

The *Gsh2* homeodomain gene controls multiple aspects of telencephalic development

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SUMMARY

Homeobox genes have recently been demonstrated to be important for the proper patterning of the mammalian telencephalon. One of these genes is *Gsh2*, whose expression in the forebrain is restricted to the ventral domain. In this study, we demonstrate that *Gsh2* is a downstream target of sonic hedgehog and that lack of *Gsh2* results in profound defects in telencephalic development. *Gsh2* mutants have a significant decrease in the expression of numerous genes that mark early development of the lateral ganglionic eminence, the striatal anlage. Accompanying this early loss of patterning genes is an initial expansion of dorsal telencephalic markers across the cortical-striatal boundary into the lateral ganglionic eminence. Interestingly, as development proceeds, there is compensation for this early

loss of markers that is coincident with a molecular re-establishment of the cortical-striatal boundary. Despite this compensation, there is a defect in the development of distinct subpopulations of striatal neurons. Moreover, while our analysis suggests that the migration of the ventrally derived interneurons to the developing cerebral cortex is not significantly affected in *Gsh2* mutants, there is a distinct delay in the appearance of GABAergic interneurons in the olfactory bulb. Taken together, our data support a model in which *Gsh2*, in response to sonic hedgehog signaling, plays a crucial role in multiple aspects of telencephalic development.

Key words: Mouse, Telencephalon, Knockout, *Gsh2*

INTRODUCTION

The telencephalon and diencephalon, which comprise the vertebrate forebrain, arise from the anterior-most region of the neuraxis (reviewed in Fishell, 1997; Rubenstein et al., 1998). The telencephalon is subdivided into the pallial and subpallial domains. The pallium gives rise to dorsal structures, including the cerebral cortex, while the subpallium gives rise to ventral structures, including the globus pallidus and the striatum, which in combination form the majority of the basal ganglia. The basal ganglia arise from two major protrusions in the wall of the ventral telencephalon known as the medial ganglionic eminence (MGE) and the lateral ganglionic eminence (LGE), which primarily gives rise to the globus pallidus and the striatum, respectively (Smart and Sturrock, 1979; Deacon et al., 1994). The appearance of these eminences occurs sequentially during development, with the more-medial MGE arising subsequent to neural-tube closure and the more lateral LGE arising shortly thereafter. The MGE and LGE are additionally hypothesized to be the source of a majority of the interneurons found in the olfactory bulb and the cerebral cortex (reviewed in Anderson et al., 1999; Parnaveles et al., 2000).

Much work has recently been undertaken to elucidate the molecular mechanisms that pattern the telencephalon. Similar to the mechanisms that control patterning of the vertebrate spinal cord, there is strong evidence that the secreted molecule

sonic hedgehog (SHH) plays an essential role in patterning of the forebrain (Ericson et al., 1995; Chiang et al., 1996; Dale et al., 1997; Shimamura and Rubenstein, 1997; Kohtz et al., 1998; Gaiano et al., 1999). Support for this hypothesis comes from both loss- and gain-of-function studies demonstrating that SHH is both necessary for and sufficient to induce expression of ventral forebrain markers. For example, the absence of *Shh* in mice (Chiang et al., 1996) or *shh* in zebrafish (Barth and Wilson, 1995) results in the loss of expression of members of the *Nkx* and *Dlx* family of homeobox transcription factors, whose expression in the forebrain is initially restricted to the ventral domain. Furthermore, in mice lacking *Shh* there is a complete absence of ventral forebrain structures accompanied by an ectopic expression of dorsal forebrain markers. Moreover, the in vitro culture of forebrain explants in the presence of SHH results in the induction of the ventral forebrain markers *Nkx2.1* (*Titf1* – Mouse Genome Informatics), *Dlx2* and *Isl1* (Ericson et al., 1995; Dale et al., 1997; Pera and Kessel, 1997; Kohtz et al., 1998), while the in vivo misexpression of SHH in the cerebral cortex of mice results in the ectopic expression of *Dlx2*, *Nkx2.1* and the LGE-specific marker, CRBP (RBP1 – Mouse Genome Informatics) (Gaiano et al., 1999).

While these studies have clearly established an essential role for SHH in patterning of the ventral forebrain, the molecular mechanisms underlying this activity remain to be elucidated.

Recent analysis of telencephalic development in mutant mice that lack either *Nkx2.1* (Kimura et al., 1996; Sussel et al., 1999), *Mash1* (*Ascl1* – Mouse Genome Informatics) (Casarosa et al., 1999), or both *Dlx1* and *Dlx2* (Anderson et al., 1997a,b), has provided valuable insight into the genetic mechanisms that regulate ventral telencephalic development. The absence of *Nkx2.1* in mice leads to an apparent conversion of the MGE to an LGE fate, clearly demonstrating a role for this gene in the proper specification of the MGE (Sussel et al., 1999). The loss of *Mash1* results in multiple defects in telencephalic development including a significant reduction in the size of the MGE and a loss of specific subpopulations of early born striatal neurons (Casarosa et al., 1999). The combined loss of *Dlx1* and *Dlx2* in mice results in altered LGE development with a subsequent loss of later born striatal matrix neurons that are believed to be derived from the subventricular zone (SVZ) of the developing striatum (Anderson et al., 1997a). Interestingly, in each of these mutations there is a significant decrease in the numbers of interneurons found in the cerebral cortex (Anderson et al., 1997b; Casarosa et al., 1999; Sussel et al., 1999), underlining the importance of ventral to dorsal cell migration in the establishment of dorsal interneuron populations.

In addition to the importance of the *Nkx*, *Dlx* and *Mash* genes in telencephalic development, two related *Gsh* genes have been implicated as important regulators of ventral forebrain development (Hsieh-Li et al., 1995; Valerius et al., 1995; Li et al., 1996; Szucsik et al., 1997). While there are 10 genes with a *Gsh* nomenclature, only *Gsh1* and *Gsh2* are related by sequence (Singh et al., 1991). *Gsh1* and *Gsh2* are expressed exclusively in the ventricular zone (VZ) in a widely overlapping pattern in the forebrain (Hsieh-Li et al., 1995; Valerius et al., 1995). Although both genes are similarly expressed in the ventral diencephalon, MGE and septum, in the LGE, expression of *Gsh1* and *Gsh2* is only partially overlapping. *Gsh1* is expressed in the most ventral and caudal region of the LGE, whereas *Gsh2* is expressed throughout the LGE. The loss of *Gsh1* in mice results in abnormal pituitary development, with the ventral telencephalon being apparently unaffected (Li et al., 1996). Previous analysis of mutant mice lacking *Gsh2* demonstrated a reduction in the size of the LGE and a loss of *Dlx2* expression in the LGE (Szucsik et al., 1997).

The vertebrate *Gsh1* and *Gsh2* genes are the mammalian homologs of the *Drosophila* intermediate neuroblasts defective (*ind*) gene that encodes a homeodomain protein essential for the establishment of dorsal-ventral cell fate in the *Drosophila* nervous system (Weiss et al., 1998). Based on gene expression patterns, the *Drosophila* neuroectoderm is divided into three domains or columns: ventral, intermediate and dorsal (reviewed in Arendt and Nübler-Jung, 1999; Cornell and Van Ohlen, 2000). *ind* is expressed exclusively within the intermediate domain and has been shown to control the establishment of intermediate cell fate in this domain. In the absence of *ind*, a majority of intermediate domain neuroblasts fail to develop. Those that develop lose their intermediate identity, as evidenced by the loss of Eve, a marker for the RP-2 neuron population normally found in this domain, and are respecified to either a ventral or dorsal fate. Instead, these cells express *achaete*, which is normally absent from the intermediate column but is expressed in both the dorsal and ventral columns, and either *vnd* or *msh*, markers of ventral and

dorsal column cells, respectively. Conversely, ectopic expression of *ind* in either the ventral or dorsal neuroblast domains results in suppression of ventral or dorsal fates (C. Doe, personal communication).

In this study, we have further explored the role that *Gsh2* plays in development of the ventral telencephalon. We tested whether *Gsh2* is a downstream target of SHH and found that *Gsh2* is both lost in the forebrain of *Shh* mutant mice and can be ectopically induced by SHH in vivo. Furthermore, we have found that *Gsh2* is essential for the early expression of a number of genes that mark various aspects of specification and differentiation of the LGE. Interestingly, *Gsh2* is also essential for the establishment of the molecularly defined boundary near the cortical-striatal sulcus, the border between the developing cortex and the LGE. Notably, while *Gsh2* is ultimately expressed in many developing structures of the ventral telencephalon, during early telencephalic development its expression is restricted to an area corresponding to the prospective LGE. In accordance with this, the most prominent effect of the loss of *Gsh2* is on development of the LGE. Moreover, the appearance of interneurons is delayed in the olfactory bulb but unaffected in the cerebral cortex, suggesting that *Gsh2* plays an early role in the differentiation and/or migration of cells from the LGE to the olfactory bulb, but not to the cerebral cortex. Thus, *Gsh2* is both a target of SHH signaling and plays an essential role in multiple aspects of ventral telencephalic development, acting both in the establishment of regional borders and the specification of distinct cell populations.

MATERIALS AND METHODS

Animals, virus preparation and injection

All animals used in these studies were maintained according to protocols approved by the Institutional Animal Care and Use Committee at NYU School of Medicine. Wild-type, heterozygous and homozygous *Gsh2* (Szucsik et al., 1997) and *Shh* (Chiang et al., 1996) mutant embryos were obtained from intercrosses of *Gsh2*^{+/-} or *Shh*^{+/-} mice. For staging of embryos, midday of the vaginal plug was considered as embryonic day 0.5 (E0.5). For viral injection studies, Swiss Webster mice (Taconic Farms, Germantown, New York) were used. Virus preparation and ultrasound surgery were both performed as previously described (Gaiano et al., 1999). Concentrated stocks of CLES (CLE virus expressing SHH) and CLEG (CLE virus expressing GSH2) were injected at titers of 2-5×10⁸.

