

# A role for *Gsh1* in the developing striatum and olfactory bulb of *Gsh2* mutant mice

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Accepted 31 August 2001

## SUMMARY

We have examined the role of the two closely related homeobox genes *Gsh1* and *Gsh2*, in the development of the striatum and the olfactory bulb. These two genes are expressed in a partially overlapping pattern by ventricular zone progenitors of the ventral telencephalon. *Gsh2* is expressed in both of the ganglionic eminences while *Gsh1* is largely confined to the medial ganglionic eminence. Previous studies have shown that *Gsh2*<sup>-/-</sup> embryos suffer from an early misspecification of precursors in the lateral ganglionic eminence (LGE) leading to disruptions in striatal and olfactory bulb development. This molecular misspecification is present only in early precursor cells while at later stages the molecular identity of these cells appears to be normalized. Concomitant with this normalization, *Gsh1* expression is notably expanded in the *Gsh2*<sup>-/-</sup> LGE. While no obvious defects in

striatal or olfactory bulb development were detected in *Gsh1*<sup>-/-</sup> embryos, *Gsh1/2* double homozygous mutants displayed more severe disruptions than were observed in the *Gsh2* mutant alone. Accordingly, the molecular identity of LGE precursors in the double mutant is considerably more perturbed than in *Gsh2* single mutants. These findings, therefore, demonstrate an important role for *Gsh1* in the development of the striatum and olfactory bulb of *Gsh2* mutant mice. In addition, our data indicate a role for *Gsh* genes in controlling the size of the LGE precursor pools, since decreasing copies of *Gsh2* and *Gsh1* alleles results in a notable decrease in precursor cell number, particularly in the subventricular zone.

Key words: Lateral ganglionic eminence, Subventricular zone, *Gsh*, Homeobox genes, Mouse

## INTRODUCTION

The mammalian striatum is the major component of the basal ganglia and serves an essential function in processing cortical information concerning cognition and voluntary movement [for review, see Gerfen (Gerfen, 1992)]. The neurons that comprise the striatum have their origin in two prominent elevations called the medial and lateral ganglionic eminences (MGE and LGE, respectively). The GABAergic medium-sized spiny projection neurons, which constitute the vast majority of striatal neurons, are generated from the LGE (Pakzaban et al., 1993; Deacon et al., 1994; Olsson et al., 1995; Olsson et al., 1998). Conversely, the majority of striatal interneurons appear to be generated from the MGE (Olsson et al., 1998; Marin et al., 2000).

Patterning of the LGE during early telencephalic development is not, as yet, fully understood. Formation of its ventromedial border with the MGE is dependent upon the expression of *Nkx2.1* (now known as *Titf1*) (Sussel et al., 1999). Furthermore, the dorsolateral limit of the LGE is controlled by LGE expression of *Gsh2* (Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001) and cortical expression of *Pax6* (Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001). Once the domain of LGE progenitors is established,

a number of genes, such as *Dlx1/2* (Anderson et al., 1997), *Mash1* (also known as *Ascl1*) (Casarosa et al., 1999) and *Ebf1* (Garel et al., 1999) are known to be required for these cells to give rise to the striatal complex. The LGE, or a domain continuous with the LGE, is also the source of olfactory bulb interneurons (Wichterle et al., 1999) which are generated from late embryonic stages into adulthood (Altman, 1969; Lois and Alvarez-Buylla, 1994; Luskin, 1993). In keeping with this, many mutants with an LGE/striatal phenotype also display defects in the olfactory bulb interneuron populations, e.g. *Dlx1/2* mutants (Bulfone et al., 1998) and *Mash1* mutants (Casarosa et al., 1999).

As mentioned above, the homeobox gene *Gsh2* is important for establishing the boundary between the LGE and cortical ventricular zones (VZ). Mutations of *Gsh2* leads to the generation of a considerably smaller striatum (Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001), with the olfactory tubercle being particularly affected (Toresson et al., 2000). This defect depends to a large extent on the ectopic expression of *Pax6* in early LGE precursor cells, since removal of this gene on the *Gsh2*<sup>-/-</sup> background significantly improves striatal development (Toresson et al., 2000). *Gsh2* is thus playing an important role in striatal development by maintaining the correct molecular identity of early LGE precursors. At later

stages of striatal development the molecular identity of *Gsh2*<sup>-/-</sup> precursors appears to normalize. This result could indicate that *Gsh2*-independent mechanisms are important for late stages of striatal neurogenesis. However, before making this conclusion one must take into consideration the possibility of compensatory mechanisms. In this respect, the closely related gene, *Gsh1* (Valerius et al., 1995), is normally expressed in a small subset of the *Gsh2*-expressing LGE cells at the border with the MGE. Thus it is possible that this gene might compensate for the loss of *Gsh2* in the generation of striatal and olfactory bulb neurons.

Interestingly, we observe a dramatic expansion of the *Gsh1* expression domain in the *Gsh2* mutant LGE. In order to examine the role of *Gsh1* in striatal and olfactory bulb development, we studied *Gsh1* single and *Gsh1/2* double homozygous mutants. Although the striatum and olfactory bulb of *Gsh1* mutants was not noticeably different from wild types, these structures were more severely disturbed in the *Gsh1/2* double mutants than in *Gsh2* single mutants. These findings demonstrate that *Gsh1*, which is not normally required for striatal and olfactory bulb development, plays an important role in the development of these forebrain regions in *Gsh2* mutant embryos.

## MATERIALS AND METHODS

### Genotyping of animals

Genotyping of *Gsh1* (Li et al., 1996) and *Gsh2* (Szucsik et al., 1997) mouse embryos and adults was carried out by PCR. The *Gsh1* wild-type allele was detected using the primers *gsh1*-5': GAAAATAAACCCGAAAAGGGAAGAGGAGGG and *gsh1*-3': ATCCGAAGGGAGGGAAGGACGCCTTGAGTA, which give an approximately 700 bp band. The targeted *Gsh1* allele was detected using the primers *gsh1*-5' (above) and TGCTAAAGCGCATGCTCC-AGACTGCCTT (giving a 300 bp band). The *Gsh2* gene was detected using the primers ATGGATGTGTTGGGTTAGACTGGGTTCTGG and TGCTTCACGCGACGGTTCTGAAACCATATT giving a 280 bp band. The targeted *Gsh2* allele was detected with primers neo5': ACTGGGCAACAGACAATCGGCT and neo3': TATTCGGCAAGC-AGGCATCGCCAT (500 bp band). All embryos that were determined to be double homozygotes by PCR were also confirmed by GSH2 immunohistochemistry and in situ hybridization for *Gsh1* on sections. Note, the targeted *Gsh1* locus lacks almost the entire coding sequence (Li et al., 1996).

### Histological analysis

Embryos were fixed and sectioned as previously described (Toresson, 2000).

### Immunohistochemistry

Immunostaining was performed on slide-mounted sections. Briefly,

after quenching in 3% H<sub>2</sub>O<sub>2</sub> the primary antibody was added to the slides in KPBS with 2% normal swine serum and 0.25% Triton X-100. Incubation was overnight at room temperature (RT). Following rinses in KPBS, biotin-conjugated swine anti-rabbit secondary antibodies (1:200, DAKO) were incubated for 2 hours at RT before rinsing in KPBS. The ABC elite kit (Vectastain) was used for detection with 3',3' diaminobenzidine (DAB) as the chromogen. Primary antibodies used were: ISL1 (1:400, provided by T. Edlund), SOX1 (1:500, provided by R. Lovell-Badge), DARPP-32 (PPP1R1B; 1:1000, Chemicon),  $\mu$ -opiate receptor ( $\mu$ -OR) (1:2000, IncStar), CaBP (1:1000, provided by P. Emson), DLX (1:400, provided by G. Panganiban), GAD<sub>67</sub> (1:5000, Chemicon), TH (1:5000, Chemicon), PAX6 (1:500, provided by S. Wilson), MASH1 (1:2500, provided by M. Nakafuku), NGN2 (ATO4; 1:3000, provided by M. Nakafuku), NKX2.1 (TITF1; 1:1300, provided by R. DiLauro) and Ki67 (1:100, Dianova). All primary antisera were raised in rabbits.

### In situ hybridization

In situ hybridization was performed as previously described (Toresson et al., 1999). Probes used were; *Gsh1*: full-length cDNA (provided by S. S. Potter) and *Raldh3* (EST-clone, GenBank accession no. AA790530; HGMP Resource Centre).

