gsh2 expression in the dorsal LGE (dLGE). This is clearly seen in (d), in which images of Pax6 (green) and Gsh2 (red) expression from adjacent sections are superimposed using Adobe Photoshop 5.5. Overlapping Pax6/Gsh2 expression domain appears yellow. (B) Expression of Dbx1 (a), Tbr2 (b), Ngn2 (c) and Er81 (d) at E11.5. Comparison of Tbr2/Dbx1 (e) and Tbr2/Gsh2 (f) expression on adjacent sections show that Dbx1 expression lies in the ventral-most part of the pallium that we define as the VP. Arrowheads point to PSB. (e) Tbr2 (green) and Dbx1 (red) have overlapping expression. (f) Tbr2 (green) and Gsh2 (red) expressions abut at the PSB. (C) On the left is a schematic drawing of a coronal section through the right telencephalic vesicle of a E11.5 mouse showing, in different colors, the hypothesized D/V progenitor zone subdivisions. A bar code representing these subdivisions is in the middle. On the right are bar graphs representing gene expression patterns. Red indicates subpallial expression; blue represents pallial expression. Different levels of expression are indicated by different shading.

A horizontal broken line represents the PSB. AEP, anterior entopeduncular area; DP, dorsal pallium; dLGE, dorsal lateral ganglionic eminence; vLGE, ventral ganglionic eminence; LP, lateral pallial mantle; MGE, medial ganglionic eminence; MP, medial pallium; PD, palladium; POa, preoptic area; PSB, pallial-subpallial boundary; St, striatum; VP, ventral pallium.
overlapped (Fig. 2Bg,h). Streams of Lhx2- and Emx1-positive postmitotic cells were continuous with LP and VP progenitors, respectively, at E11.5, E12.5 and E14.5 (Figs 1Ab; 2Af-h,Bf-h; 3Bc; 4Ae,f). The topographic relationship of these streams to the VP and LP progenitor zones suggests that Lhx2 and Emx1 are useful markers for identifying postmitotic cells from the VP and LP, respectively.

dLGE mantle cells express an unique combination of molecules: Gad67, Pax6, Six3 (Fig. 2Aa,b,c,h,Ba-c and data not shown; Oliver et al., 1995; Puelles et al., 2000). Streams of Pax6- and Six3-positive postmitotic cells were continuous with the dLGE progenitor zone E11.5, E12.5 and E14.5 (Figs 2Ab,c,h,Bb,c; 3Bf; see Figs 6Bf; 7Ab). In contrast, the mantle of the vLGE expressed Gad67 (Fig. 2Aa,Ba), and very few scattered Pax6-positive cells (Figs 2Ab,h,Bb; 4Ab; see Fig. 6Bf).

The overlapping expression of Gsh2 and Pax6 in the lateral telencephalon (Fig. 1Ad) suggests that these genes may have important roles in regulating the PSB and the development of the subdivisions that flank it. To test this hypothesis, we analyzed the phenotypes of mice that were homozygous for loss-of-function alleles of Gsh2 and Pax6.

dLGE progenitor cells express markers of the VP during early telencephalic development in Gsh2 mutants

Gsh2 mutants were reported to have a small LGE at E12.5 (Szucsik et al., 1997). To elucidate the basis for this phenotype, we studied molecular properties of the LGE at earlier stages in the Gsh2 mutants. At E10.5 there were subtle molecular changes in the LGE progenitor zone. Expression of Mash1 in the dLGE was reduced (Fig. 3Ae,e'), whereas expression of Ngn2 expanded ventrally into the LGE (Fig. 3Ac,c'). Pax6 and Tbr2 expression are similarly affected, while Emx1 expression appears grossly normal (Fig. 3Aa,a',b,b',f,f').

By E11.5, the defects in the Gsh2 mutants were more apparent. The LGE was smaller than in wild-type littersmates, and pallial marker expression (Dbx1 and Tbr2) extended through the LGE progenitor zone (Fig. 3Ba,a',b,b'). Strikingly, the expression of the VP marker, Dbx1, spread throughout most of the subpallium (Fig. 3Ba,a'). However, the ventral boundary of Emx1 expression (which marks the ventral limit of the LP) appeared grossly normal (both in the VZ and mantle, data not shown).
shown). Consistent with the hypothesis that the progenitor population in the dLGE is respecified into VP-like progenitors, the number of *Lhx2*-positive postmitotic neurons was increased as early as E11.5 (indicated by arrows in Fig. 3Bi,i’).