RNA in situ hybridization

Whole embryos (E9.5 and E10.5), whole heads (between E12.5 and E15.5) or brains (E18.5) were fixed at 4°C in 4% paraformaldehyde for 1-4 hours, rinsed in phosphate-buffered saline (PBS), cryoprotected overnight in 30% sucrose in PBS and embedded in HistoPrep (Fisher Scientific). Embedded tissues were sectioned on a cryostat at 20 µm. In situ hybridization of sections was performed as described in (Schaeren-Wiemers and Gerfin-Moser, 1993; Wilkinson and Nieto, 1993) using non-radioactive DIG-labeled probes for *Gsh2* (Hsieh-Li et al., 1995), *Gad67* (*Gad1* – Mouse Genome Informatics) (Behar et al., 1994), *Dlx1* (Price et al., 1991), *dopamine receptor 2* (*Drd2* – Mouse Genome Informatics) (Montmayeur et al., 1991), *enkephalin* (preproenkephalins 1 and 2; *Penk1* and *Penk2* – Mouse Genome Informatics) (Song and Harlan, 1994), *Dlx2* (Porteus et al., 1991), *Mash1* (Guillemot and Joyner, 1993), *Ebfl* (Garel et al., 1997), *Pax6* (Walther and Gruss, 1991), *Ng2* (*Atoh4* – Mouse Genome Informatics) (Gradwohl et al., 1996), *Math2* (*Atoh2* – Mouse Genome

Informatics) (Bartholomä and Nave 1994; Shimizu et al., 1995), *NeuroD* (Lee et al., 1995) and *Msx1* (MacKenzie et al., 1991).

Staining of tissue sections

Tissue was processed for immunohistochemistry and PLAP histochemistry as described above. The following antibodies were used for immunofluorescence: mouse anti-PAX6 (1:1000, gift of A. Kawakami), sheep anti-PLAP (1:200, American Research Products), mouse anti-NKX2.1 (1:2000, gift of J. Whitsett), rabbit anti-RBP (1:400, gift of U. Eriksson, Stockholm, Sweden), rabbit anti-DARPP32 (1:10,000, gift of P. Greengard), rabbit anti-GSH2 (1:2000, gift of K. Campbell), rabbit anti-calbindin (1:5000, SWANT), rabbit anti-GABA (1:5000, Sigma), and rabbit anti- β -galactosidase (1:250, Cappel). Secondary antibodies used were: FITC-conjugated donkey anti-sheep, FITC-conjugated donkey anti-rabbit, Cy3-conjugated donkey anti-rabbit, Cy3-conjugated donkey anti-mouse (all from Jackson ImmunoResearch, West Grove, PA). Sections were washed in PBS, blocked for 1 hour with PBS containing 10% donkey serum and 0.2% Triton X-100. Sections were incubated in primary antibodies diluted in block (with 10% serum) overnight at 4°C, then washed three times in PBS and incubated with secondary antibodies diluted in PBS containing 1% donkey serum and 0.2% Triton X-100 for 1–2 hours at room temperature in the dark. Fluorescent images were obtained using either a cooled-CCD camera (Princeton Instruments) and Meta-morph software (Universal Imaging, West Chester, PA) or a confocal microscope (Leica). For histochemical detection of PLAP, staining was carried out as previously described (Gaiano et al., 1999).

X-Gal staining

Tissue was prepared as described above. Sectioned tissue was washed with 100 mM phosphate buffer, 2 mM MgCl₂ and 5 mM EGTA for 30 minutes, at room temperature; followed by two washes of 5 minutes at room temperature with 100 mM phosphate buffer (pH 7.4), 2 mM MgCl₂, 0.01% sodium desoxycholate, and 0.02% Nonidet-P40. The blue precipitate was generated by exposure overnight in the dark at 37°C to 100 mM phosphate buffer [pH 7.4], 2 mM MgCl₂, 0.01% sodium desoxycholate, 0.02% Nonidet-P40 with 5 mM potassium-ferricyanide and 1 mg/ml of X-gal.

RESULTS

Gsh2 is expressed in the ventral forebrain and is a downstream target of SHH

Previous studies have shown that expression of *Gsh2* is restricted in the mouse forebrain to the ventral domain (Hsieh-Li et al., 1995; Szucsik et al., 1997). *Gsh2* is first detected between E9

and E10 in the forebrain and is expressed in the later developing ventral thalamus, hypothalamus, MGE, LGE and caudal ganglionic eminence (CGE). To better understand the localization of *Gsh2*, we assayed its expression at a variety of stages of telencephalic development. At E9.5, *Gsh2* mRNA expression was localized to the ventrolateral telencephalon, a region from where the LGE will putatively emerge (Fig. 1A). Interestingly, at E9.5, although a few GSH2-expressing cells appeared to intermingle with those expressing NKX2.1, GSH2