### Toluidine Blue staining and counting of mitotic figures

Slides were rapidly immersed in a solution of 0.5% Toluidine Blue in dH<sub>2</sub>O and subsequently washed in dH<sub>2</sub>O until staining was considered optimal. Slides were then quickly taken through a series of washes of increasing ethanol concentration and finally in xylene. Every twelfth section was analysed and cells in all stages of mitosis were included. Mitotic figures were assigned to the ventricular zone (VZ) if they were located at the luminal (i.e. ventricular) surface. All mitotic figures found deeper were assigned to the subventricular zone (SVZ).

### TUNEL

TUNEL analysis was performed on E12.5, E16.5 and E18.5 embryos using the in situ Cell Death Detection Kit (Boehringer Mannheim/Roche).

### Volumetric analysis

The volume of each striatum was estimated by stereology using the Castgrid software (Olympus, DK). The area positive for ISL1 or SOX1 was delineated on each section and the area measured by the computer. The striatal volume of each section was determined by multiplying the measured area by the thickness of the section (i.e. 12  $\mu$ m). The total volume was then determined by adding the volume of each section and then multiplying by 12 (since every 12<sup>th</sup> section was measured).

## RESULTS

### *Gsh2*<sup>-/-</sup> embryos exhibit disruptions in the development of the striatum and olfactory bulb

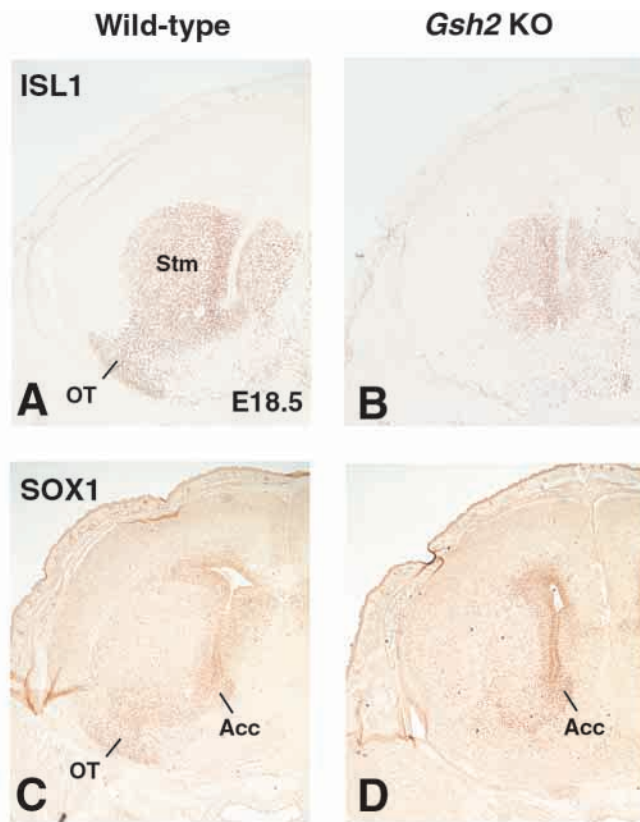
*Gsh2* gene function has recently been shown to be required for

**Table 1. Striatal volumes in *Gsh1* and *Gsh2* single and double homozygous mutants at E18.5**

	Wild type volume in mm <sup>3</sup> (% of wild type)	<i>Gsh2</i> <sup>-/-</sup> volume in mm <sup>3</sup> (% of wild type)	<i>Gsh1</i> <sup>-/-</sup> volume in mm <sup>3</sup> (% of wild type)	<i>Gsh1</i> <sup>-/-</sup> ; <i>Gsh2</i> <sup>-/-</sup> volume in mm <sup>3</sup> (% of wild type)
ISL1 volume (i.e. striatal complex)	1.99±0.08 (100)	0.90±0.02* (45)	1.84±0.09 (93)	0.41±0.02* <sup>‡</sup> (21)
SOX1 volume (i.e. ventral striatum)	0.51±0.05 (100)	0.16±0.01* (31)	0.43±0.01 (84)	0.06±0.01* <sup>‡</sup> (12)
ISL1 <sup>+</sup> SOX1 <sup>-</sup> volume (i.e. dorsal striatum)	1.48±0.13 (100)	0.74±0.02* (50)	1.41±0.08 (95)	0.35±0.03* <sup>‡</sup> (24)

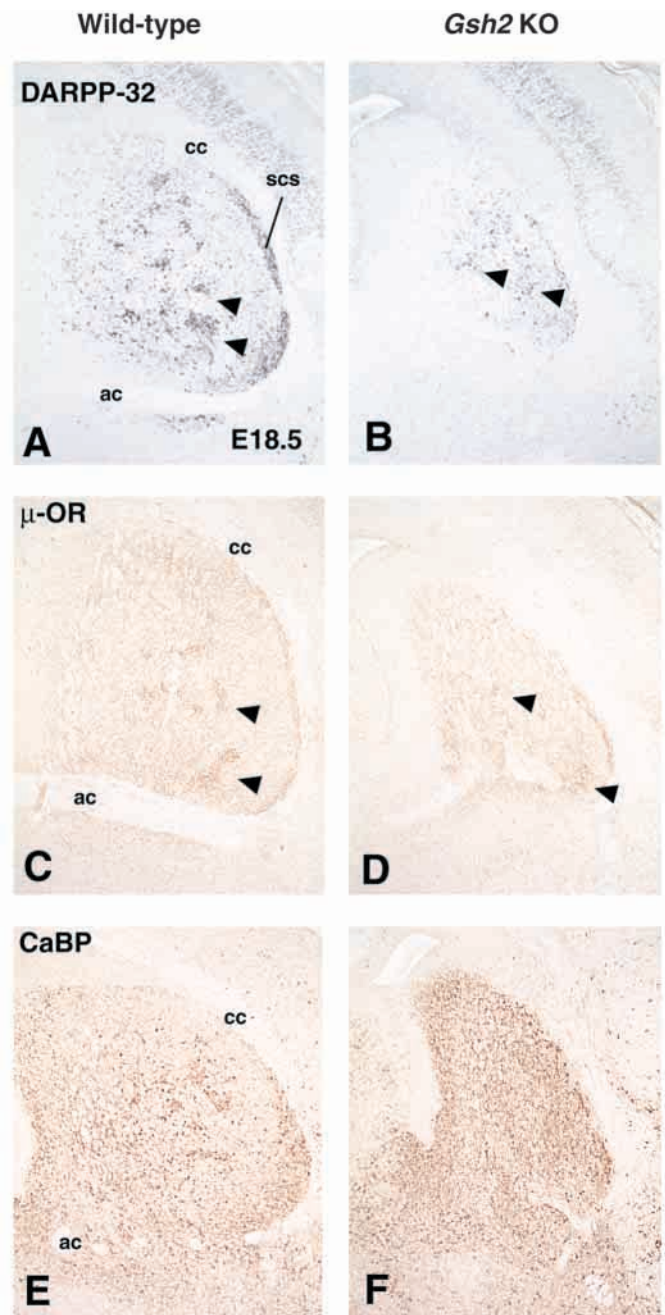
*n*=4 for each allele. The ISL1<sup>+</sup> SOX1<sup>-</sup> volume was determined by subtracting the SOX1<sup>+</sup> volumes from the ISL1<sup>+</sup> region.

\*Significant from wild type at 99%; <sup>‡</sup>significant from *Gsh2*<sup>-/-</sup> at 99%, one way ANOVA with the Fisher PLSD post-hoc test.



**Fig. 1.** The striatal complex at E18.5 in wild types and *Gsh2* homozygous mutants. Immunostaining for ISL1 (A,B) delineates the entire striatal complex while SOX1 staining (C,D) selectively marks the olfactory tubercle and portions of the nucleus accumbens (Acc) (i.e. ventral striatum). The *Gsh2* mutant displays a significant reduction in size of the striatal complex (B), which is most prominent in the ventral striatum, in particular within the olfactory tubercle (OT) region (B,D). Stm, striatum.

the correct molecular identity of LGE precursor cells at early stages of striatal development (Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001). Loss of *Gsh2* gene function leads to developmental abnormalities in the structures that comprise the striatal complex, including the dorsal striatum, nucleus accumbens and olfactory tubercle. The LIM-homeodomain protein, Islet 1 (ISL1) marks the overwhelming majority of neurons of the striatal complex (Fig. 1A). Quantification of ISL1-expressing cells showed a 55% reduction in the volume of the striatal complex in the E18.5 *Gsh2* mutant, as compared to wild types (Fig. 1B; Table 1). Our previous study demonstrated that the olfactory tubercle is particularly defective in the *Gsh2* mutants (Toresson et al., 2000). Here we have examined the expression of the HMG-box protein SOX1, which marks cells in the ventral striatum (including both the nucleus accumbens and olfactory tubercle) (Fig. 1C). *Gsh2* mutants displayed a 69% reduction in the region of SOX1-expressing cells as compared to wild types (Table 1). Interestingly, the expression of SOX1 in the nucleus accumbens region appears to be less affected than that in the olfactory tubercle (Fig. 1D). Although the ventral striatum is most severely reduced in the *Gsh2* mutants, the ISL1-positive, SOX1-negative region (i.e. dorsal striatum) is also significantly reduced (50% of wild type) (Table 1).