At E11.5, the expression of subpallial markers (*Mash1*, *Dlx2* and *Six3*) in the LGE was greatly reduced in the *Gsh2* mutants and their expression was limited to vLGE (Fig. 3Bd,d’,f,f’ and not shown). *Gsh1* expression appears slightly stronger than in normal mice, with its dorsal boundary in the vLGE intact (Fig. 3Be,e’). Together with the expansion of the VP marker expression, these observations suggest that the dLGE is respecified into a VP-like neuroepithelium. Consistent with this idea, *Six3* and *Pax6* expression in the SVZ/mantle of dLGE is greatly reduced (see arrows in Fig. 3Bf,f’ and data not shown).

Between E12.5 and E14.5 the molecular defects in the LGE become less obvious. At E14.5 pallial gene expression (e.g. *Ngn2*, Fig. 4Ah,‘h’) recedes dorsally, coinciding with the position of ectopic *Gsh1* expression (arrow, Fig. 4Ai,i’). The fact that ectopic *Gsh1* expression temporally and spatially correlates with the restoration of LGE and striatal properties (e.g. *Mash1*, *GAD67* and µ-opioid receptor expression, Figs 4a,a’,c,c’,g,g’; 5b,b’,c,c’) suggests that *Gsh1* may compensate for *Gsh2* function during later developmental stages.

In sum, the molecular defects in progenitor cells suggest that there is a transient (from ~E10.5-E13.5) ventral expansion of VP properties and a complementary loss of dLGE properties in *Gsh2* mutants. Consistent with the model that the dLGE acquires properties of the VP, this region of the dLGE lacks a secondary proliferative population (subventricular zone, SVZ) (Fig. 3Bi,i’), which at this age is characteristic of the LGE and not the pallium (Sheth et al., 1997). The lack of the SVZ cells at early ages may explain why the LGE is smaller in *Gsh2* mutants, especially since we did not detect excessive cell death in the *Gsh2* mutants (Fig. 3Bg,g’). To further test the model that the dLGE is respecified in *Gsh2* mutants, we studied the molecular identities of postmitotic cells that are produced from

**Fig. 3.** Pallial gene expression in neuroepithelial progenitors extends ventrally in *Gsh2* mutants. (A) Expression of transcription factors in neuroepithelial progenitors shown by in situ RNA hybridization of telencephalic coronal sections (only one hemisphere shown) from E10.5 wild-type (wt, left panel) and *Gsh2* mutant (−/−, right panel) mice. Expression of *Pax6* (a,a’), *Tbr2* (b,b’) and *Ngn2* (c,c’) show ventral expansion (compare arrows). Expression of *Gsh2* (d,d’) can not be detected in the *Gsh2* mutant using a probe that overlaps with a region of the transcription unit that was not deleted. In the mutants, strong expression of *Mash1* (e,e’) slightly recedes ventrally (compare arrows). Expression of *Emx1* (f,f’) does not appreciably change. (B) Expression at E11.5. In the mutants *Dbx1*, *Tbr2*, *Lhx2* extends ventrally whereas *Mash1* and *Six3* recede ventrally (a-d’,f,f’). *Gsh1* expression may extend slightly more dorsally (e,e’). Note that *Gsh2* mutants show increased numbers of *Lhx2*+ VP mantle cells (c,c’, arrows), and show a loss of *Six3* expression in the dLGE mantle zone (f,f’, arrow). The yellow lines in panels (f,f’) represent the dorsal boundary of *Nkx2.1* expression. TUNEL analysis shows no changes in apoptosis between the wild-type and mutant tissues (g,g’). An M-phase cell cycle marker (phosphorylated histone III) shows no major changes in the mitotic index in the VZ and a slight decrease in the SVZ at E11.5 (h,h’). A 30 minute pulse of BrdU at E12.5 reveals a reduction in the number of proliferating cells in the SVZ in the mutant LGE (i,i’, arrow). (C) Bar graph schema summarizing *Gsh2*−/− progenitor cell defects at E11.5. (Left) Telencephalic progenitor domains in wt and *Gsh2* mutants (−/−). We interpret that the mutant lacks the dLGE and has an expansion of the VP. Stippling of the vLGE reflects its abnormal properties. (Right) Interpretation of gene expression patterns in the wt and *Gsh2* mutants. Blue is pallial gene expression; red is subpallial gene expression. See legend to Fig. 1 for abbreviations.
the progenitor zones flanking the PSB between E11.5 and E18.5.