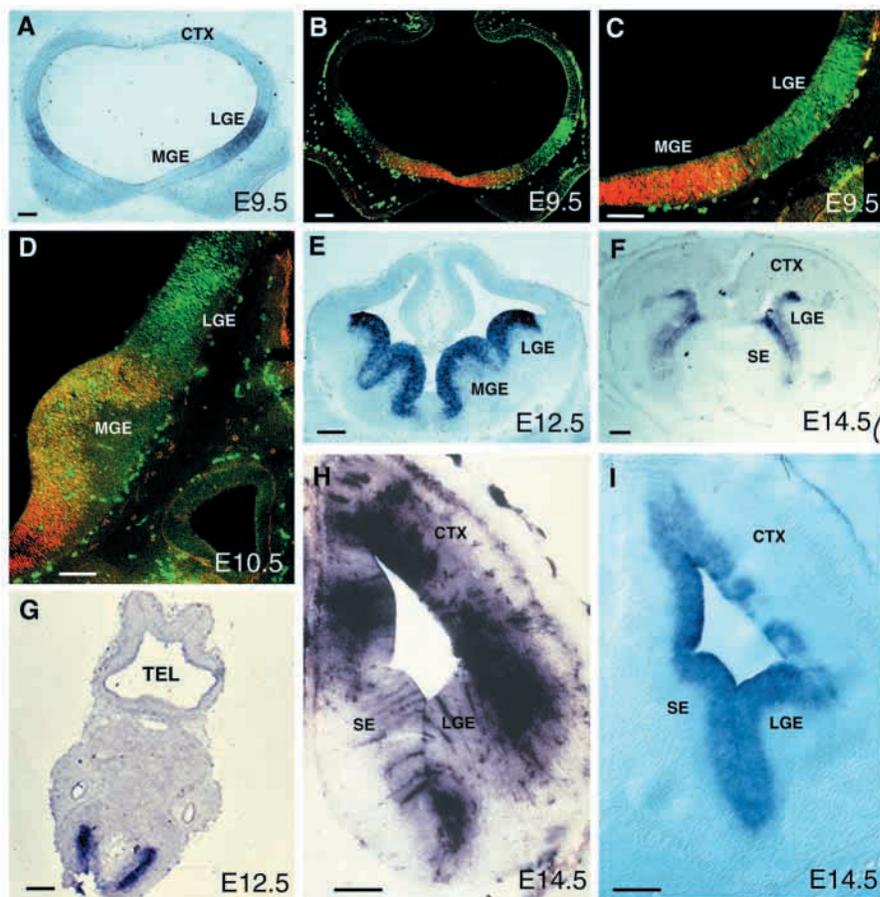


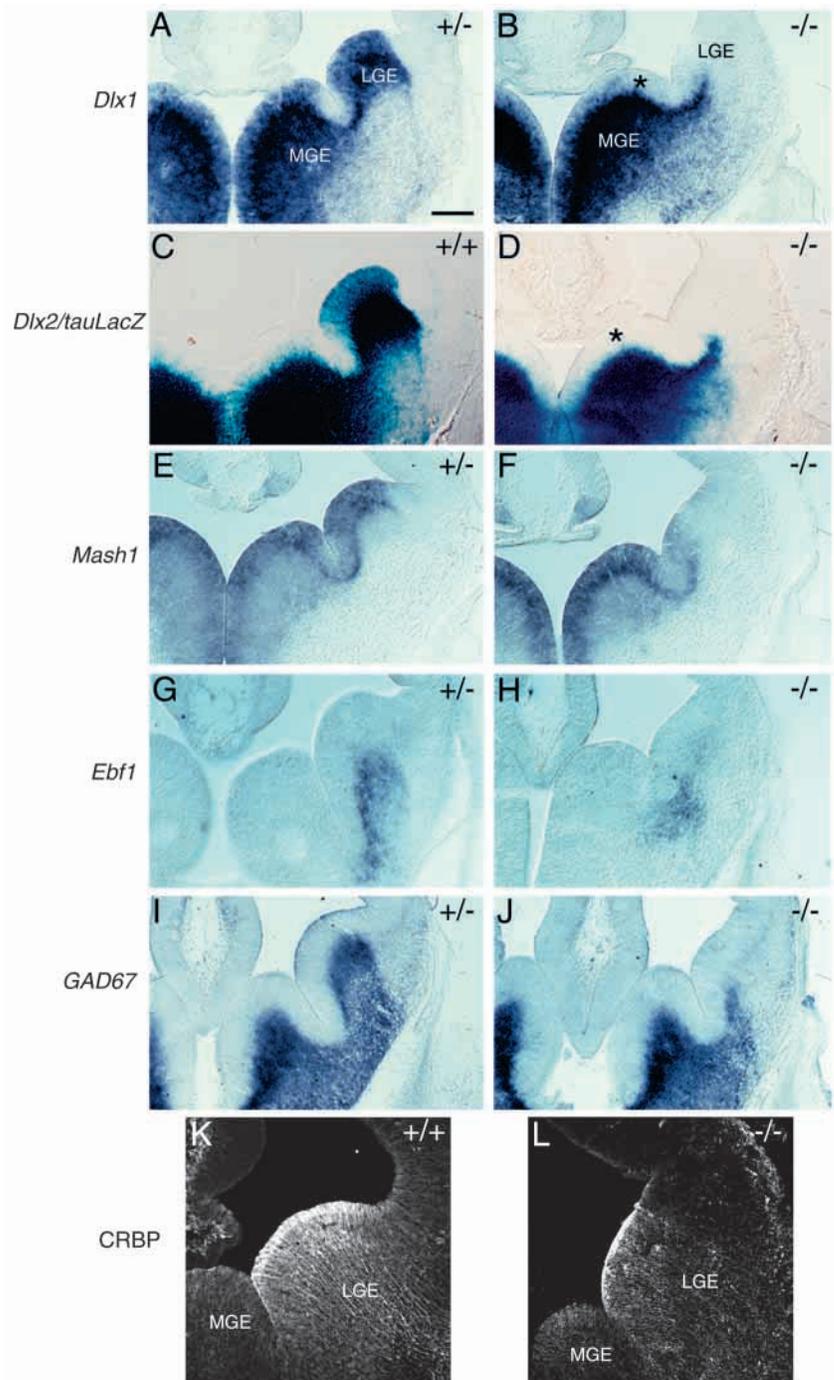
Fig. 1. Expression of *Gsh2* in the developing telencephalon and regulation by SHH. (A–F) In situ hybridization and immunocytochemical localization of *Gsh2* mRNA and protein expression on coronal sections of the developing mouse telencephalon. Expression of *Gsh2* mRNA (A) and protein (green) (B,C) is observed in the ventrolateral telencephalon at E9.5, corresponding to the presumptive LGE region. (B,C) Double immunofluorescence for both NKX2.1 (red) protein and GSH2 (green) protein at E9.5 reveals NKX2.1 and GSH2 expression in complementary, non-overlapping domains in the ventral telencephalon. (D) Coincident with the appearance of the MGE at E10.5, expression of GSH2 (green) overlaps with NKX2.1 (red) in the developing MGE (yellow). *Gsh2* is shown in both the MGE and LGE at E12.5 (E) and the developing LGE and septum at E14.5 (F). (G) Coronal sections from E12.5 heads taken from a *Shh* knockout mouse demonstrate a loss of *Gsh2* mRNA expression in the ventral forebrain. Note the normal expression of *Gsh2* in the non-neural tissue. (H,I) SHH was misexpressed in the telencephalon at E10.5 by use of high-titer retroviruses expressing both SHH and human placental alkaline phosphatase (PLAP) as a reporter. PLAP histochemical staining of coronal sections at E14.5 reveals multiple viral infection sites throughout the telencephalon (H). *Gsh2* in situ hybridization of a section adjacent to (H) reveals normal *Gsh2* expression in the septum and LGE and ectopic *Gsh2* expression (I) in the cerebral cortex, corresponding with sites of viral infections (H). CTX, cerebral cortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; SE, septum; TEL, telencephalon. Scale bars; 50 μ m (A–D,H,I); 250 μ m (E–G).

and NKX2.1 were predominantly expressed in separate domains (Fig. 1B,C). By E10.5, coincident with the emergence of the MGE, GSH2 expression extended into the MGE and overlapped with NKX2.1 expression (Fig. 1D). By E12.5, *Gsh2* was expressed in the MGE, LGE (Fig. 1E) and septum (shown at E14.5, Fig. 1F). At E12.5, *Gsh2* was also expressed in the CGE and the ventral diencephalon (data not shown). Expression of *Gsh2* mRNA was restricted to the VZ and was not expressed in the SVZ or differentiated structures of the developing ventral telencephalon.

SHH is expressed in both the ventral forebrain and the underlying prechordal plate (Echelard et al., 1993; Roelink et al., 1994; Marti et al., 1995), and is known to have ventralizing activity within the forebrain (Ericson et al., 1995; Dale et al., 1997; Pera and Kessel, 1997; Kohtz et al., 1998; Gaiano et al., 1999). The ventral restricted expression of *Gsh2* and the loss of ventral structures in *Shh* knockout mice suggests that expression of *Gsh2* may be regulated by SHH signaling. To test this hypothesis, we undertook *Shh* loss- and gain-of-function analyses. In mutant embryos lacking *Shh*, the forebrain exists as a single fused vesicle at E11.5 with a complete absence of ventral forebrain structures and the entire telencephalon expresses the dorsal marker *Emx1* (Chiang et al., 1996). In mice lacking *Shh* function, normal expression of *Gsh2* at E12.5 was not detected in the forebrain (Fig. 1G). For gain-of-function experiments, we used ultrasound backscatter microscopy to visualize the early mouse embryo (Olsson et al., 1997; Lui et al., 1998), which allowed us to injected high titer, SHH-expressing retrovirus (Gaiano et

al., 1999) into the developing forebrain at E8.5, E9.5 and E10.5. Embryos were subsequently assayed 4 days later at E12.5, E13.5 and E14.5, respectively, for viral expression and ectopic *Gsh2* expression. As shown at E14.5 (Fig. 1H,I) the misexpression of SHH within the telencephalon resulted in the ectopic expression of *Gsh2* dorsally. This ectopic expression of *Gsh2* was observed at all ages assayed (data not shown). In contrast, expression of virally derived SHH did not have an effect on the normal expression of *Gsh2* in the septum or the LGE. Control virus expressing the PLAP reporter construct alone did not result in ectopic expression of *Gsh2* (data not shown). Therefore, SHH is both necessary

Fig. 2. Early patterning defects in the absence of *Gsh2*. Coronal sections from control (wild type or heterozygous) and *Gsh2* knockout embryos were analyzed for a number of markers expressed in the ventral telencephalon at E12.5. For each of the experiments either wild-type or heterozygous animals were used as controls, since no heterozygous phenotype is observed. Analysis of *Dlx1* (A,B), *Dlx2/tauLacZ* (C,D), *Mash1* (E,F), *Ebf1* (G,H) and *Gad67* (I,J) mRNA expression is shown. For *Dlx1*, *Ebf1*, *Mash1* and *Gad67*, expression of mRNA was determined by in situ hybridization. In this and subsequent experiments, expression of *Dlx2* mRNA was determined by X-gal staining of sections taken from a strain of mice in which the *tauLacZ* gene has been targeted to the *Dlx2* locus (J. G. C., S. Nery, A. L. and G. F., unpublished). Shown here, expression of *Dlx1*, *Dlx2/tauLacZ*, *Mash1* and *GAD67* (A-F,I,J) is similarly lost in all but the ventral-most aspect of the LGE of *Gsh2* mutant embryos. *Dlx1* and *Dlx2/tauLacZ* expression is also reduced in the VZ of MGE of *Gsh2* mutant embryos (asterisks) (B,D). *Ebf1* expression in the SVZ of the LGE is also significantly reduced (G,H). Expression of RBP (CRBP) protein, as demonstrated by immunocytochemistry, remains in the radial glia of the mutant LGE (K,L). Note the significant reduction in the size of the LGE in all cases. Scale bar: 250 μ m.



for and sufficient to induce expression of *Gsh2* in the telencephalon at the ages examined.

Patterning defects associated with the loss of *Gsh2*

Previous work has demonstrated that the absence of *Gsh2* results in a morphological reduction in the size of the LGE and a loss of *Dlx2* in the LGE at E12.5 (Szucsik et al., 1997). To further examine the role of *Gsh2* on ventral telencephalic patterning, we assayed the expression of a number of genes implicated in this process. The homeodomain transcription factors *Dlx1* and *Dlx2* are expressed in the VZ and SVZ of multiple ventral forebrain structures including the MGE, LGE, CGE and ventral diencephalon (Price et al., 1991; Porteus et al., 1991; Bulfone et al., 1993). At E12.5, in embryos lacking *Gsh2* there was an absence of both *Dlx1* and *Dlx2* expression in all but the most ventral aspect of the LGE (Fig. 2A-D). Expression of *Mash1*, a bHLH transcription factor essential for proper striatal development (Casarosa et al., 1999), showed a similar reduced expression pattern (Fig. 2E,F). Furthermore, expression of *Ebf1*, a gene essential for the transition of cells from the SVZ to the striatal mantle (Garel et al., 1999), was significantly reduced in the mutant LGE (Fig. 2G,H), as was *Gad67* (Fig. 2I,J), the precursor enzyme that catalyzes the formation of the neurotransmitter GABA (Behar et al., 1994). Notably, the expression of RBP (retinol binding protein), normally expressed in retinoid-producing radial glial cells of the LGE (Toresson et al., 1999), remained in the LGE (Fig. 2K,L). While the domain of RBP expression appeared normal, a slight decrease in the level of expression was observed, although at this level of analysis it is difficult to determine if the radial glia are disorganized. Taken together, these observations suggest that, although the LGE appears to be at least partially specified, the normal patterning of this structure is severely affected by the loss of *Gsh2*. In addition to the striking effects on LGE development, there appears to be additional, although less severe, patterning defects in the MGE and CGE. Most notable

was the significant decrease in *Dlx1* and *Dlx2* expression in the VZ of the MGE of *Gsh2* mutant embryos (Fig. 2A-D). In addition, the CGE was slightly reduced in size, a change accompanied by a decrease in the expression of *Dlx2* (data not shown).