**Fig. 2.** Patch-matrix organization at E18.5 in wild types and *Gsh2* homozygous mutants. DARPP-32 immunohistochemistry outlines the dorsal striatum and shows intense labeling in striatal patches and the subcallosal streak (scs) of the wild type (A). Labeling can still be detected in the *Gsh2* mutant but patches are fewer and smaller and labeling of the scs is weaker (B). At this stage, immunohistochemistry for the  $\mu$ -OR shows patchy distribution and scs labeling in the wild type (C) and to a lesser extent in the *Gsh2* mutant (D). Arrowheads in A-D show representative patches labeled with either DARPP-32 or  $\mu$ -OR. CaBP expression in the *Gsh2* mutant striatum (F) appears to be increased as compared to wild type (E). ac, anterior commissure; cc, corpus callosum.

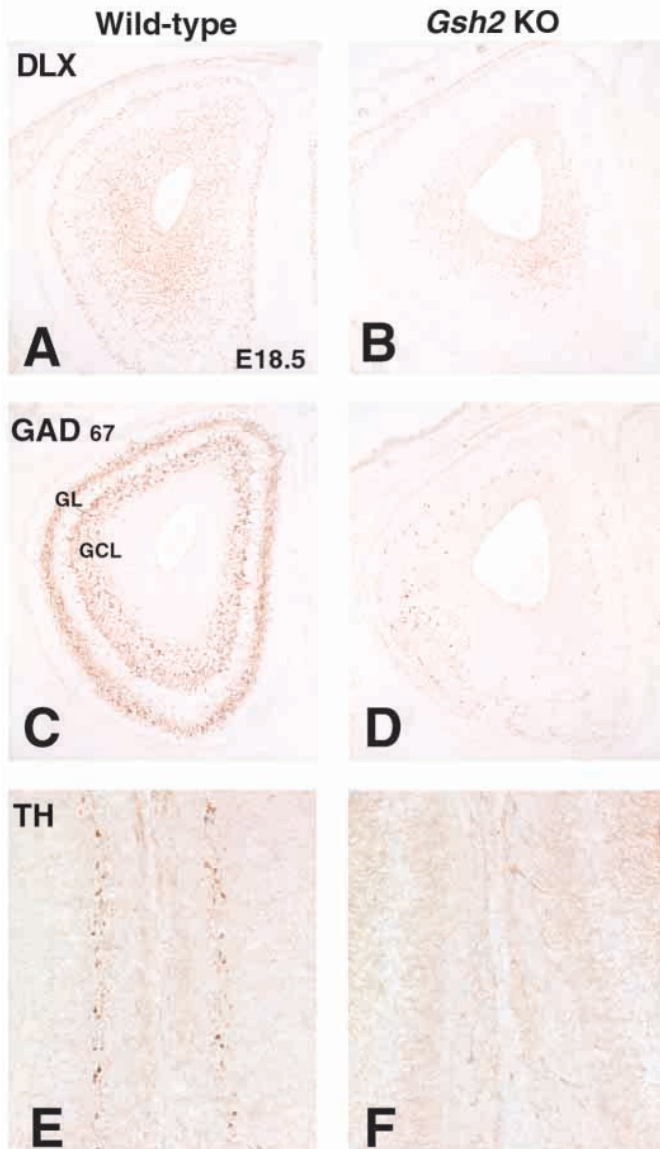
The striatum is composed of two distinct neuroanatomical and neurochemical compartments termed the patch and matrix [for review, see Gerfen (Gerfen, 1992)]. In the adult, DARPP-



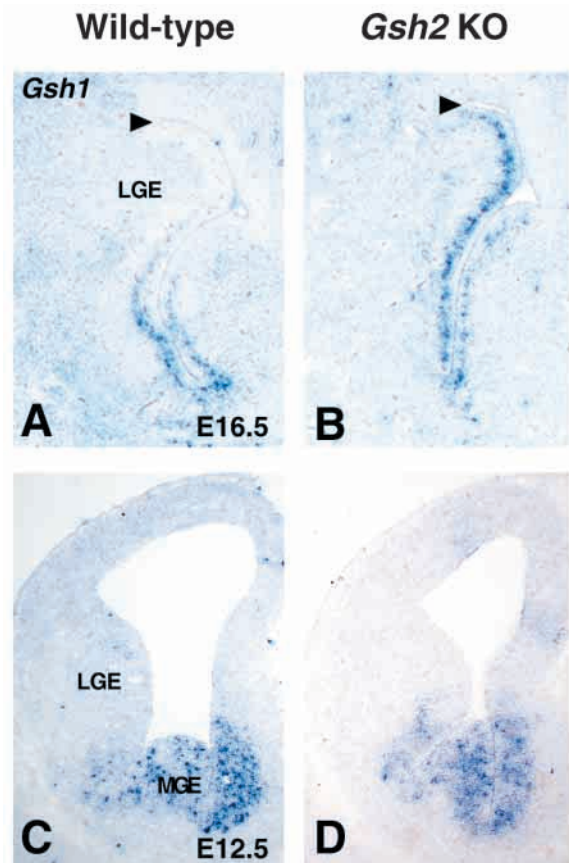
32 is expressed by essentially all striatal projection neurons (i.e. in both the patch and matrix compartments) (Anderson and Reiner, 1991) while, at prenatal stages, it is largely restricted to patch neurons (Foster et al., 1987). In the *Gsh2*<sup>-/-</sup> striatum, at E18.5, there is a clear reduction in the number of DARPP-32 positive cells (Fig. 2B) and the patches formed are smaller and fewer than in the wild type (Fig. 2A). In addition, staining

for the  $\mu$  opiate receptor ( $\mu$ -OR), which is a reliable patch marker even into adulthood (Moon Edley and Herkenham, 1984), confirms the DARPP-32 result demonstrating the existence of smaller patches in the E18.5 *Gsh2* mutant striatum (Fig. 2D). The matrix compartment, as marked by Calbindin (CaBP) expression (Gerfen et al., 1985), appears to be less affected in the *Gsh2* mutant. Indeed, it seems that more neurons express high levels of CaBP in *Gsh2* mutants than in wild types (Fig. 2E,F). Thus both patch and matrix neurons are formed in the *Gsh2* mutant striatum, however, their proportions are altered as compared to that in wild types.

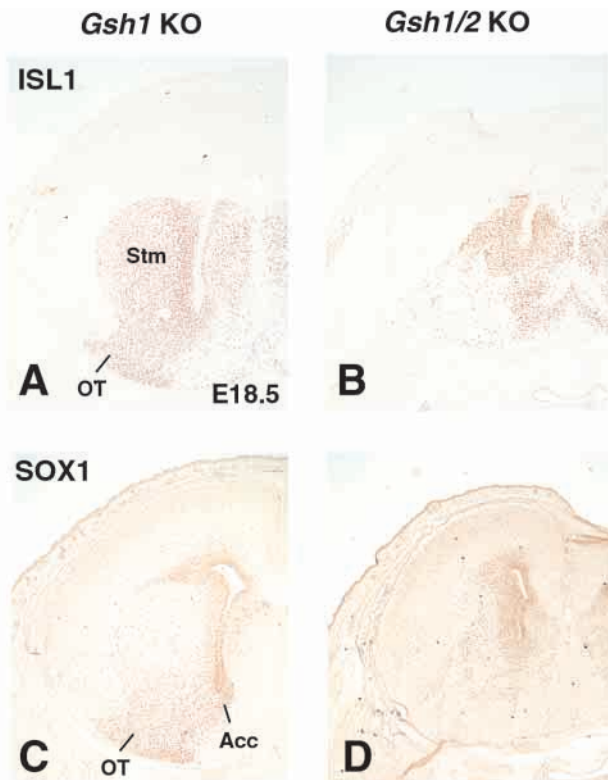
The striatum is not the sole derivative of the LGE. GABAergic and dopaminergic interneurons of the olfactory bulb (Shipley et al., 1995) are also thought to derive from the LGE at embryonic stages (Wichterle et al., 1999) and the lateral wall of the telencephalic ventricle (i.e. the subventricular zone, SVZ) at postnatal and adult stages (Altman, 1969; Lois and Alvarez-Buylla, 1994; Luskin, 1993). Progenitors of these olfactory bulb interneurons express DLX proteins while migrating along the rostral migratory stream (Porteus et al., 1994). At E18.5, DLX expression in the *Gsh2*<sup>-/-</sup>