**Evidence that respecification of dLGE progenitors leads to an expansion of VP and a reduction of dLGE postmitotic neurons in Gsh2 mutants**

In Gsh2 mutants, expression of Tbr1, a general pallial mantle marker, expanded ventrally, while expression of Gad67, a general subpallial mantle marker, retracted correspondingly (Fig. 4Aa,a’,B). To characterize the molecular properties of the supernumerous pallial cells in the Gsh2 mutants, we examined the expression of Emx1 and Lhx2, markers of postmitotic cells in the LP and VP, respectively. At E11.5 and E14.5, the domain of Lhx2 expression in the VP mantle was clearly expanded in the Gsh2 mutants (Figs 3Bc,c and 4Af,f). However, there is no detectable expansion of Emx1-positive LP cells (Fig. 4Ae,e’).

Correspondingly, Gsh2 mutants had fewer dLGE-type mantle cells in the subpallium, as shown by the greatly reduced number of Pax6- and Six3-expressing cells at E14.5 (Figs 4Ab,b’ and not shown). However, other LGE mantle properties (Gerfen and Wilson, 1996) are preserved, albeit in a smaller tissue. This includes the expression of µ-opioid receptor, dopamine receptor 2 (Drd2) and substance P (Tac1) at E14.5 and E18.5 (Figs 4Ac,c’; 5c,c’ and not shown). Together, these observations suggest that Gsh2 is mainly required to produce dLGE-derived cells.

The fact that LGE development is severely affected at early stages led us to investigate the development of the olfactory bulb (OB), which obtains a large fraction of its GABAergic interneurons from the region of the LGE (Goldman and Luskin, 1998; Bulfone et al., 1998) and L. Long and J. L. R. R., unpublished observations). At E14.5, Gsh2 mutants had a severe reduction in the number of Gad67- and Dlx1-positive neurons (Fig. 5Aa,a’,b,b’). In addition, the outer ring of Pax6-positive cells were undetectable in the Gsh2 mutant OB (Fig. 5Ad,d’). Interestingly, Mash1 expression in the VZ of the Gsh2 mutant OB appeared normal (Fig. 5Ac,c’). By E18.5, the mutant OB contains a large number of cells that expressed Gad67 and Dlx1 (Fig. 5Ba,a’ and not shown). The recovery of OB interneuronal marker expression at E18.5 correlates with the substantial recovery of the LGE phenotype by E18.5 (Fig. 5b,b’c,c’).

MGE morphogenesis and differentiation appeared grossly normal in the Gsh2 mutants. For example, Nkx2.1a homeobox gene essential for MGE specification (Susse1 et al., 1999), was expressed normally in Gsh2 mutants (data not shown). Its dorsal limit is indicated by the yellow lines in Fig. 3Bf,f’. The MGE produces cells that tangentially migrate into the striatum and cortex (Lavdas et al., 1999; Wicherte et al., 1999; Sussel et al., 1999; Martin et al., 2000; Anderson et al., 2001). Lhx6 expression, which marks some of those migrating neurons, appeared normal in Gsh2 mutants, supporting the idea that Gsh2 function is not essential for many aspects of MGE development (data not shown). Therefore, aside from the ectopic expression of Dbx1 in the MGE (Fig. 3Ba,a’), we have found no other defects in this structure in Gsh2 mutants.
In sum, Gsh2 appears to function in dLGE development in part by repressing the expression of genes that are strongly expressed in VP progenitors (Dbx1, Ngn2 and Tbr2) and by defining dLGE identity with other subpallial transcription factors (Mash1, Er81, Dlx2) and with Pax6. In the next section, we describe experiments that investigate the function of Pax6 in regulating regionalization around the PSB, and provide evidence that Pax6 and Gsh2 have complementary functions.