Recovery of patterning defects in *Gsh2* mutants by mid-neurogenesis

Although at E15.5 a reduction in the size of the developing striatum remained, in *Gsh2* mutants there was a striking recovery in the expression of each of the genes analyzed throughout the rostral to caudal extent of the telencephalon. Whereas expression of *Dlx1* and *Dlx2* was lost in the majority of the LGE at E12.5, by E15.5, while still reduced, both genes were expressed in the developing striatum (Fig. 3A-D). Interestingly, similar to the absence of expression in the VZ of the MGE at E12.5, expression of *Dlx1* and *Dlx2* was absent in the VZ of the LGE in *Gsh2* mutants at E15.5. By E18.5, however, expression of *Dlx1* and *Dlx2* in the VZ had recovered and appeared normal (data not shown). By E15.5, expression of *Mash1* appeared at normal levels within the VZ of the developing striatum (Fig. 3E,F). Although still reduced compared with controls, expression of *Ebf1* (Fig. 3G,H) and *Gad67* (Fig. 3I,J) had also significantly recovered by E15.5.

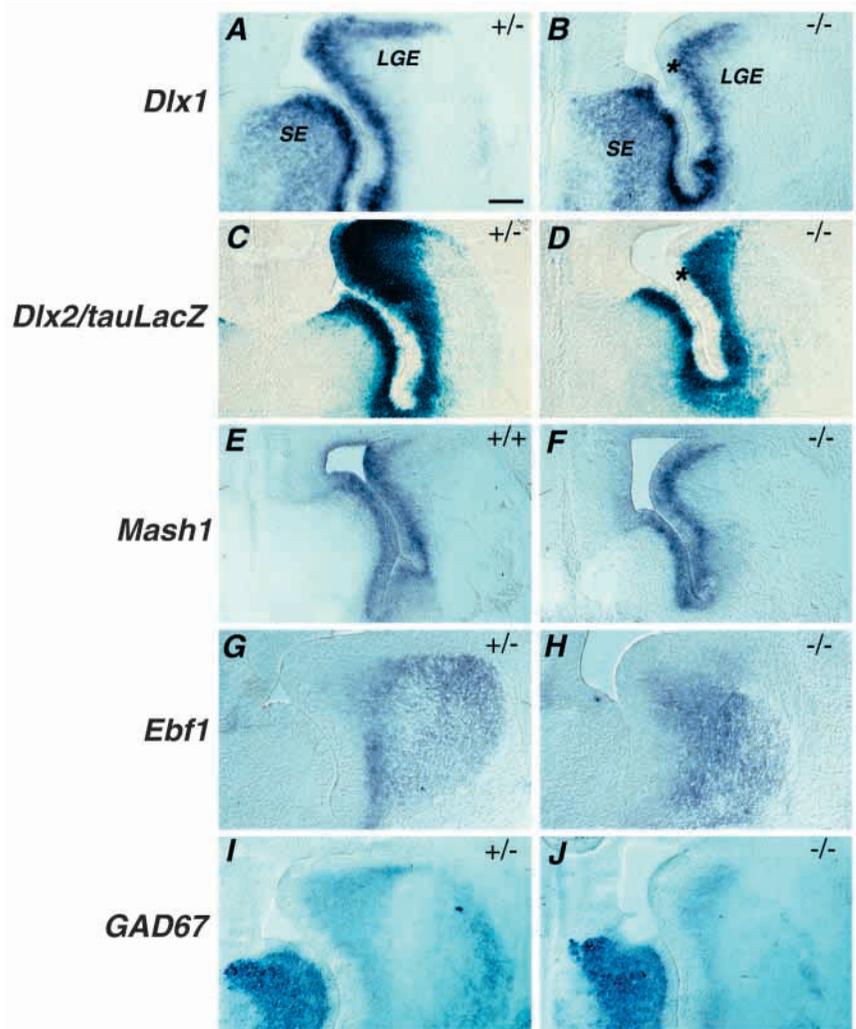


Fig. 3. Ventral telencephalic defects recover in *Gsh2* mutants by mid-neurogenesis. In situ hybridization (A,B,E-J) and X-gal staining (C,D) analysis of coronal sections at E15.5 reveals that the expression of a number of patterning genes that are lost at E12.5 show a dramatic recovery in the LGE/developing striatum by E15.5. In the SVZ of the developing striatum expression of *Dlx1* (A,B) and *Dlx2/tauLacZ* (C,D) significantly recovers in the SVZ. However, expression of *Dlx1* and *Dlx2/tauLacZ* remains absent in the VZ of the mutant LGE (asterisks) (B,D). In the VZ of the mutant LGE, expression of *Mash1* (E,F) appears to be at near normal levels. Furthermore, although expression of *Ebf1* is still reduced in the SVZ of the developing mutant striatum, there is a significant recovery of expression by E15.5 (G,H). Expression of *Gad67* is also significantly recovered in the striatal SVZ and mantle (I,J). Scale bar: 100 μ m.

Early born striatal neuron populations are affected in embryos lacking *Gsh2*

Previous work has suggested that *Mash1*, *Dlx1* and *Dlx2* control the development of subsets of early born and later born striatal matrix neurons, respectively (Casarosa et al., 1999; Anderson et al., 1997a). Since *Gsh2* is required for the early expression of these genes, the status of specific striatal cell populations was investigated in *Gsh2* mutant embryos. For these experiments, the expression of a number of striatal markers, including DARPP32 (*Ppp1r1b* – Mouse Genome Informatics) (Langley et al., 1997), *enkephalin* (*Enk*) (Song and Harlan, 1994) and the *dopamine receptor 2* (*Drd2*) (Mansour and Watson, 1995), which mark populations of early born striatal neurons, as well as calbindin (Liu and Graybiel, 1992) and *Ebf1* (Garel et al., 1999), which mark later developing striatal matrix neurons, was examined. *Mash1* mutant embryos displayed a significant reduction in *Enk* and *Drd2* expression in both the nucleus accumbens and the ventrolateral aspect of the telencephalon (Casarosa et al., 1999), regions that are believed to be derived from early born striatal cells (Hinds and Angevine, 1965; Bayer, 1986; de Carlos et al., 1996). Similarly, at E18.5, we observed a reduction of *Enk* expression in the most ventral aspect of the nucleus accumbens and ventrolateral (perirhinal) region of the ventral telencephalon in *Gsh2* mutant embryos (Fig. 4A,B). Expression of *Drd2* was also missing in both the nucleus accumbens and perirhinal regions at this age (Fig. 4C,D). There was also a partially penetrant (in 2 of 3 *Gsh2* mutant embryos analyzed) loss of DARPP32-positive cells in the striatal SVZ (Fig. 4E,F) further suggesting a loss of subpopulations of early born striatal neurons. In contrast, development of later born neurons that comprise the striatal matrix appeared unaffected. *Ebf1* expression, although reduced at E15.5 (Fig. 3G,H) and at E18.5 (data not shown), was present in the presumptive striatal matrix in *Gsh2* mutants. Normal expression of calbindin within the striatal matrix (Fig. 4G,H) further suggests this aspect of striatal development was unaffected by the loss of *Gsh2*. These results suggest that, despite the recovery of early patterning defects by later stages of telencephalic development, the generation of specific subpopulations of early, but not late born, striatal neurons is permanently affected.

Dynamic changes in the cortical-striatal boundary in *Gsh2* mutants

The boundary at the junction of the cortex and the LGE (cortical-striatal boundary) is defined by the restricted expression of several genes. Expression of *Pax6*, a paired homeodomain gene essential for the proper development of cortical progenitors (Götz et al., 1998) and *Ngn2*, a gene that encodes a bHLH transcription factor necessary for determination of the dorsal telencephalic phenotype (Fode et al., 2000), is restricted to proliferating precursors in the developing cortex. While expression of each of these genes normally extends slightly beyond the cortical-striatal sulcus into the LGE, a sharp boundary divided expression of *Pax6* and *Ngn2* from the ventrally restricted expression of *Dlx1*, *Dlx2* and *Mash1* (compare Fig. 2A,C,E with Fig. 5A,C,E) (see also Stoykova et al., 1996; Torii et al., 1999; Fode et al., 2000). As shown in Fig. 2, the loss of *Gsh2* resulted in a regression of *Dlx1*, *Dlx2* and *Mash1* expression to the most ventral aspect of the LGE at E12.5. The loss of *Gsh2* further resulted in a