**Fig. 3.** Olfactory bulb interneurons at E18.5 in wild types and *Gsh2* mutants. DLX immunohistochemistry reveals positive cells in the SVZ, granule cell layer (GCL) and glomerular layer (GL) of the wild type (A). In the *Gsh2* mutant (B) fewer than normal cells are positive for DLX and these cells are mostly confined to the SVZ. GABAergic interneurons of the granule cell layer and glomerular layer in the wild type (C) are revealed by immunohistochemistry for GAD<sub>67</sub>. In the *Gsh2* mutant (D), there is a dramatic reduction in the number of GAD<sub>67</sub> cells and most are found in the granule cell layer. E and F show the medial portion of the olfactory bulbs at higher magnification to visualize the few TH neurons present in the glomerular layer. Interneurons positive for TH are detected in the glomerular layer of wild-type (E) but not *Gsh2* mutant (F) olfactory bulbs.



**Fig. 4.** *Gsh1* expression in wild types and *Gsh2* mutants. At E16.5, *Gsh1* expression in the wild type is confined to the ventralmost VZ corresponding to the former MGE and medialmost LGE (A). In *Gsh2*<sup>-/-</sup> embryos at this stage, *Gsh1* transcripts are detected at a high level throughout the LGE (B). Arrowheads in A and B indicate the LGE-cortex boundary. At E12.5, wild-type *Gsh1* expression is observed in the MGE and medialmost LGE (C). A slight expansion of the *Gsh1* expression domain is evident in the *Gsh2* mutant at this stage (D).

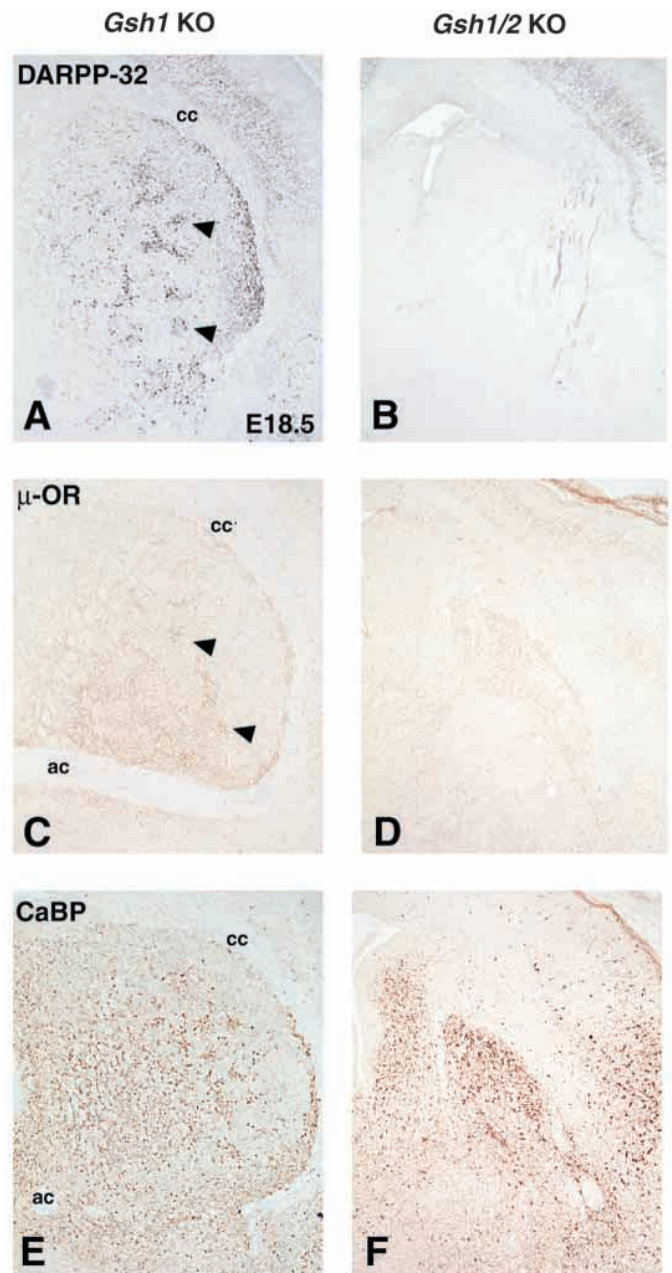


**Fig. 5.** The striatal complex at E18.5 in *Gsh1* and *Gsh1/2* homozygous mutants. No abnormalities can be seen in ISL1 (A) or SOX1 (C) expression in the *Gsh1* mutant striatum (Stm). The *Gsh1/2* double mutant has only a rudimentary striatum as revealed by ISL1 (B) and nearly absent SOX1 (D) staining. Acc, nucleus accumbens; OT, olfactory tubercle.

olfactory bulb is notably reduced and mainly confined to the olfactory bulb SVZ (Fig. 3B) as compared to wild types where strong staining is detected also in the granule cell layer and glomerular layer (Fig. 3A). Furthermore, the proportion of cells expressing glutamic acid decarboxylase (GAD<sub>67</sub>), a synthetic enzyme for GABA production, is also considerably reduced and largely confined to the granule cell layer (Fig. 3C,D). Dopaminergic olfactory bulb interneurons constitute a smaller population than their GABAergic counterparts (Shipley et al., 1995) and can be marked by the expression of tyrosine hydroxylase (TH, the rate limiting enzyme in dopamine production) (Fig. 3E). TH-positive neurons are not detected in the E18.5 *Gsh2*<sup>-/-</sup> olfactory bulb (Fig. 3F).

#### The expression domain of *Gsh1* is expanded in the *Gsh2* mutant LGE

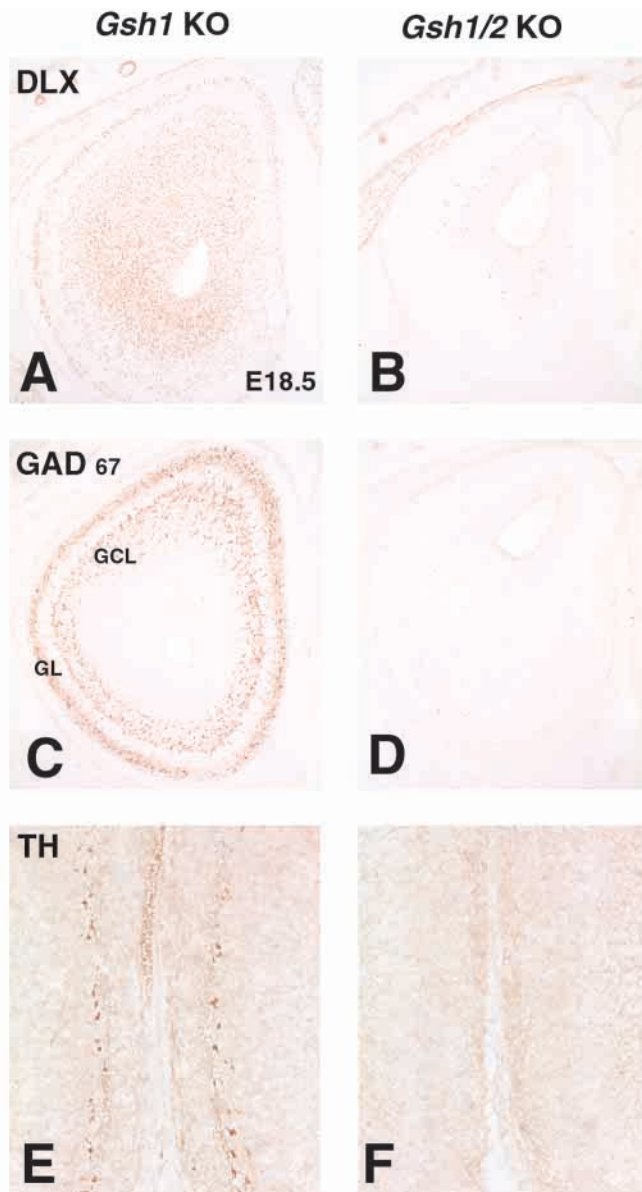
It is interesting to note that the molecular identity of *Gsh2*<sup>-/-</sup> LGE precursors normalizes at later stages (i.e. between E14–15) of striatal neurogenesis (Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001). The mechanisms underlying this delayed recovery are at present unknown, however, they are likely to account for the striatal structures that exist in these mutants. *Gsh2* has a close family member *Gsh1*, the expression of which overlaps with *Gsh2* expression in the MGE but is only expressed in a small subset of LGE precursors in the ventromedial portion (Fig. 4A,C). Interestingly, we find that