Evidence that pallial progenitor cells take on subpallial properties in Pax6 mutants

Previous studies of Pax6 mutants have demonstrated that the pallium expresses subpallial genes (Stoykova et al., 1996; Stoykova et al., 1997). These studies suggest that the expression of subpallial genes in the cortex is due to a disruption of the ’cortico-strial boundary’, leading to increased tangential migration of subpallial cells (Stoykova et al., 1996; Stoykova et al., 1997; Chapouton et al., 2000). Here we provide evidence that respecification of pallial progenitors may also lead to expression of subpallial genes in the Pax6 mutant cortex.

Analysis of Pax6 mutants at E10.5 showed a slight reduction of Ngn2 expression in pallial progenitor cells near the PSB (Fig. 6Af,f'). Correspondingly, there was a subtle dorsal expansion of Gsh2 and Mash1 expression (Fig. 6Aa,a',b,b'). The fact that Emx1 expression appears normal (Fig. 6Ae,e') suggests that the pallial defect primarily affects the VP and had less of an effect on the LP and DP at this age. Supporting this idea, the gap between the Emx1-positive LP and Gsh2-positive dLGE, which corresponds to VP, is lost in Pax6 mutants (Fig. 6Ac,c'). However, the mutant Pax6 RNA, which continues to be transcribed in these animals, exhibits uniformly high levels of expression in the MP, DP, LP and VP, unlike the graded expression found in wild-type embryos (Fig. 6Ad,d') suggesting a more general defect throughout the pallium.

By E11.5, pallial defects near the PSB were more apparent in the Pax6 mutants. Tbr2 and Ngn2 expression in the progenitor zone was markedly reduced in the region of the VP and LP while their expression in the DP was less severely affected (Fig. 6Ba,a' and not shown). Furthermore, Dlx1 expression, which marks the VP, was undetectable (Fig. 6Bb,b'). At the same time, there was a dorsal expansion of subpallial gene expression, particularly into the region of the VP (see Gsh2, Mash1, Brn4 (Pou3f4 - Mouse Genome Informatics), Er81, Dlx2 in Fig. 6Bc,c',d,d',e,e' and data not shown). Cells in the pallium that ectopically express the subpallial marker DLX2 were mitotically active, based on their expression of the proliferative cell nuclear antigen (PCNA) (Fig.7B). Normally, Dlx2-positive tangentially migrating subpallial cells are not mitotically active (Anderson et al., 2001). This result, together with the reduction of Ngn1, Ngn2, Tbr1 and Dlx1 (pallial marker) expression (in Fig. 6Ba,a', 6Bb,b', 7Ah,h' and not shown), suggests that at least some of the cells ectopically expressing subpallial markers are mis-specified pallial progenitors. The expansion of the dorsal limit, but not the ventral limit, of ER81 (a dLGE marker) in progenitor cells (Fig. 6Bc,c') suggests that the VP takes on properties of the dLGE. On the other hand, the lack of ectopic Six3 expression in the ventricular zone of the VP (data not shown) shows that not all subpallial properties move dorsally.

Together, these data demonstrate that in the Pax6 mutants most areas of the pallium were abnormal, that pallial subdivisions closest to the PSB were the most severely affected, and that the VP may be partially respecified to express some molecular characteristics of the dLGE. These findings at E10.5-E12.5 are not caused by major changes in proliferation or cell death (Fig. 6Bg,g',h,h'). To test the respecification model, we studied the molecular identities of the postmitotic cells that are produced from the progenitor zones flanking the PSB in Pax6 mutants.
Figure 6. Subpallial gene expression in neuroepithelial progenitors extends dorsally in Pax6 mutants. (A) At E10.5, the dorsal shift of ventral gene expression is already noticeable. Expression of transcription factors in neuroepithelial progenitors shown by in situ RNA hybridization of telencephalic coronal sections (only one hemisphere is shown) from E10.5 wild-type (wt, left panel) and Pax6 mutant (−/−, right panel) mice. The top row shows expression of subpallial markers (Gsh2 (a,a’) and Mash1 (b,b’)); note the subtle dorsal expansion in their expression (arrows). Parts c and c’ show the superposition of images of Gsh2 (red) and Emx1 (green) expression from adjacent sections; the left arrow shows the reduction in the VP. The bottom row shows expression of pallial markers (Pax6 (d,d’), Emx1 (e,e’) and Ngn2 (f,f’)). Pax6 transcripts are produced from the mutant gene, and continue to show lower expression in much of the LGE (arrows). Emx1 expression does not change significantly, whereas Ngn2 expression recedes dorsally (arrows). (B) At E11.5, the molecular phenotypes are much more pronounced. Note the decrease in pallial expression of Ngn2 (a,a’, arrows), and the loss of Dbx1 (b,b’) expression in the VP (arrows). Different subpallial markers expand into the pallium to different extents. Er81 (arrows in c,c’) expands primarily into the region of the VP, Gsh2 (arrows in d,d’) expands more extensively (into ~VP and LP) and Mash1 (arrows in e,e’) expression increases as a gradient from the VP through the DP. There is a large increase in the number of Pax6-positive (f,f’) postmitotic dLGE cells (arrows). TUNEL assay shows no significant changes in apoptosis between the wild-type and Pax6 mutants (g,g’). An M-phase marker (phosphorylated histone III) reveals no major change in mitotic index in the lateral telencephalon in the Pax6 mutant (compare h with h’). (C) Bar graph summarizing Pax6−/− progenitor cell defects at E11.5. (Left) Telencephalic progenitor domains in wt and Pax6 mutants (−/−). The mutant is interpreted as having a severe reduction in the VP and a reduction in the LP. The entire pallium also expresses some molecular features of the subpallium (e.g. high Mash1); this is indicated by stippling of the LP and DP. (Right) Interpretation of gene expression patterns in the wt and Pax6 mutants. See legend to Fig. 1 for abbreviations.