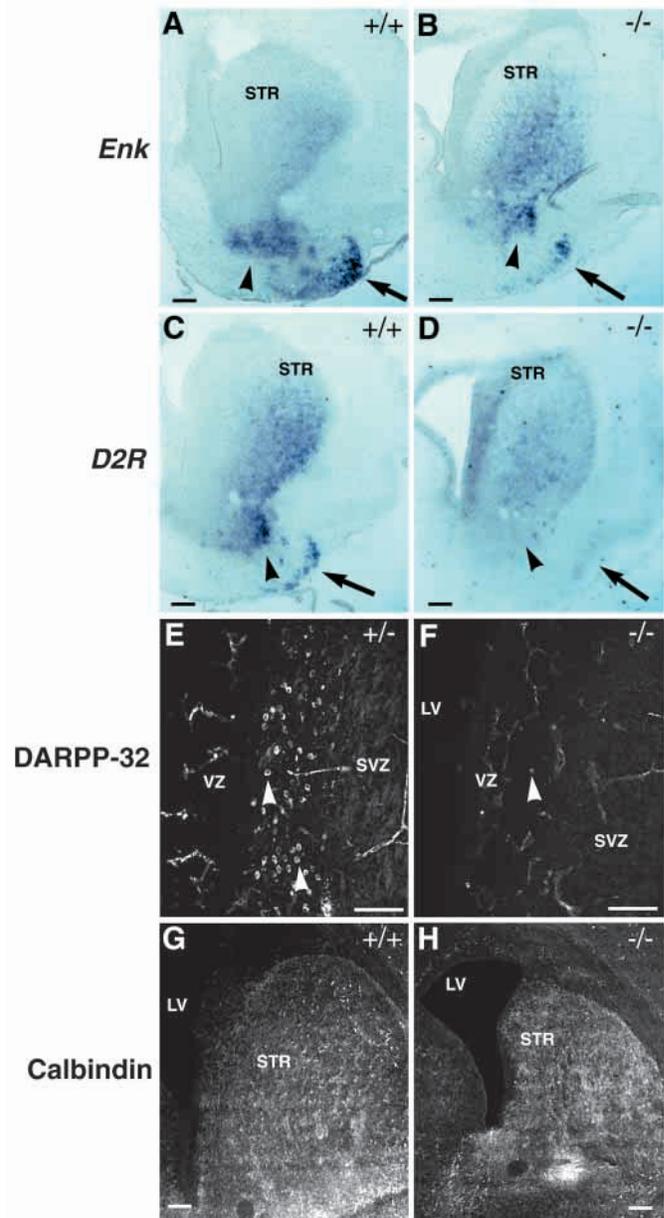
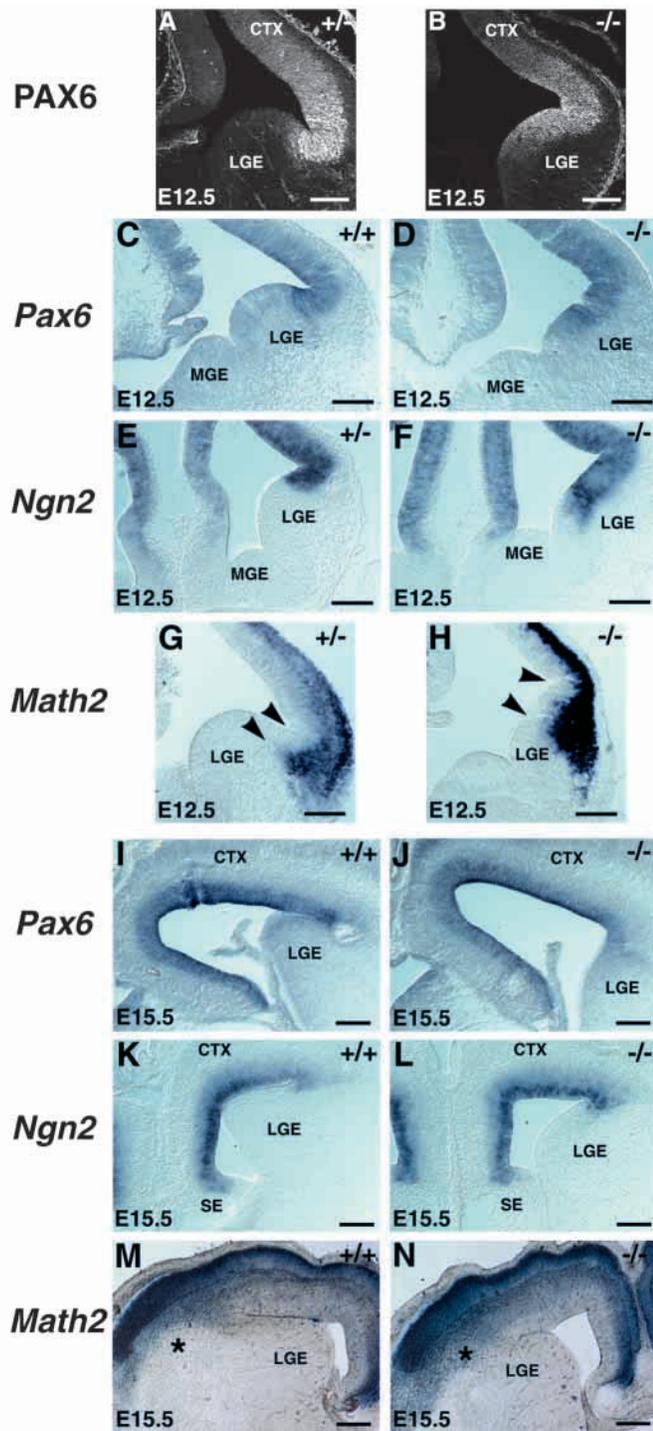


Fig. 4. The absence of *Gsh2* affects the generation of early born striatal cells. In situ hybridization (A–D) and immunofluorescent (E–H) analysis of coronal sections from E18.5 brains reveals a reduction in the presence of early, but not late born, striatal cells. (A,B) A reduction in *Enk* mRNA expression is observed in the ventral nucleus accumbens (arrowheads) and the perirhinal region of the ventral telencephalon (arrows). (C,D) *Drd2* (*D2R*) mRNA expression is lost in both the nucleus accumbens (arrowheads) and the perirhinal cortex (arrows). (E,F) In the SVZ of the striatum, the number of DARPP32 immuno-positive cells (arrowheads) is significantly reduced in *Gsh2* mutant brains. (G,H) Calbindin immunoreactivity, however, is similar in control and mutant brains. LV, lateral ventricle; STR, striatum; SVZ, subventricular zone; VZ, ventricular zone. Scale bars: 50 μ m.

dramatic expansion of *Pax6* and *Ngn2* expression into the VZ of the mutant LGE (Fig. 5A–F). Interestingly, the ventral-most aspect of this expansion correlated with the dorsal-most expression of *Dlx1*, *Dlx2* and *Mash1* in the mutant LGE



(compare Fig. 2B,D,F with Fig. 5B,D,F). The expansion of *Pax6* and *Ngn2* expression suggests that cells in the mutant LGE are acquiring a more dorsal fate. In support of this hypothesis, the expression of *Math2* (Fig. 5G,H) and *NeuroD* (data not shown), markers of more differentiated cortical cells (Lee, 1997), was present more ventrally in the mutant LGE compared with controls. Coincident with the recovery of gene expression in the striatum by E15.5, there was a molecular re-establishment of the cortical-striatal boundary in the *Gsh2* mutant embryos as demonstrated by the normal restricted

expression of *Pax6* (Fig. 5I,J) and *Ngn2* (Fig. 5K,L) to the developing cerebral cortex. Interestingly, however, in the developing striatal mantle at this age, there is an ectopic expression of *Math2* (Fig. 5M,N), suggesting at least some LGE-derived cells may have taken on a more permanent dorsal phenotype.

***Gsh2* gain-of-function analysis**

In the *Drosophila* neuroectoderm, ectopic expression of the *Gsh2* homolog *ind* in the dorsal column inhibits expression of dorsal cell markers (C. Doe, personal communication). To test whether *Gsh2* can similarly suppress the expression of dorsal cell markers in mouse embryos, we expressed GSH2 in the early developing telencephalon using high-titer retroviruses. Delivery of *Gsh2*-containing retroviruses into the developing forebrain at E9.5 did not result in the inhibition of the normal expression of *Pax6* (both mRNA and protein), *Ngn2* or *Msx1* in the dorsal telencephalon when examined at E14.5 (Fig. 6A-H). *Pax6* mRNA or PAX6 protein expression in the developing cortex at E14.5 was additionally not inhibited by viral delivery of *Gsh2* to the forebrain at E8.5 (data not shown). The ability of *Gsh2* to drive ectopic expression of the ventral markers *Dlx2*, *Mash1* and RBP was also explored. Interestingly, while misexpression of *Gsh2* did not result in the ectopic expression of either *Dlx2* (Fig. 6I,J) or *Mash1* (data not shown) in the cerebral cortex, ectopic RBP expression was observed (Fig. 6K,L). Therefore, while *Gsh2* alone neither inhibited the expression of the dorsal markers *Pax6*, *Ngn2* and *Msx1*, nor drove ectopic expression of the ventral markers *Dlx2* or *Mash1*, it could induce RBP expression dorsally.

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Migration to the cerebral cortex and olfactory bulb in *Gsh2* mutants

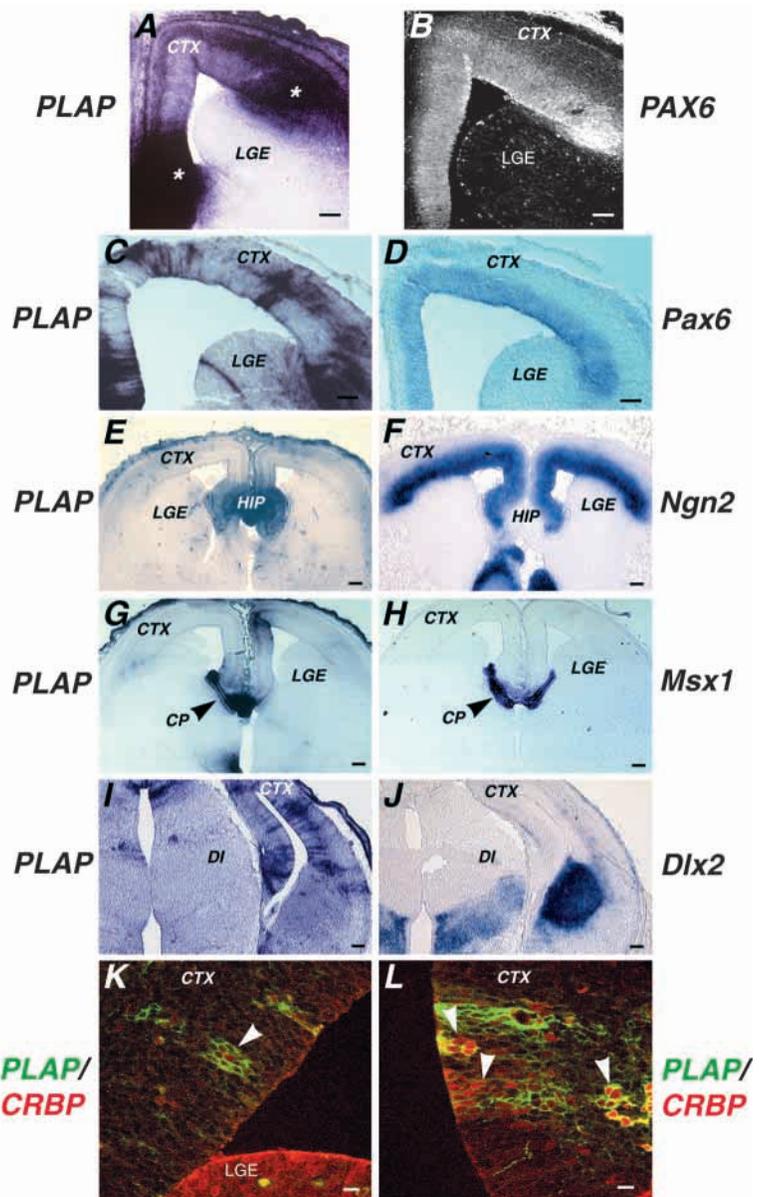
Both cell-tracing and genetic mutant analyses have recently lead to the hypothesis that the ventral telencephalon is the source of a majority of the interneurons found in the cerebral cortex and the olfactory bulb (reviewed in Anderson et al., 1999; Parnaveles et al., 2000). There are two main routes of migration that these cells are known to take. Starting late embryonically, striatal SVZ cells migrate to the olfactory bulb via the rostral migratory stream, where they differentiate into the interneurons of the periglomerular and granule cell layers