**Fig. 6.** Patch-matrix organization at E18.5 in *Gsh1* and *Gsh1/2* double homozygous mutants. The *Gsh1* mutant striatum appears identical to wild type for DARPP-32 (A), μ-OR (C) and CaBP (E). The *Gsh1/2* double mutant striatum is devoid of DARPP-32-positive neurons (B) and no μ-OR patches can be detected (D). Arrowheads in A and C point to patches. Neurons expressing CaBP are detected in the rudimentary double mutant striatum (F). ac, anterior commissure; cc, corpus callosum.

concomitant with the normalization of molecular identity in LGE precursors at E16.5 there is an expansion of *Gsh1* expression throughout the mutant LGE VZ up to the cortical-striatal boundary (Fig. 4B). This expansion appears to be only starting at earlier stages (e.g. E12.5) (Fig. 4D) when significant misspecification remains evident in the *Gsh2*<sup>-/-</sup> LGE (Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001).





**Fig. 7.** Olfactory bulb interneurons at E18.5 in *Gsh1* and *Gsh1/2* homozygous mutants. DLX (A), GAD<sub>67</sub> (C) and TH (E) expression appear normal in *Gsh1* mutants. The *Gsh1/2* double mutant has very few DLX (B) labeled cells in the SVZ. The double mutants are also completely devoid of GAD<sub>67</sub> staining (D) and lack TH-positive cells (F). GL, glomerular layer; GCL, granule cell layer.

#### The *Gsh1/2* double mutant striatum and olfactory bulb are more severely defective than in the *Gsh2* mutants

To examine whether *Gsh1* has a significant role in striatal and olfactory bulb development on its own, we analyzed *Gsh1*<sup>-/-</sup> embryos. Using the same markers as for the *Gsh2* mutant embryos, we could not detect any significant abnormalities in striatal development (Fig. 5A,C; Table 1). The expression of the striatal markers DARPP-32,  $\mu$ -OR and CaBP in *Gsh1*<sup>-/-</sup> embryos (Fig. 6A,C,E) were indistinguishable from wild types (Fig. 2A,C,E). Additionally, *Gsh1*<sup>-/-</sup> mutants did not exhibit detectable olfactory bulb interneuron defects (Fig. 7A,C,E).

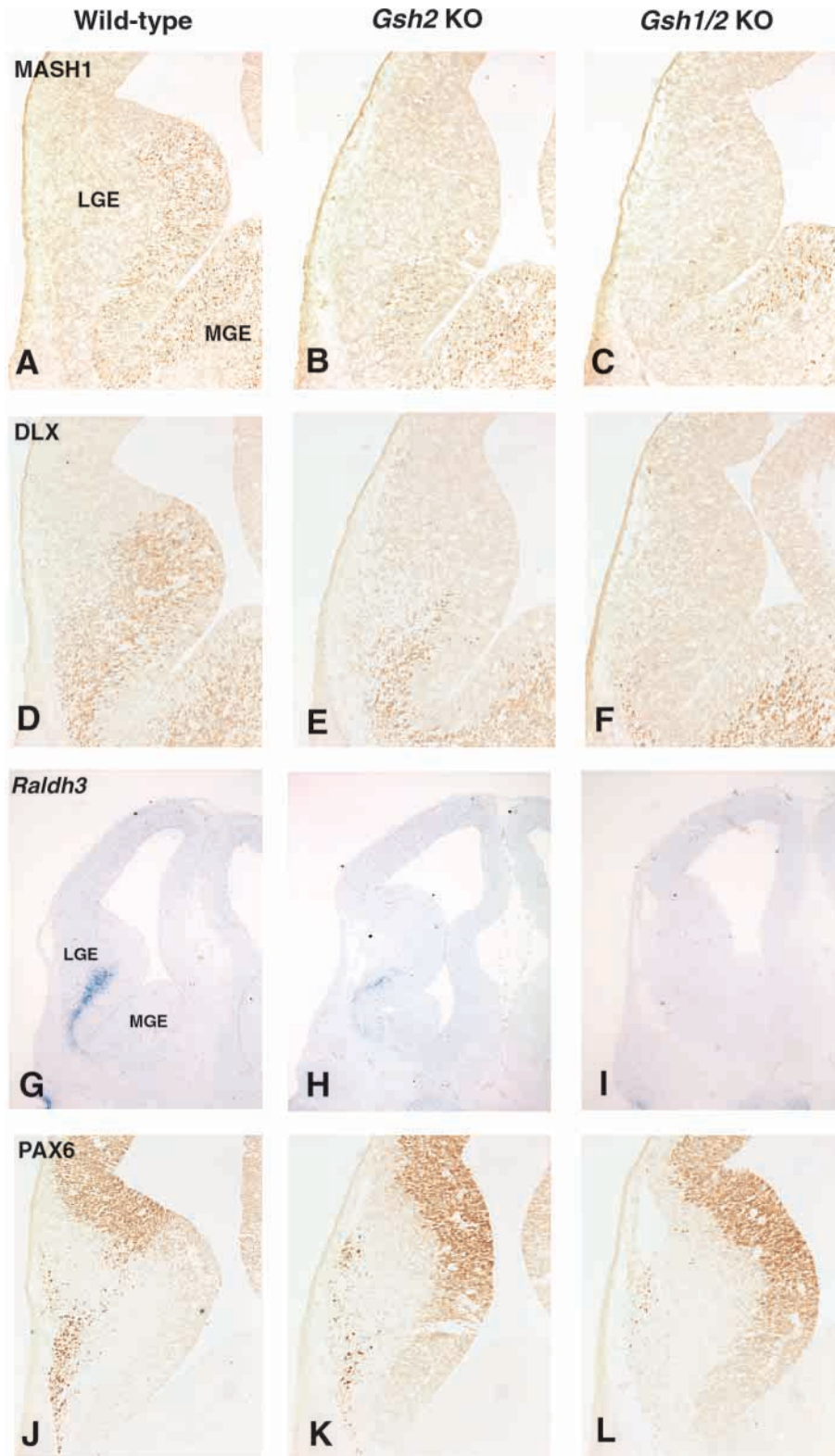
These results argue that, in the presence of functional *Gsh2* alleles, *Gsh1* is not required for striatal projection neuron and olfactory bulb interneuron development. This notion is also supported by the expression pattern of *Gsh1* itself (Fig. 4A,C).

The expanded expression domain of *Gsh1* in the *Gsh2*<sup>-/-</sup> telencephalon, however, suggests that it may have a role in striatal and olfactory bulb development when *Gsh2* is missing. To determine this, we generated *Gsh1/2* double homozygous mutants. At E18.5, the *Gsh1/2* double mutant striatum, as marked by ISL1 expression, is dramatically reduced (54% smaller than in the *Gsh2* mutant), being only 21% of the wild-type striatum (Fig. 5B; Table 1). Again, the ventral striatum demarcated by SOX1 staining is severely affected in the double mutant being 37% of that in the *Gsh2* mutants and only 12% of wild types (Fig. 5D; Table 1). In addition to this considerable reduction in size, removal of both *Gsh1* and *Gsh2* genes has more severe effects on striatal neuron differentiation since no DARPP-32-positive cells can be detected at E18.5 (Fig. 6B). Moreover, no  $\mu$ -OR positive patches can be seen in the double mutant striatum (Fig. 6D). These results indicate that the patch compartment is completely deficient even though ISL1 staining (Fig. 5B) indicates the presence of a rudimentary striatum. Expression of the early neuronal marker  $\beta$ -III-tubulin is present within the double mutant striatum (data not shown) as well as Calbindin (CaBP) (Fig. 6F). Therefore the loss of *Gsh1* and *Gsh2* function does not appear to affect all neuronal differentiation in the mutant striatum. The lack of DARPP-32 and  $\mu$ -OR expression, however, demonstrates a requirement for at least one of either *Gsh2* or *Gsh1* to be present for the differentiation of striatal projection neurons in the patch compartment to occur. Our data further show that MGE-derived striatal (NKX2.1<sup>+</sup>) and cortical interneurons (DLX<sup>+</sup> and CaBP<sup>+</sup>) (Anderson et al., 1999; Marin et al., 2000) are generated in significant numbers. Many NKX2.1-positive cells are seen in the *Gsh1/2* mutant striatum (Fig. 9L) as well as DLX- and CaBP-positive cells in the cortex (Fig. 9I) (data not shown), similar to that in wild types. As in the *Gsh2* mutants, we did not detect an increase in TUNEL-positive cells within the LGE or the striatal complex of the *Gsh1/2* double mutants. The lack of data supporting cell death in the *Gsh2* single mutant (Toresson et al., 2000; Yun et al., 2001) and *Gsh1/2* double mutant striata, suggests that their reduction in size could be due to proliferation defects.