**Ventral respecification of pallial progenitors is correlated with an expansion of subpallial neurons and a reduction of pallial neurons in Pax6 mutants**

Consistent with the molecular changes in the progenitor zones, markers of the VP mantle were the most severely affected in the Pax6 mutants. At E11.5, Tbr1 and Lhx2 expression in postmitotic cells was greatly reduced (not shown). By E14.5, Tbr1 and Lhx2 expression in the mantle of the VP was almost entirely eliminated (Fig. 7Ad,f,f’). LP postmitotic markers (Emx1) continued to label a laminar structure in the position of the olfactory cortex, but showed that this structure was hypoplastic (Fig. 7Ae,e’ and not shown), dLGE mantle markers (Pax6, Six3, Gad67) expanded into the regions of the VP and perhaps the LP mantle (Fig. 6Bf,f’, 7Aa,a’,b,b’, and not shown). In addition, defects in the DP were demonstrated by the ectopic expression of some dLGE SVZ markers (ER81, Dlx2, Mash1, and GAD67) and the abnormally thin Tbr1/Emx1-positive cortical plate (Fig. 7Aa’a’,g,g’,i,i’, and not shown). Similar phenotypes are present at E18.5 in the Pax6 mutants (not shown). Together, these results suggest that Pax6 is required for patterning of the VP. In the absence of Pax6, the VP progenitor zone becomes ventralized and there is a corresponding reduction/lack of VP mantle and an expansion of the LGE mantle.

**DISCUSSION**

Herein, we report that the Pax6 and Gsh2 homeobox genes have complementary roles in patterning the telencephalic...
subdivisions flanking the PSB. These results have implications for elucidating the molecular mechanisms that regulate dorsoventral regional specification in the telencephalon, and for gaining insights into the mechanisms that underlie CNS evolution.

**Evidence for molecularly distinct progenitor subdivisions flanking the pallial-subpallial boundary (PSB)**

Our results support the idea that there is an evolutionary conservation in the expression of homologous transcription factors that define pallial and subpallial subdivisions (Smith-Fernandez et al., 1998; Puelles et al., 2000). According to the model of Puelles et al., the pallium has four major progenitor zones: MP, DP, LP, and VP. Previously, VP progenitors were defined by their lack of *Emx1* expression and their strong expression of *Pax6*, and VP neurons were defined by their lack of *Emx1* expression and their expression of *Tbr1* (Puelles et al., 2000). Here we show that *Dbx1* expression marks VP progenitor cells (Figs 1Ba, 3Ba, 6Bb) and that *Lhx2* expression marks the VP mantle cells (Figs 2Ag,h,Bg,h; 3Bc). We wish to point out, that in the absence of fate mapping data, our conclusions regarding the relationships of progenitors and their derivatives are provisional. However, the analysis of *Gsh2* and *Pax6* mutants provides genetic evidence that supports our proposed, molecularly-defined subdivisions.