(reviewed in Alvarez-Buylla, 1997; Goldman and Luskin, 1998). Additionally, shortly after the appearance of the ganglionic eminences, large streams of cells are observed to leave the ventral telencephalon and tangentially migrate dorsally to populate the cortical plate and marginal zone of the developing cerebral cortex (de Carlos et al., 1996; Tamamaki et al., 1997; Meyer et al., 1998; Lavdas et al., 1999; J. G. C., S. Nery and G. F., unpublished). While it is currently not clear which ventral structures generate these distinct cell populations, a number of indirect lines of evidence suggest that the LGE is the major source of interneurons in the olfactory bulb while the MGE and CGE are the major sources of cerebral cortical interneurons (Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 1999; H. Wichterle, S. Nery, M. Alvarez-Dolado, L. Erskin, C. Mason, G. F., D. Turnbull and A. Alvarez-Buylla, unpublished). Analysis of *Gsh2* mutants revealed that the appearance of the interneuron population in the olfactory bulb was significantly delayed, while the generation of cerebral cortical interneurons was apparently unaffected (Fig. 7). Since *Gsh2* mutant mice display abnormal ventral telencephalic development, using a number of markers that are expressed by both cortical and olfactory bulb interneurons (*Gad67*, GABA, *Dlx1*, *Dlx2* and calbindin), we examined whether the loss of *Gsh2* affected the generation of either of these cell populations.

First, to determine whether development of interneurons in the olfactory bulb was affected in *Gsh2* mutants, we examined expression of *Dlx1*, *Dlx2*, *Gad67* and GABA in the olfactory bulb at E15.5 and E18.5. At E15.5, there was a significant reduction in the level of *Dlx1* (data not shown), *Dlx2* (Fig. 7A,B), *Gad67* (Fig.

7C,D) and GABA (data not shown) expression in the olfactory bulb. By E18.5, expression of *Dlx1* (data not shown), *Dlx2* (Fig. 7E,F) and *Gad67* (Fig. 7G,H) in the olfactory bulb of *Gsh2* mutants had almost completely recovered. However, expression of GABA (Fig. 7I,J) remained absent in the olfactory bulb. The appearance of *Gad67* and not GABA at E18.5 may reflect a delay in the differentiation to a mature GABAergic phenotype. While expression of *Dlx1*, *Dlx2* and *Gad67* was absent in the olfactory bulb of *Gsh2* mutants at E15.5, expression of these genes had significantly recovered in the developing striatum of *Gsh2* mutants (Fig. 3A-D,I,J). By E18.5, the age at which *Dlx1*, *Dlx2* and *Gad67* expression has achieved normal levels in the striatum in *Gsh2* mutants (data not shown), immature interneurons, as evidenced by the significant expression of *Dlx1*, *Dlx2* and *Gad67*, are now present in the olfactory bulb. These results suggest that the loss of *Gsh2* results in a delay in the ability of these cells to migrate to the olfactory bulb and differentiate into GABAergic interneurons.

Fig. 6. *Gsh2* misexpression does not inhibit the expression of dorsal markers but induces ectopic expression of RBP (CRBP). Gain-of-function analysis was undertaken using high-titer dicistronic retroviruses co-expressing both GSH2 and human placental alkaline phosphatase (PLAP) as a reporter. In all cases shown retrovirus was delivered to the developing forebrain at E9.5 and analysis carried out at E14.5. (A-J) Corresponding coronal sections were assayed for PLAP expression and expression of several genes expressed in the developing telencephalon. PLAP expression was determined by histochemical staining (A,C,E,G,I) or by localization of PLAP protein by immunocytochemistry (K,L). Expression of PAX6 (B) and RBP (K,L) protein was assayed by immunofluorescence. Expression of *Pax6* (D), *Ngn2* (F), *Msx1* (H) and *Dlx2* (J) mRNA was assayed by in situ hybridization. Despite viral expression in the cerebral cortex (A,C,I), hippocampus (E) or choroid plexus (arrowhead, G), expression of PAX6 protein (B), *Pax6* mRNA (D), *Ngn2* (F) or *Msx1* (arrowhead, H) was not repressed, while *Dlx2* was not ectopically expressed in the dorsal telencephalon (J), note normal *Dlx2* expression in the CGE and dorsally migrating cortical interneurons. (K,L) Confocal images of PLAP immunostaining (green) reveals that GSH2 misexpression results in the ectopic expression of RBP (red, white arrowheads) in the developing cortex just dorsal to the LGE. Notice the level of RBP is variable and typically less than the endogenous level found in the LGE (K), also shown in red. CGE, caudal ganglionic eminence; CP, choroid plexus; DI, diencephalon; HIP, hippocampus. Scale bars: 250 μ m (A-H); 50 μ m (I,J).