Since *Gsh1* is required for striatal development in the *Gsh2*<sup>-/-</sup> telencephalon, could it have a similar role in olfactory bulb neurogenesis? As described above, the *Gsh2*<sup>-/-</sup> olfactory bulb displays a significant reduction in DLX- and GAD<sub>67</sub>-expressing cells (Fig. 3B,D). In the *Gsh1/2* double mutant, there is an even greater reduction in DLX staining (Fig. 7B) and a complete lack of GAD<sub>67</sub>-positive (Fig. 7D) and TH-positive cells (Fig. 7F). These findings also demonstrate a role, albeit less pronounced, for *Gsh1* in olfactory bulb neurogenesis of *Gsh2*<sup>-/-</sup> mice.

#### Lack of a complete respecification of striatal progenitors in the *Gsh1/2* double mutant LGE

Based on the expansion of the *Gsh1* expression domain in the *Gsh2* mutant, along with the severely disturbed striatal development observed in the *Gsh1/2* double mutant, we would expect a more severe misspecification of striatal progenitors in the *Gsh1/2* double mutant from that seen in the *Gsh2* mutant.



**Fig. 8.** Progenitor cell specification at E12.5 in *Gsh2* and *Gsh1/2* double homozygous mutants. MASH1 protein is detected in many cells of the MGE and LGE VZ of the wild type (A). In the *Gsh2* mutant the LGE level of MASH1 is lower and confined to the ventromedial VZ (B). In the *Gsh1/2* double mutant, only a few MASH1-positive cells are seen in the LGE, close to the MGE (C). The majority of VZ and SVZ cells of the wild type are positive for DLX (D). In the *Gsh2* mutant, only cells of the medialmost LGE SVZ express DLX proteins (E) while DLX-positive cells in the *Gsh1/2* double mutant LGE are very rare (F). In situ hybridization for *Raldh3* marks cells in the ventromedial LGE of the wild type (G). *Raldh3* expression is present at low levels in the same region of the *Gsh2*<sup>-/-</sup> LGE (H) but completely absent in the double mutant (I). High levels of PAX6 protein show a sharp boundary in the dorsal LGE of the wild type (J). Ectopic PAX6 is found in a large region of the LGE VZ in both *Gsh2* mutants (K) and *Gsh1/2* double mutants (L).

LGE/MGE sulcus in *Gsh1/2* double mutants (Fig. 8C,F). For the analysis of these mutants the expression domain of the gene for the retinoic acid synthesizing enzyme, *retinaldehyde dehydrogenase 3* (*Raldh3*; *Aldh1a3*) (Li et al., 2000), is particularly interesting being confined to the ventromedial half of the LGE (Fig. 8G). *Raldh3* transcripts can be detected at low levels in the *Gsh2* mutant LGE (Fig. 8H) but are absent from the double mutant LGE (Fig. 8I). Despite the more severe alterations in ventral gene expression seen in *Gsh1/2* double mutants, the ectopic expression of the dorsal regulators PAX6 and NGN2 in the double mutant LGE (Fig. 8L) (and data not shown) was not noticeably different from that in the *Gsh2* mutant (Fig. 8K) (and data not shown). Thus, the slightly expanded expression domain of *Gsh1* at E12.5 (Fig. 4D) appears to be necessary for the little correct specification present in the *Gsh2* mutant LGE at this stage. Contrary to the defects observed in the double mutant LGE at E12.5, the MGE appears very similar to wild types (Fig. 8), as does the expression of the MGE-marker NKX2.1 (data not shown).

Somewhat surprisingly, however, at E16.5 the molecular identity of VZ cells

At E12.5, the morphology of the double mutant LGE was similar to that of the *Gsh2* mutant (Fig. 8), however, the molecular identity of the double mutant LGE is more severely disturbed than that of the *Gsh2* mutant. MASH1 and DLX, which both cover around one third of the E12.5 *Gsh2* mutant LGE (Fig. 8B,E), are only detected in a few cells close to the

in the double mutant LGE appears partially restored in that the domains of NGN2 (Fig. 9C) and PAX6 (Fig. 9F) expression are similar to that in *Gsh2* single mutants (Fig. 9B,E) and wild types (Fig. 9A,D). Only a few cells within the dorsolateral LGE of *Gsh1/2* double mutants still express low levels of NGN2 and PAX6. Moreover, MASH1 is expressed in the VZ of the double





mutant LGE, albeit at lower levels (data not shown). The SVZ was more severely disturbed in the double mutant since DLX proteins (Fig. 9I) were detected in significantly fewer cells than in *Gsh2* mutants (Fig. 9H) or wild types (Fig. 9G). This phenotype is restricted to the double mutant LGE since DLX expression appears at normal levels in the ventral-most telencephalic SVZ corresponding to the MGE as marked by NKX2.1 (Fig. 9I,L).

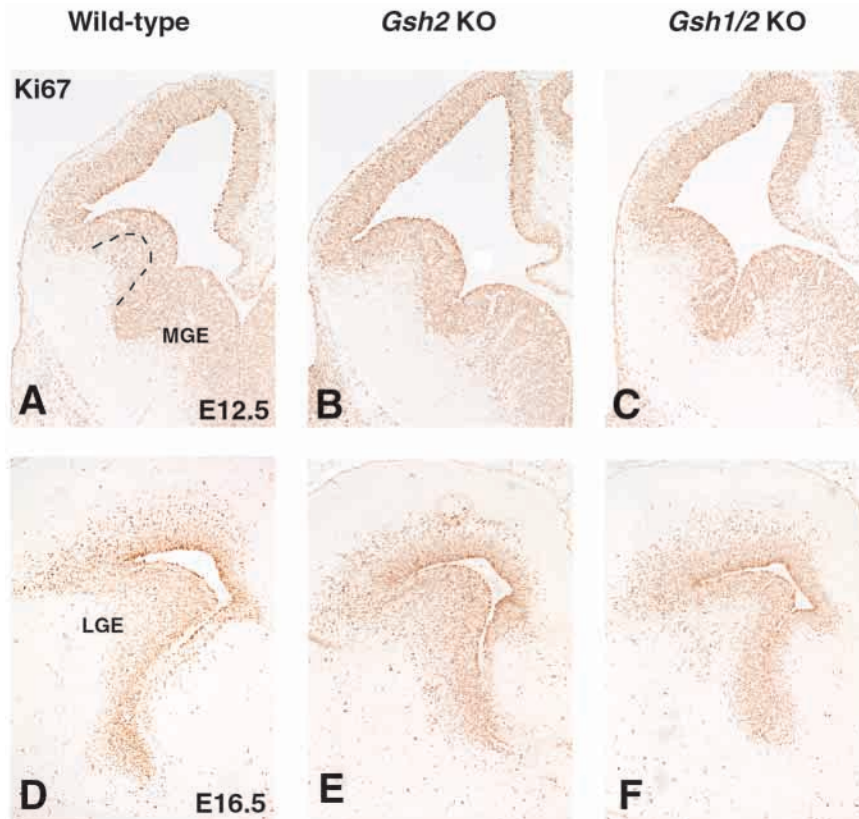
**Fig. 9.** Progenitor cell specification in *Gsh2* and *Gsh1/2* double homozygous mutants at E16.5. (A,B) NGN2 expression is detected in many cells of the cortical VZ but not in the LGE of the wild type (A). The *Gsh2* mutant looks similar to the wild type at this stage (B). In the *Gsh1/2* double mutant (C), high levels of NGN2 are detected only in the cortical VZ but low levels can also be detected in a few cells in the lateral LGE. (D-F) The expression of PAX6 is also similar in wild type (D), *Gsh2* single (E) and *Gsh1/2* double mutants (F). (G-I) In the wild type, high levels of DLX protein can be detected in the VZ, SVZ and mantle of the ventral telencephalon (G). The *Gsh2* mutant shows less labeling in the VZ but otherwise looks similar to the wild type (H). Considerably fewer cells in the *Gsh1/2* double mutant LGE SVZ express DLX (I). (J-L) NKX2.1 expression defines the remnant of the MGE (asterisk in J) at this stage and clearly helps to visualize the shortening of the LGE VZ in the *Gsh2* mutant (K) and *Gsh1/2* double mutant (L) compared to the wild type (J). The NKX2.1-positive region in L is larger than in J and K, this is because the section in L is somewhat more caudal than the others; we do not notice an increase in MGE size in the double mutant. Arrows in I and L shows the boundary between the MGE and LGE. GP, globus pallidus.