In addition to the previously proposed subpallial primordia (Fig. 1C; Puelles et al., 2000), we provide evidence that the LGE domain is further subdivided into dorsal (dLGE) and ventral (vLGE) progenitor zones. Although both of these zones have some common molecular properties (e.g. express *Gad67*; Fig. 2Aa,Ba), they can be distinguished by the expression of distinct combinations of transcription factors. Progenitors in the dLGE express *Pax6*high, *Gsh2*high, and *ER81*, whereas progenitors in the vLGE express *Gsh1*, *Pax6*low, *Gsh2*low (Fig. 1Aa-e,Bd). Consistent with this model is the observation that postmitotic cells that appear to be produced by the dLGE can be distinguished from those produced by the vLGE by their expression of *Pax6* and *Six3* (Figs 2Ab,c,h,Bb,c; 3Bf; 6Bf; 7Ab). While vLGE-derived cells contribute to the striatum, we are uncertain of the fates of dLGE cells. Our previous study suggested that these postmitotic *Pax6*-positive cells in the mouse contribute to the nucleus accumbens, striatum (laterostriatal stripe), olfactory tubercle, piriform cortex and basal amygdala (Puelles et al., 2000), consistent with observations made by other workers (Stoykova et al., 1996, Smith-Fernandez et al., 1998).

**Analysis of the *Gsh2* and *Pax6* mutants provides evidence that these genes have essential roles in patterning the dLGE and VP, respectively**

Gene expression boundaries in the progenitor cells of the embryonic telencephalon become progressively distinct
identities around the PSB. The opposing genetic programs that specify the progenitor cell increase/decrease in the number of their postmitotic contraction of progenitor zones leads to a corresponding (Figs 3Bg,g,h,h,h). We found no evidence for changes in cell death or selective apoptosis or decreased proliferation for several reasons. We found no evidence for changes in cell death or mitotic index in the ventricular zones of the mutants (Figs 3Bg,g,h,h,h, 6Bg,g,h,h,h). In addition, the expansion/contraction of progenitor zones leads to a corresponding increase/decrease in the number of their postmitotic derivatives. These evidences support the model that Gsh2 and Pax6 regulate telencephalic regionalization through regulating the opposing genetic programs that specify the progenitor cell identities around the PSB.

Pax6 and Gsh2 may control telencephalic patterning through regulating Mash1, Ngn1 and Ngn2 expression

Ngn1, Ngn2 and Mash1 are b-HLH transcription factors that are implicated in regulating telencephalic D/V fates (Fode et al., 2000). Ngn1 and Ngn2 are expressed in the progenitor zones of the DP, LP, and VP while Mash1 is expressed in the LGE and MGE as well as the MP. Loss of function mutations show that Ngn1 and Ngn2 are necessary for the specification of pallial properties and for the repression of subpallial properties including Mash1 expression (Fode et al., 2000). In contrast, Mash1 is necessary for the development of some early-born subpallial neurons (Casarosa et al., 1999; Horton et al., 1999; Marin et al., 2000) but it is not required for the repression of Ngn1 and Ngn2 expression or other pallial properties (Casarosa et al., 1999; K. Y. and J. L. R. R., unpublished observations). Interestingly, ectopic expression of Mash1 in the pallium is sufficient to induce subpallial characteristics in the cerebral cortex (Fode et al., 2000).

Early dLGE phenotypes in the Gsh2 mutants are the ectopic expression of Ngn1 and Ngn2, and the reduction of Mash1 expression at E10.5 (Fig. 3Ac,c,e,e and not shown). These defects are present less than 24 hours after the time when Gsh2 expression normally begins (data not shown), suggesting that Ngn1 and Ngn2 may be targets of Gsh2 regulation. From the proposed functions and genetic interactions among the Ngn1, Ngn2 and Mash1 (Fode et al., 2000), we hypothesize that derepression of Ngn1 and Ngn2 expression in the subpallium may be a primary defect that leads to the loss of subpallial properties in the Gsh2 mutant. As opposed to Gsh2 mutants, Pax6 mutants have ectopic expression of Mash1 in the pallium and reduction in Ngn1 and Ngn2 expression (Fig. 6Ba,a,e,e and not shown). Pax6 mutants exhibit cortical expression of subpallial markers (Gsh2, Gad67, Er81, Dlx2, Dlx1, Brn4 and Six3) (Figs 6Bd,d,e,e, 7Aa,a, 7B, and not shown; Stoykova et al., 1996), a phenotype that is similar to that seen in Ngn1 and Ngn2 mutants (Fode et al., 2000). These results suggest that Pax6 may regulate pallial development in part by positively regulating Ngn1 and Ngn2 expression, which in turn suppresses subpallial properties (e.g. Mash1 and Gsh2 expression) in the pallium.