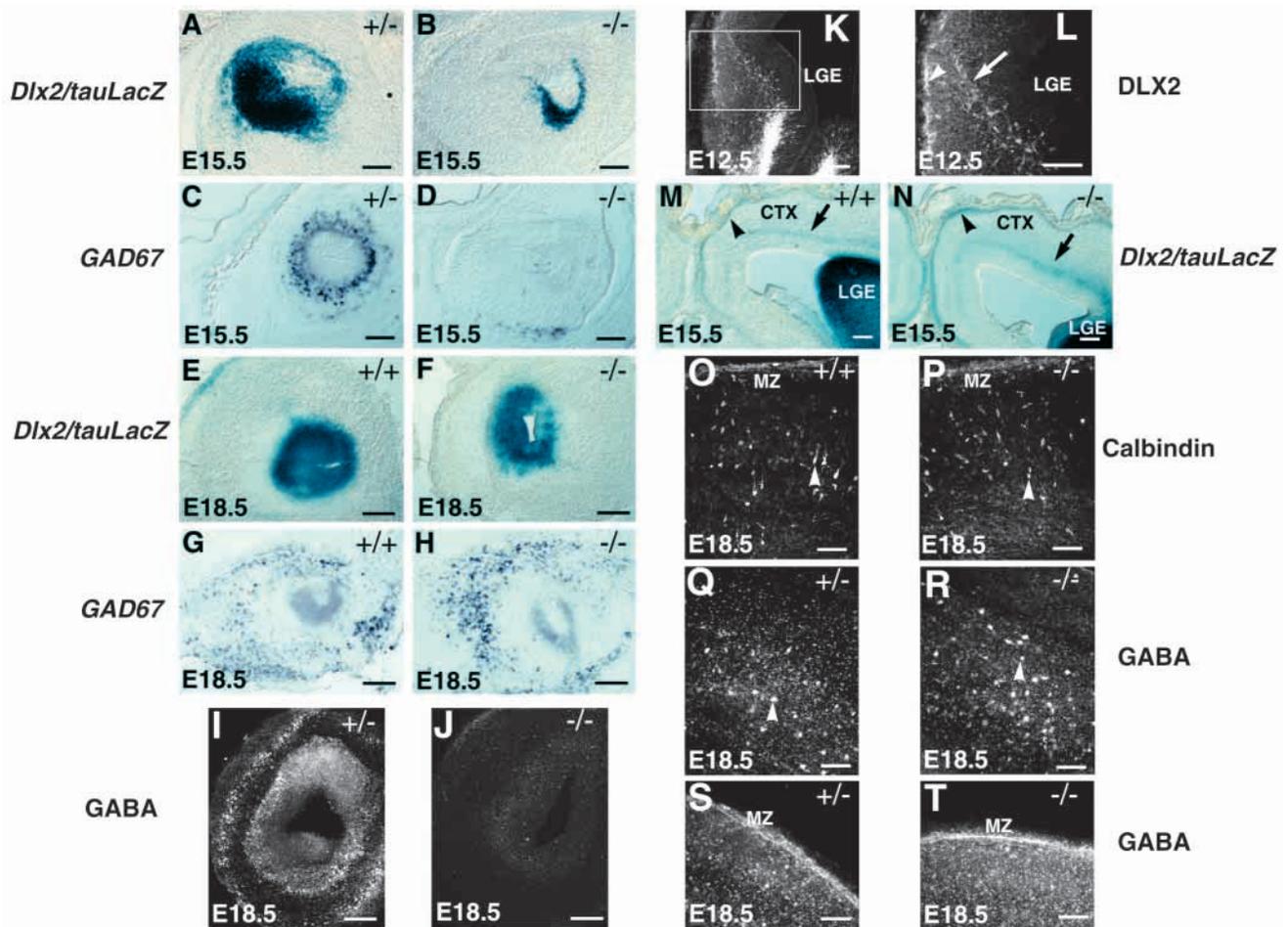


Fig. 7. A delay in the appearance of interneurons in the olfactory bulb is observed in *Gsh2* mutants. The absence of *Gsh2* has a profound effect on the timing of the appearance of the interneuron population within the olfactory bulb, but does not affect the appearance of the cortical interneuron or neocortical marginal zone populations. Analysis of coronal sections from the olfactory bulb (A–J) or the cerebral cortex (K–T) of control and mutant embryos is shown here. At E15.5, there is a significant reduction in the level of *Dlx2/tauLacZ* expression in the SVZ of the olfactory bulb, as revealed by X-gal staining (A,B) and *Gad67* (C,D) expression in the granule cell layer of the olfactory bulb, as revealed by in situ hybridization. At E18.5, *Dlx2/tauLacZ* expression is near normal levels (E,F), while no difference in *Gad67* expression is observed between controls and mutant embryos (G,H). (I,J) At E18.5, however, mature interneurons, as revealed by GABA immunostaining, remain absent from the olfactory bulb. (K,L) By contrast, at E12.5, significant migration to the dorsal telencephalon in *Gsh2* mutants is still observed. Many DLX2/ β -galactosidase-positive cells are seen putatively leaving the MGE/ventral LGE migrating through the mutant LGE dorsal to the developing cerebral cortex (K). Higher magnification of the boxed area (shown in L) reveals cells migrating to the SVZ/IZ (arrow) and MZ (arrowhead) of the cortex. Additionally, comparable levels of *Dlx2/tauLacZ* are observed in both the MZ (arrowheads) and SVZ/IZ (arrows) of the developing cortex at E15.5 (M,N). In the neocortex, equivalent numbers of both calbindin (O,P) and GABA (Q,R) immunopositive cells (arrowheads) are observed at E18.5. At E18.5, expression of calbindin (O,P) and GABA (S,T) in the neocortical MZ is unaffected. MZ, marginal zone. Scale bars: 200 μ m (A–N); 50 μ m (O–T).

In contrast, by a number of criteria, migration of interneurons to the cerebral cortex appeared unaffected. Expression of *Dlx2*, calbindin and GABA, markers of the developing cortical interneuron populations, was examined. At E12.5, during the earliest stage of ventral to dorsal migration, in the *Gsh2* mutants, many positive cells were observed apparently migrating from the ventral telencephalon through the mutant LGE to both the SVZ/intermediate zone (IZ) of the developing cortex and the neocortical marginal zone (MZ) (Fig. 7K,L). At E15.5, many *Dlx2*-positive cells were also observed leaving the developing striatum and apparently migrating dorsally along the SVZ/IZ boundary in both control and mutant embryos (Fig. 7M,N). Numerous *Dlx2*-positive

cells were also observed in the neocortical MZ at this age (Fig. 7M,N). Consistent with the observation that a robust migration of *Dlx2*-positive cells occurred in the absence of *Gsh2*, numerous calbindin-positive (Fig. 7O,P) and GABA-positive (Fig. 7Q,R) cells were also observed in the cortex of mutant embryos at E18.5. Quantitation of these cell populations at E18.5 revealed approximately equal numbers of calbindin-positive cells in control (mean 35.17 ± 11.51 cells/field) and mutant (mean 31.67 ± 5.39 cells/field) brains, as well as approximately equal numbers of GABA-positive cells in control (mean 25.83 ± 5.88 cells/field) and mutant (mean 27.17 ± 4.26 cells/field) brains. Additionally, at E18.5, the GABA-positive (Fig. 7S,T) and calbindin-positive (Fig. 7O,P)

populations in the neocortical MZ appear unaffected. Taken together, these results suggest that, while *Gsh2* is required for the normal development of olfactory bulb interneurons, it is not required for the generation of the majority of ventrally derived interneurons in cortical plate and MZ of the cerebral cortex.

DISCUSSION

In this study we demonstrate that *Gsh2* is downstream of SHH signaling and is a key regulator of multiple aspects of ventral telencephalic development. The early absence of *Dlx1*, *Dlx2*, *Mash1* and *Ebf1* expression in the LGE supports the hypothesis that *Gsh2* lies high in the genetic hierarchy controlling the early specification and development of the LGE. Our evidence also suggests that *Gsh2* is required for the early specification of GABAergic interneurons in the LGE and their subsequent migration to the developing olfactory bulb. Moreover, *Gsh2* is essential for the establishment of the molecularly defined border between the intermediate (LGE) and dorsal (cortical) domains of the telencephalon.

Conservation of neural patterning mechanisms in *Drosophila* and mice

The striking sequence homology between the *Drosophila vnd*, *ind* and *msh* genes with the vertebrate *Nkx*, *Gsh* and *Msx* genes, respectively, has previously raised the interesting possibility that aspects of the mechanism establishing domains within the neuraxis is conserved across species (D'Alessio and Frasch, 1996; Isshiki et al., 1997; Chu et al., 1998; McDonald et al., 1998; Weiss et al., 1998). Consistent with this hypothesis, in the vertebrate spinal cord, the mammalian homologs *Nkx2.2*, *Gsh1* and *Msx1* are expressed in a non-overlapping pattern resembling the ventral to dorsal expression of their *Drosophila* homologues. Investigation of the role that *Nkx* genes play in development of the mammalian nervous system has lent further support to this hypothesis. In the mouse spinal cord, the loss of *Nkx2.2* results in an expansion of the adjacent dorsal motoneuron population into the more ventral V3 interneuron domain which normally expresses *Nkx2.2* (Briscoe et al., 1999). Although, it is currently not known whether the loss of *Nkx2.2* also results in a predicted expansion of *Gsh1* in the spinal cord. In the forebrain, the loss of *Nkx2.1* results in an apparent transformation of the ventral MGE to a more intermediate LGE fate (Sussel et al., 1999). Interestingly, we show that *Nkx2.1* and *Gsh2* are expressed in complementary domains early in telencephalic development prior to the appearance of the ganglionic eminences. We are currently exploring whether, similar to *vnd* and *ind* in *Drosophila*, *Nkx2.1* and *Gsh2* also interact in the establishment of the ventral (MGE)/intermediate (LGE) boundary in the mammalian telencephalon.

Although our early expression data suggests that *Gsh2* may be involved in the establishment of the MGE/LGE boundary, we clearly demonstrate that *Gsh2* is necessary for the establishment of the dorsal/intermediate (cortical-striatal) boundary in the vertebrate telencephalon. Similar to the expansion of the dorsal column into the intermediate column in the *Drosophila ind* mutant (Weiss et al., 1998), an expansion of cortical markers into the LGE occurs in the absence of *Gsh2*. Notably, however, expression of both RBP and *Ebf1*, although

reduced, is maintained in the LGE, suggesting that the transformation to a dorsal fate is not complete. Consistent with previous work (D'Alessio and Frasch, 1996; Isshiki et al., 1997; Chu et al., 1998; McDonald et al., 1998; Weiss et al., 1998), these results support the hypothesis that aspects of dorsal/ventral patterning and the establishment of gene expression domains throughout the neuraxis are at least partially conserved through evolution.

Comparison of spinal cord and forebrain patterning mechanisms

The mechanism by which SHH patterns the ventral neuraxis in vertebrates has recently been a subject of much investigation (reviewed in Tanabe and Jessell, 1996; Rubenstein et al., 1998). In the vertebrate spinal cord, a model has been put forth suggesting that the generation of some specific cell types within the ventral spinal cord is established via cross-repressive interactions between a distinct set of homeodomain proteins that are expressed in overlapping domains (Briscoe et al., 2000). These proteins fall into two categories: those induced by SHH in a concentration dependent manner (NKX2.2 and NKX6.1), and those repressed by SHH (PAX6, PAX7, IRX3, DBX1 and DBX2). The combinatorial interactions of these proteins in the five defined ventral domains of the spinal cord are hypothesized to result in the generation of the five distinct classes of ventral neurons. While members of the *Nkx* family of homeodomain genes appear to play similar roles in the establishment of the ventral-most boundary in both the spinal cord and forebrain (Briscoe et al., 1999; Sussel et al., 1999), it is currently not clear whether the *Gsh1* and *Gsh2* homeodomain genes, which are expressed in the intermediate domain of the spinal cord, (Hsieh-Li et al., 1995; Valerius et al., 1995; Szucsik et al., 1997) function analogously throughout the neural tube. Thus, it will be interesting to determine whether these genes are also involved in boundary formation in the spinal cord.

Although the forebrain may be at least partially patterned via the mutually repressive interactions of homeodomain patterning genes, patterning of the forebrain appears to be more complex. Recent analysis suggests that the actions of the homeodomain genes are intertwined with the actions of *Mash1* and *Ngn* genes that encode bHLH transcription factors (Fode et al., 2000), which are expressed in ventral and dorsal domains in the telencephalon, respectively. Interestingly, the loss of either *Ngn2* alone, or both *Ngn1* and *Ngn2*, leads to a partial respecification of dorsal cortical cells to a more ventral fate. This conversion appears to be dependent on the abnormal expression of *Mash1* dorsally (Fode et al., 2000). Therefore, within the forebrain interactions between both homeodomain (e.g. GSH2 and NKX2.1) and bHLH (e.g. MASH1 and NGN2) proteins may be necessary for proper patterning and regionalization. However, the interactions between these genes are likely to be complex, as suggested by the seemingly paradoxical results obtained by both the *Gsh2* analysis undertaken here and the previous analysis of *Mash1*. While *Gsh2* is necessary to inhibit *Ngn2* expression in the LGE, in our viral gain-of-function assays *Gsh2* is not sufficient to inhibit *Ngn2* expression dorsally. Furthermore, while ectopic *Mash1* expression in the dorsal telencephalon can impose a ventral telencephalic phenotype on cells previously programmed to a dorsal fate (Fode et al., 2000), there is not an

expansion of *Ngn2* into the LGE in the absence of *Mash1* (Casarosa et al., 1999). Therefore, it is apparent that a complete understanding of dorsal-ventral patterning cannot be explained by the known patterning molecules and clearly awaits the identification of novel genes or, at the very least, a better understanding of known candidates.