#### Loss of *Gsh* gene function results in fewer LGE precursor cells

In the *Gsh2* mutant, as well as in the *Gsh1/2* double mutant, the LGE is significantly reduced in size from that in the wild type (Fig. 9). As mentioned earlier, this reduction does not seem to result from increased cell death, suggesting that reduced proliferation may contribute to the phenotype. Indeed, previous studies have shown that at E12.5 in the *Gsh2* mutant, the SVZ of the LGE does not form properly (Toresson et al., 2000; Yun et al., 2001). Staining for the cell cycle marker Ki67 (Schlüter et al., 1993) at E12.5 shows very few cells in the SVZ of either the *Gsh2* mutant (Fig. 10B) or the *Gsh1/2* double mutant (Fig. 10C) as compared to wild types (Fig. 10A). At E16.5, proliferation within the *Gsh2* mutant SVZ appears to have resumed since Ki67 staining indicates the presence of numerous precursor cells in the SVZ (Fig. 10E). In the E16.5 double mutant, however, SVZ proliferation (as marked by Ki67 staining) remains deficient (Fig. 10F). To

complement this data, we did Toluidine Blue stains on sections from E18.5 brains and counted mitotic figures (i.e. dividing precursors) in the VZ and SVZ. The data show that the loss of *Gsh2* as well as *Gsh1* genes results in a significant decrease in the number of dividing striatal precursors, both in the VZ and the SVZ (Table 2). The reduction is most severe in the SVZ as indicated by the ratio of mitotic figures in the SVZ versus the VZ in each of the wild-type, *Gsh2* and *Gsh1/2* alleles. Taken





**Fig. 10.** Proliferating cells at E12.5 and E16.5 in *Gsh2* and *Gsh1/2* double homozygous mutants. Immunohistochemistry for the cell cycle marker Ki67 shows strong labeling in the VZ and SVZ (separated by the dashed line) of the wild-type LGE and MGE (A). Labeling in the *Gsh2* mutant is normal in the VZ and MGE SVZ but significantly lower in the LGE SVZ (B). The *Gsh1/2* double mutant (C) is similar to the *Gsh2* mutant. At E16.5, the SVZ of the *Gsh2* mutant LGE (E) has improved, showing numerous Ki67-positive cells which is not the case in the SVZ of the double mutant LGE (F).

together with the reduction of DLX expression in the SVZ of the double mutant LGE (Fig. 9I), these findings indicate that *Gsh2* or *Gsh1* (in the absence of *Gsh2*) is required for the correct formation of the LGE SVZ precursor population.

## DISCUSSION

### The role of *Gsh1* in the *Gsh2* mutant telencephalon

As would be expected from the expression pattern of *Gsh1* in the wild-type telencephalon, the development of striatal projection neurons and olfactory bulb interneurons does not normally seem to be dependent on this gene product. Despite this, our data clearly demonstrate that in the absence of *Gsh2*, *Gsh1* is required for much of the striatal development observed. What makes this particularly interesting is the fact that *Gsh1* is normally only expressed in a very small subpopulation of precursors confined to the ventromedial limit of the LGE. In *Gsh2* mutants, the *Gsh1* expression domain expands considerably, so that it covers the entire mutant LGE

VZ by late stages of striatal neurogenesis. Although the extent of *Gsh1* expression in the early LGE (i.e. E12.5) of the *Gsh2* mutant is minimal as compared to later stages, the present results show that it is nonetheless required for the little correct LGE specification present at that time-point. These results suggest that *Gsh2* gene function is required for both early and late stages of striatal development. In the absence of *Gsh2*, *Gsh1* gene function can, at least in part, direct later stages of striatal development. Interestingly, in the absence of both *Gsh1* and *Gsh2* the ventral regulators *Mash1* and *Dlx* genes are observed in the late VZ and SVZ of the mutant LGE. Their expression at this stage, however, is greatly reduced and only scattered DLX-positive cells are observed. Thus, *Gsh* gene function does not appear to be required for the establishment and/or induction of molecular identity within LGE precursors but rather for its maintenance.

We previously proposed a model for the molecular specification of striatal and cortical progenitors (Toresson et al., 2000) based on the results from *Gsh2*, *Sey*, *Sey/Gsh2* and *Neurogenin* (Fode et al., 2000) mutants. According to this model cortical and striatal progenitors have the ability to express either a ventral (DLX, MASH1) or a dorsal (NGN) developmental program and the choice between the two is under *Pax6* and *Gsh2* control. This model explains nicely the early misspecification in the *Gsh2*<sup>-/-</sup> LGE and in the *Sey* pallium. The recovery of molecular identity in the *Gsh2* mutant LGE at later stages, however, was suggested to depend on *Gsh1*. Although *Gsh1* appears to possess similar functions to *Gsh2* in maintaining ventral specification of LGE precursors, it is not required to repress dorsal gene expression in LGE precursors, as is the case for *Gsh2*. Indeed, the present results show that removal of *Gsh1* from the *Gsh2*<sup>-/-</sup> background did not result in an expansion of the ectopic *Pax6* and *Ngn* expression domain from that observed in *Gsh2* mutants, even at E12.5. Unlike that observed in the *Gsh2* mutants, however, in *Gsh1/2* double mutants very little *Mash1* or *Dlx* gene expression was detected in the ventromedial LGE at E12.5. These new findings limit the validity of the previously proposed model (Toresson et al., 2000) to the early stage, dorsolateral LGE cells and suggests a *Ngn*-independent regulation of *Mash1* and *Dlx* gene expression in the ventromedial LGE. Interestingly, Yun et al. (Yun et al., 2001), have recently suggested that the LGE may be divided into

**Table 2. Mitotic figures in the VZ and SVZ of wild type, *Gsh2* single and *Gsh1/2* double homozygous mutants at E18.5**

	Wild type	<i>Gsh2</i> <sup>-/-</sup>	<i>Gsh1</i> <sup>-/-</sup> ; <i>Gsh2</i> <sup>-/-</sup>
Ventricular zone (VZ)	219±19.8	138±10.4*	144±13.0*
Subventricular zone (SVZ)	1101±89.9	486±37.5*	291±27.0*
Ratio SVZ/VZ	5.1±0.3	3.6±0.5*	2.2±0.2*‡

*n*=4 for each allele.

\*Significant from wild type at 95%; ‡significant from *Gsh2*<sup>-/-</sup> at 95%, one way ANOVA with the Fisher PLSD post-hoc test.

dorsal (dorsolateral) and ventral (ventromedial) domains based on restricted gene expression patterns. If this is the case, then perhaps a unique function of *Gsh2* is to repress *Pax6* and *neurogenin* gene expression in the dorsolateral LGE domain while other factors restrict their expression from the ventromedial LGE domain. This apparent difference in *Gsh2* and *Gsh1* function may, however, be due to the fact that *Gsh1* is not expressed in the dorsolateral LGE of *Gsh2*<sup>-/-</sup> mutants at an early enough time-point. Genetic replacement of *Gsh2* with the *Gsh1* gene is needed to adequately address this issue.