Gsh2 mutants generate LGE-derived structures at later developmental stages

Through -E14.5, Gsh2 mutants exhibit clear evidence for molecular defects in the dLGE progenitor zone and in its derived neurons. These mutants lack expression of dLGE postmitotic cell markers (Pax6, Six3), suggesting that the dLGE mantle is not produced (Fig. 4Ab,b,C). Furthermore, at E14.5 the striatum is small with fewer cells expressing the gene for the µ opioid receptor and other striatal mantle markers compared to wild-type littermates (Fig. 4Ac,c and not shown). In addition, markers of olfactory bulb (OB) GABAergic local circuit neurons (Gad67 positive and Dlx1 positive) are missing (Fig. 5Aa,a,b,b). Many of these neurons are thought to be derived from the region of the LGE (Anderson et al., 1997a; Goldman and Luskin, 1998).

By E18.5, however, the morphology and histology of both the striatum and OB in the Gsh2 mutants are more normal than at E14.5 (Figs 4, 5). We are uncertain of the precise mechanism(s) that account for this recovery, but there are several potential explanations. First, Gsh2 mutants ectopically express Gsh1, a homolog of Gsh2, in the LGE at later stages of development (e.g. at E14.5, see Fig. 4Ai,i). We suggest that the increase in Gsh1 expression may compensate for the lack of Gsh2 function. An alternative possibility is that the Gsh2 mutation may affect the dLGE more severely than the vLGE. Thus, while the expression of dLGE mantle markers is lost (Pax6 and Six3), the expression of striatal mantle markers (e.g. µ opioid receptor) is preserved albeit in a smaller region (Fig. 5Bc,c'). Thus, the recovery in striatal phenotype may reflect differentiation from the vLGE. Corollaries of this model are that the vLGE is the major source of striatal projection neurons and that the vLGE develops later than the dLGE. This hypothesis is consistent with the observation that dLGE mantle cells (expressing Pax6 and Six3) are present very early (E10.5/E11.5). Smith-Fernandez et al. used BrdU-birthdating to also-provided evidence that these Pax6+ cells are born early (~E11.5; Smith-Fernandez et al., 1998). Furthermore, there is evidence that the majority of LGE cells born before E12.5 do not contribute to the neostriatum (van der Kooy and Fishell, 1987; K. Y., unpublished observations). To test the hypothesis that Gsh1 can functionally compensate for Gsh2, we are analyzing Gsh1/2 double mutants. To test the hypothesis that the vLGE and dLGE differentially contribute to the striatum, we are performing several experiments, including birth-dating studies, the identification of additional markers that distinguish dLGE and vLGE postmitotic cells, and lineage analyses.

Pax6 is required for dorsalizing most of the telencephalon

We suggest that different levels of Pax6 expression are important for regulating regionalization within both pallium
and subpallium. Thus, although we propose that the VP is the most severely affected region of the telencephalon, there are ventralization defects in most of the Pax6

and telencephalon. Like the VP, the DP and LP express low levels of Ngn1 and Ngn2 in Pax6 mutants (Figs 6Af,f,Ba,a; 7Ah,h and not shown), and they ectopically express Mash1 and Dlx2 in pallial progenitor cells (Figs 6Ab,b,Ba,e; 7Ag,g,B). Furthermore, neurogenesis from the LP and DP is reduced based on the thinner Emx1/Tbr1-positive olfactory cortex and neocortical plate (Fig. 7Ae,e,f,f'). These results are consistent with previous studies that provided evidence for hypoplasia and mispatterning of the neocortex and olfactory cortex in Pax6 mutants (e.g. see Stoykova et al., 1996; Stoykova et al., 1997; Stoykova et al., 2001; Caric et al., 1997; Warren et al., 1999; Bishop et al., 2000; Jimenez et al., 2001). As noted earlier, part of the LGE is also ventralized, based on the ectopic expression of the MGE marker Nkx2.1 in the ventricular zone (Stoykova et al., 2001; O. Marin, K. Y. and J. L. R., unpublished observations). Thus, Pax6 has an important role in dorsal specification in most of the telencephalon. Not surprisingly, this is similar to its requirement in patterning in other CNS regions (Ericson et al., 1997b; Grindley et al., 1997; Mastick et al., 1997; Osumi et al., 1997; Warren and Price, 1997; Wawersik et al., 1999; Valverde et al., 2000).