Genetic interactions governing early telencephalic development

The morphological reduction in the size of the LGE in *Gsh2* mutants that is accompanied by the initial loss of genes that mark early LGE development suggests that *Gsh2* lies high in the genetic cascade controlling LGE specification and development. Notably, early in LGE development, there is a striking loss of *Dlx1*, *Dlx2*, *Mash1*, *Ebf1* and *Gad67* expression in the LGE of *Gsh2* mutant embryos. Loss-of-function mutants of *Mash1* (Casarosa et al., 1999), or the combination of *Dlx1* and *Dlx2* (Anderson et al., 1997a), have revealed distinct roles for each of these genes in striatal development. The loss of *Mash1* results in a reduction in the generation of early born striatal neurons generated in the VZ that comprise the perirhinal and nucleus accumbens regions of the basal ganglia (Casarosa et al., 1999). The combined loss of *Dlx1* and *Dlx2* conversely results in a failure to generate later born striatal matrix neurons that are hypothesized to be derived from the striatal SVZ (Anderson et al., 1997a). Interestingly, in the absence of *Gsh2* there is an apparent defect in the generation of early born striatal neurons, while development of later born striatal matrix neurons appears unaffected. This result might be explained by our observation that there is an early loss of *Mash1* in the absence of *Gsh2*, but that recovery of *Dlx1* and *Dlx2* expression by E15.5 in the striatal SVZ is perhaps sufficient to allow for the generation of later born matrix neurons.

The early loss of *Gad67* expression in the LGE also suggests that *Gsh2* is required, either directly or indirectly, for the initial appearance of the GABAergic phenotype. It has been speculated that members of the *Dlx* family, perhaps in combination with bHLH genes such as *Mash1*, control specification of the GABA neurotransmitter phenotype in the ventral telencephalon (Anderson et al., 1999; Fode et al., 2000). Gain-of-function experiments have shown that ectopic expression of either *Dlx2* in vitro (Anderson et al., 1999) or *Mash1* in vivo (Fode et al., 2000) induces markers of GABAergic interneurons. However, in the absence of *Mash1*, or *Dlx1* and *Dlx2*, GABAergic cells are still generated. Therefore, while these genes may be sufficient to induce this cell type, it does not appear that either *Mash1*, or *Dlx1* and *Dlx2*, are necessary for the specification of all interneurons. Perhaps the early failure of GABAergic specification in the absence of *Gsh2* is simply a result of the early absence of *Dlx1*, *Dlx2* and *Mash1* expression in the LGE. Alternatively, other transcription factors may be regulated by *Gsh2* and act in coordination with *Dlx1*, *Dlx2* and/or *Mash1* in the specification of ventral interneurons.

Although it is clear that *Gsh2* is essential for the proper early patterning of the telencephalon, the mechanism(s) by which this occurs is not as well understood. The results presented here suggest two potential pathways of genetic interaction. The first model proposes that *Mash1*, *Dlx1* and *Dlx2* lie directly downstream of *Gsh2*. Alternatively, the expression of *Mash1*,

Dlx1 and *Dlx2* may be downstream of SHH, but only indirectly dependent on *Gsh2* gene function. In this second model, the SHH-mediated induction of *Mash1*, *Dlx1* and *Dlx2* in the LGE is repressed by the expansion of dorsal inhibitory factor(s), normally excluded from the LGE by *Gsh2*.

One potential candidate that could contribute to the negative regulation of *Mash1*, *Dlx1* and *Dlx2* is *Ngn2*. Consistent with this hypothesis, the ventral expansion of *Ngn2* in the mutant LGE is accompanied by a complementary regression of *Mash1*, *Dlx1* and *Dlx2* expression. Furthermore, in embryos lacking *Ngn2* there is conversion of dorsal telencephalic cell fate to a more ventral fate, as evidenced by the ectopic expression of *Mash1*, *Dlx1* and *Dlx2* within the developing cortex (Fode et al., 2000), further suggestive of an inhibitory interaction between these ventral patterning genes and *Ngn2*. The recent studies by Toresson et al. (2000) demonstrate that in the absence of both *Gsh2* and *Pax6*, LGE expression of both DLX (as recognized by a pan-DLX antibody) and MASH1 is rescued, and *Ngn2* is no longer expanded into the mutant LGE at E12.5. This observation is consistent with the hypothesis that *Mash1* and *Dlx2* are repressed by the expansion of dorsal genes, such as *Pax6* and *Ngn2*, and only indirectly controlled by *Gsh2*. Moreover, the lack of ectopic *Dlx2* and *Mash1* expression in the *Gsh2* gain-of-function experiments is also consistent with the hypothesis that induction of *Dlx2* and *Mash1* is regulated only indirectly by *Gsh2*. Indeed, we have shown that SHH can induce both *Dlx2* (Gaiano et al., 1999) and *Mash1* (S. Nery and G. F., unpublished) when ectopically expressed in the developing cerebral cortex in vivo, suggesting that these genes are also downstream targets of SHH. A further test of these two models would be to examine if SHH can induce expression of *Mash1* and *Dlx2* in the absence of *Gsh2*. The combinatorial knockouts of *Gsh2* and the *Ngn* genes may also be informative in elucidation of the interactions between these genes.

Clearly, in addition to *Gsh2*, other genes play crucial roles in striatal development. Although *Gsh2* has the capacity to induce expression of RBP, a marker restricted to the LGE, its expression is maintained in the *Gsh2* mutant LGE. This suggests that the regional specification of radial glia in the LGE can occur in the absence of *Gsh2*. The absence of RBP expression (J. G. C. and G. F., unpublished) and the loss of a striatum (Chiang et al., 1996) in *Shh* mutant embryos suggests that genes downstream of SHH, other than *Gsh2*, are required for RBP expression. Furthermore, while there are profound early defects in patterning of the telencephalon in the absence of *Gsh2*, by E15.5 there is a dramatic compensation for the early loss of patterning genes in the LGE, as well as a re-establishment of markers that define the cortical-striatal boundary. It is currently unclear what the molecular mechanism governing this recovery is. Identification of other genes involved in striatal development may shed light on this phenomenon.

Appearance of interneurons is delayed in the olfactory bulb but unaffected in the cerebral cortex in *Gsh2* mutants

The delay in the appearance of interneurons in the olfactory bulb may reflect either a delay in their migration or, if these cells are born locally in the olfactory bulb, a defect in their regional generation and/or differentiation. Although the rostral

migratory stream has been described as arising late in embryonic development (reviewed in Alvarez-Buylla, 1997; Goldman and Luskin, 1998), we observe interneurons in the olfactory bulb by as early as E15.5. Recent experiments have shown that the embryonic LGE also has the capacity to contribute to cells of the adult rostral migratory stream (Wichterle et al., 1999), suggesting that the olfactory bulb interneurons normally observed at E15.5 may also arise via this route. Despite the absence of *Dlx1*, *Dlx2* and *Gad67* expression in the mutant olfactory bulb at E15.5, expression of these genes has significantly recovered in the mutant developing striatum. In *Gsh2* mutants at E18.5, an age at which *Dlx1*, *Dlx2* and *Gad67* expression has achieved normal levels in the mutant striatum, *Gad67*-positive cells are present in the mutant olfactory bulb, coincident with the presence of *Dlx1*- and *Dlx2*-positive cells. This lag in the appearance of interneurons in the olfactory bulb, combined with the evidence suggesting that the rostral migratory stream is established by mid-neurogenesis, support the hypothesis that there is a delay in the migration of interneurons from the LGE/developing striatum to the olfactory bulb.

In contrast, migration of cells to the cerebral cortex from the ventral telencephalon is apparently unaffected in *Gsh2* mutants. Since the LGE is primarily affected in embryos lacking *Gsh2*, this result is consistent with recent genetic mutant and cell transplantation studies suggesting that the migration of the majority of cortical interneurons in the cortex arises not from the LGE, but from other ventral structures, particularly the MGE and CGE (Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 1999; H. Wichterle, S. Nery, M. Alvarez-Dolado, L. Erskin, C. Mason, G. F. D. Turnbull and A. Alvarez-Buylla, unpublished). Our analysis of the ventral-to-dorsal migration of calbindin and GABA-positive interneurons in *Gsh2* mutant embryos, however, does not rule out the possibility that specific subpopulations of ventrally derived cells within the developing cortex may be affected. Future analysis of the relative contributions of the MGE, LGE, CGE and other ventral regions of the telencephalon should be fruitful towards understanding the origins of the heterogeneous interneuron populations in the cerebral cortex.

Conclusions

In summary, our results demonstrate that *Gsh2* is a key regulator of downstream SHH patterning events in the ventral forebrain. Additionally, similar to its *Drosophila* homolog, *ind*, *Gsh2* functions in an apparent evolutionarily conserved manner in the molecular establishment of boundaries in the nervous system. Interestingly, *Gsh2* is also essential for the generation of early born subpopulations of striatal neurons, and the proper specification of LGE generated interneurons and their subsequent migration to the olfactory bulb. While *Gsh2* clearly plays an important role in ventral forebrain development, a more complete understanding of the multiple aspects of development of the LGE awaits the identification of additional regulatory genes.

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