The mechanism by which *Gsh1* functions in the *Gsh2* mutant telencephalon is, as yet, unclear. The simplest explanation would be that in the absence of *Gsh2*, the small domain of LGE precursors that normally expresses *Gsh1* expands dramatically and constitutes the entire LGE at later stages. This is not a typical situation where compensation through genetic redundancy has previously been proposed (e.g. *Otx* or *En* genes) (Acampora et al., 1997; Joyner, 1996; Suda et al., 1997). In these previous examples compensation occurs where the homologous genes overlap in their expression patterns. In the case of the *Gsh* genes, the region of telencephalon where *Gsh1* and *Gsh2* expression overlaps (i.e. the MGE) appears to be least affected by the removal of both genes. Instead, it is the mostly *Gsh2*-positive; *Gsh1*-negative LGE that requires *Gsh1* function in the absence of *Gsh2*. Some mechanism may therefore exist that allows for the enlargement of the ventromedial population in the *Gsh2* mutant. One possibility is that the dorsolateral LGE has a negative influence on the proliferation of ventromedial LGE cells and in the *Gsh2*<sup>-/-</sup> LGE this inhibition is lost. Support for this model, comes from the expression profile of DLX proteins in the *Gsh2* mutant LGE. DLX proteins are normally expressed at high levels in VZ precursors of the dorsolateral LGE while their expression is found deeper in cells of the SVZ at more ventromedial locations (see Fig. 8D, Fig. 9G) (Eisenstat et al., 1999). In the *Gsh2* mutant, the DLX expression profile appears to be more similar to that in ventromedial regions of the wild-type LGE (see Fig. 8E, Fig. 9H) (Corbin et al., 2000) suggesting that the *Gsh2* mutant LGE possesses characteristics typical of the ventromedial LGE.

### ***Gsh* genes are required for maintaining the LGE precursor pool**

One of the most striking defects in the *Gsh2* single and *Gsh1/2* double homozygous mutants is the reduction in the volume of the striatal complex. This effect could be caused by at least two different mechanisms, increased cell death or a reduced proliferation of striatal precursors. As was the case in the *Gsh2* mutant (Toresson et al., 2000; Yun et al., 2001), we did not detect an increase in TUNEL labelling in the *Gsh1/2* double mutant striatum, indicating that increased cell death does not contribute significantly to this phenotype. The reduction in striatal volume is therefore most likely caused by a proliferative deficit within the progenitor pool. Indeed, the *Gsh2* mutant LGE does not appear to establish a proliferative SVZ at E12.5 (Toresson et al., 2000; Yun et al., 2001). This is, at least in part, ameliorated at later stages of striatal neurogenesis in the *Gsh2* mutant, but remains significantly reduced in the *Gsh1/2* double mutant. The present results, therefore, indicate that *Gsh2* (or *Gsh1* in its absence) function is required for the proliferation of LGE precursors both in the

VZ and SVZ. *Gsh* gene function appears to be particularly important for the establishment of the SVZ since the ratio of precursors in the SVZ versus the VZ decreases significantly when going from wild type to *Gsh2* mutant to *Gsh1/2* double mutant. Moreover, the loss of both *Gsh1* and *Gsh2* leads to a severe reduction in DLX-expressing SVZ cells. The fact that *Gsh* genes control both the molecular identity of LGE precursors and aspects of their proliferation is interesting in the light of results, primarily from *Drosophila* studies, showing that the cell cycle control machinery in many cases is linked to cell fate decisions (Edgar and Lehner, 1996).

### ***Gsh* genes and clonal relationships in the striatum and olfactory bulb**

Recent studies have shown that the majority of striatal interneurons are generated from the MGE (Olsson et al., 1998; Marin et al., 2000) while the different classes of striatal projection neurons are generated in the LGE (Pakzaban et al., 1993; Deacon et al., 1994; Olsson et al., 1995; Olsson et al., 1998). The clonal relationships, however, of striatal projection neurons are unknown. The major subdivisions of the striatal complex (i.e. caudate-putamen, nucleus accumbens and olfactory tubercle) display many similarities based on their morphology and the expression of different neurochemicals (Heimer et al., 1995). They differ, however, in afferent and efferent connections as well as in their compartmentalization (Heimer et al., 1995), making it possible that they are generated from different progenitors within the LGE, separated spatially and/or temporally. Our studies of *Pax6/Gsh2* double mutants (Toresson et al., 2000), indicated that the failure to properly generate the olfactory tubercle in *Gsh2* mutants is due to the misspecification of precursor cells in the dorsolateral LGE at an early stage of striatal development. This finding suggests that olfactory tubercle neuronal precursors are normally located in this portion of the LGE, predominantly at early stages. The neurons of the dorsal striatum and nucleus accumbens are largely generated at later stages than those in the olfactory tubercle (Bayer and Altman, 1995) and therefore benefit, at least partially, from the *Gsh1* expansion in the *Gsh2* mutant. Therefore, there may be both spatial and temporal domains of distinct striatal precursor cells for the olfactory tubercle, dorsal striatum and nucleus accumbens positioned along the dorsolateral to ventromedial extent of the LGE.

Another subdivision of striatal neurons is the patch-matrix distinction (Gerfen, 1992). Patch neurons make up 15% of total striatal volume and are generated at a low but steady level from the earliest time-points of striatal neurogenesis until the end (van der Kooy and Fishell, 1987). The generation of matrix neurons however, starts later than patch neurogenesis and they are born in increasing numbers as striatal development proceeds (van der Kooy and Fishell, 1987). In the *Gsh2* mutant, there is a clear reduction of striatal patches, which is likely due to the early misspecification of LGE precursors. The matrix compartment must also be affected, since it normally constitutes 85% of the striatum (Lanca et al., 1986) and the *Gsh2* mutant striatum is reduced to 45% of the wild type. Moreover, in the *Gsh1/2* double mutant we observe a specific lack of patch neurons and the small rudimentary striatum formed is positive only for the matrix marker CaBP. Thus *Gsh2* (or *Gsh1* in its absence) are absolutely required for the formation of the striatal patch compartment. It has been



suggested previously that striatal patch neurons derive from the VZ while the SVZ contributes to the formation of the matrix compartment (Anderson et al., 1997; van der Kooy and Fishell, 1987). Although only correlative, our data fit with this model: the disturbances in patch formation correlates with the altered gene expression in the VZ, while the reduction in the matrix compartment could be associated with the observed decrease in SVZ precursors.

Interestingly, the progressive loss of DARPP-32-positive patches not only correlates with decreasing copies of *Gsh* alleles but also with a reduction in the expression of the retinoic acid synthesizing enzyme, *Raldh3* (Li et al., 2000). Our previous studies have implicated LGE-derived retinoids in the development of the striatal projection neurons (Toresson et al., 1999). Thus, the altered *Raldh3* expression in the *Gsh2* single and *Gsh1/2* double mutants may contribute to the striatal phenotype via a reduction in retinoid synthesis.

In addition to the striatal defects observed, abnormalities in olfactory bulb interneuron development are also detected in *Gsh2* single (Corbin et al., 2000; Yun et al., 2001) and *Gsh1/2* double mutants. Olfactory bulb interneurons can be divided into two distinct morphological subtypes, the granule neurons and the periglomerular neurons. The granule cells are GABAergic while the periglomerular cells can be GABAergic, dopaminergic or both (Shipley et al., 1995). The generation of these neurons begins at late embryonic stages and continues into adulthood. Previous studies (Corbin et al., 2000; Yun et al., 2001) have demonstrated that *GAD67* gene expression in the olfactory bulb appears to be deficient at E15 but returns to normal at E18.5. In spite of this, GABA-expressing neurons remain deficient (Corbin et al., 2000). The paucity of *GAD67* protein-expressing cells observed here is likely to account for this. Thus the defect in granule cell development in the *Gsh2* mutant may be due to a developmental delay (Corbin et al., 2000). This delay is likely to be dependent on *Gsh1* gene function since the *Gsh1/2* double mutants appear to have no *GAD67*-expressing neurons. While the formation of GABAergic granule cells does not absolutely require *Gsh2* gene function, periglomerular neurogenesis seems to be more sensitive to *Gsh* gene dose. Reid et al. (Reid et al., 1999), have recently performed clonal analysis of olfactory bulb interneurons and shown that when progenitors were labeled at E17 in the rat (E15 in the mouse), clones contained either granule cells or periglomerular cells. These findings indicate that from this time-point in the neurogenesis of olfactory bulb interneurons, separate precursor populations exist for the two different subtypes. In this respect, *Gsh2* appears to be essential for the generation of periglomerular interneurons.

We thank Kerstin Fogelström and Elsy Ling for excellent technical assistance. Antibodies were kindly provided by R. DiLauro, T. Edlund, P. Emson, R. Lovell-Badge, M. Nakafuku, G. Panganiban and S. Wilson. We are grateful to S. S. Potter for providing the *Gsh1* and *Gsh2* mutant mice. This study was supported by the Human Frontiers Science Organization (RG160-2000B), Cornell Foundation and the Swedish MRC (12539 and 12196).

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