Previous studies of Pax6 mutants suggested that the ectopic expression of subpallial markers in the cortex was due to defects in the ‘cortico-striatal border’. This conclusion was based on changes in the expression of the R-cadherin adhesion molecule in the region of the ‘cortico-striatal border’ (Stoykova et al., 1997), and in the structure of the radial glial (Goetz et al., 1998) in Pax6 mutants. It was argued that these defects altered the physical properties of the boundary, enabling subpallial cells to move into the cortex (Stoykova et al., 1996; Chapouton et al., 2000). A robust tangential migration of immature DLX-positive interneurons is known to occur from the subpallium to the pallium, particularly from the MGE (Nkx2.1-positive) domain (Anderson et al., 1997a; Anderson et al., 1997b; Anderson et al., 1999b; Marin et al., 2000; Anderson et al., 2001). However, these DLX-positive cells are postmitotic (Anderson et al., 2001) whereas most of the cortical cells expressing DLX2 in the Pax6 mutants at E11.5 appear to be mitotically active, based on the co-expression of PCNA and DLX2 (Fig. 7B). We suggest that most of the increase in subpallial gene expression in the cortex of Pax6 mutants, particularly in the ventricular zone, is due to the ventral respecification of the pallial progenitors. However, it is likely that the increase in subpallial gene expression in the SVZ and postmitotic layers of the cortex is contributed by the increase in tangential migration detected by Chapouton et al., 2000. This increase could be due to defects at the PSB, and/or to enlargement of the Nkx2.1-expressing domain, the source of these interneurons (Stoykova et al., 2001; Marin, K. Y. and J. L. R., unpublished observations).

**Similarities and differences in patterning of the spinal cord and telencephalon**

There is evidence that homeobox genes of the Nkx and Pax families have similar roles in D/V patterning in the spinal cord and telencephalon, based on gene expression patterns and genetic experiments (Briscoe et al., 1999; Briscoe et al., 2000; Sussel et al., 1999; Sander et al., 2000; Stoykova et al., 2001). For instance, early expression of the Nkx2 gene family is restricted to ventral CNS domains (Qiu et al., 1998). Their dorsal limit of expression is regulated by Pax6 in the spinal cord (Ericson et al., 1997b; Briscoe et al., 2000) and telencephalon (Stoykova et al., 2001; Marin, K. Y. and J. L. R., unpublished observations). Pax6 mutants also affect molecular properties of intermediate dorsoventral positions of both the telencephalon and the spinal cord, where the expression of WNT-signaling molecules is decreased (Osumi et al., 1997; Wawersik, et al, 1999; Pleasure, K. Y. and J. L. R., unpublished observations).

An additional similarity in the molecular organization of the embryonic spinal cord and telencephalon is exemplified by the expression of Dlx1 in an intermediate D/V position in both structures (Fig. 1Ba,a; Lu et al., 1992; Shoji et al., 1996; Pierani et al., 1999). In the spinal cord, Dlx1 expression is independent of sonic hedgehog function and is responsive to retinoic acid signaling (Pierani et al., 1999). Ongoing studies are aimed at testing whether telencephalic Dlx1 expression is under similar controls.

There are also major differences in D/V patterning of the spinal cord and telencephalon, which is exemplified by the expression and function of Gsh2 and Nkx6.1. In the telencephalon, Gsh2 expression and function are restricted to ventral regions, whereas in the spinal cord its expression and function are restricted to dorsal regions (Sander et al., 2000; K. Y. and J. L. R., unpublished observations). Nkx6.1 is expressed in the ventral spinal cord, but its expression has not been detected in the telencephalon (Qiu et al., 1998). Despite these differences, it is interesting to note that both genes regulate the ventral expression of Pax6 and Dlx1 (Gsh2 in the telencephalon and Nkx6.1 in the spinal cord).

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