

# Loss of Gsx1 and Gsx2 Function Rescues Distinct Phenotypes in Dlx1/2 Mutants

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## ABSTRACT

Mice lacking the Dlx1 and Dlx2 homeobox genes (Dlx1/2 mutants) have severe deficits in subpallial differentiation, including overexpression of the Gsx1 and Gsx2 homeobox genes. To investigate whether Gsx overexpression contributes to the Dlx1/2 mutant phenotypes, we made compound loss-of-function mutants. Eliminating Gsx2 function from the Dlx1/2 mutants rescued the increased expression of *Ascl1* and *Hes5* (Notch signaling mediators) and *Olig2* (oligodendrogenesis mediator). In addition, Dlx1/2;Gsx2 mutants, like Dlx1/2;Ascl1 mutants, exacerbated the Gsx2 and Dlx1/2 patterning and differentiation phenotypes, particularly in the lateral ganglionic eminence (LGE) caudal ganglionic eminence (CGE), and septum, including loss

of GAD1 expression. On the other hand, eliminating Gsx1 function from the Dlx1/2 mutants (Dlx1/2;Gsx1 mutants) did not severely exacerbate their phenotype; on the contrary, it resulted in a partial rescue of medial ganglionic eminence (MGE) properties, including interneuron migration to the cortex. Thus, despite their redundant properties, Gsx1 and -2 have distinct interactions with Dlx1 and -2. Gsx2 interaction is strongest in the LGE, CGE, and septum, whereas the Gsx1 interaction is strongest in the MGE. From these studies, and earlier studies, we present a model of the transcriptional network that regulates early steps of subcortical development. *J. Comp. Neurol.* 521:1561–1584, 2013.

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**INDEXING TERMS:** Dlx; Gsx; CGE; LGE; MGE; septum; development; mutant; interneuron; GABA; basal ganglia

Transcription programs that regulate the genetic circuits underlying regional and cell type identity in the forebrain are beginning to be elucidated. Here we focus on the development of mouse subcortical telencephalic domains, known as the lateral, medial, and caudal ganglionic eminences (LGE, MGE, and CGE, respectively), and the septum (Flames et al., 2007). These primordia generate  $\gamma$ -aminobutyric acid (GABA)-ergic projection neurons of subpallial nuclei (e.g., striatum and globus pallidus) and GABAergic interneurons of the pallium and olfactory bulb. Over 100 transcription factors have been implicated in mediating their development (Long et al., 2009a,b), including the Dlx1 and -2 and Gsx1 and -2 (Gsh1 and -2) homeobox genes. These transcription factors are expressed in primary and secondary progenitors in the ventricular and subventricular zones (VZ and SVZ, respectively); as neurons are produced, subsets continue to express Dlx1 and/or Dlx2 (Cobos et al., 2005a, 2007,

and unpublished data). Gsx1 and -2 have partially redundant functions and together promote LGE regional fate (Corbin et al., 2000; Toresson et al., 2000; Toresson and

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Campbell, 2001; Yun et al., 2001, 2003; Waclaw et al., 2004), whereas *Dlx1* and *-2*, which are linked genes, promote later steps in subcortical differentiation, in part through inducing the expression of *Arx*, *Dlx5*, and *Dlx6* homeobox genes (except in the septum; Anderson et al., 1997a; Cobos et al., 2005b; Long et al., 2007, 2009a,b).

Different *Dlx* gene dosages control different processes. *Dlx1* and *-2* mutants lack expression of *Dlx1/2/5/6*; loss of expression of these eight *Dlx* alleles uncovers the fundamental *Dlx*-mediated programs, which include 1) repressing Notch signaling and glial differentiation, through decreasing *Ascl1* (*Mash1*) and *Olig2* expression (Yun et al., 2002; Petryniak et al., 2007); 2) promoting GABAergic neural differentiation through inducing expression of glutamic acid decarboxylase (*GAD1* and *-2*) and vesicular GABA transporter (Anderson et al., 1997a; Long et al., 2009a,b); and 3) promoting neuronal migration through repressing neurite outgrowth and *Pak3* kinase expression and by maintaining expression of *Cxcr4* and *-7* cytokine receptors (Anderson et al., 1997b; Long et al., 2007; Cobos et al., 2007; Wang et al., 2011). More subtle phenotypes arise in *Dlx1*<sup>-/-</sup>;*Dlx2*<sup>+/-</sup> mutants, which have defects in synapse development (Stanco, Cobos, and Rubenstein, unpublished). *Dlx1*<sup>-/-</sup> mutants show defects in survival of a subset of subcortically derived cortical interneurons (dendrite innervating interneurons; Cobos et al., 2005a). Loss of *Dlx5* and *-6* results in defects in interneuron migration and differentiation (Wang et al., 2010). Reduced *Dlx* dosage (*Dlx1*<sup>-/-</sup>, *Dlx5/6*<sup>+/-</sup>) results in viable mice that have abnormal cortical function, epilepsy, and fear behaviors (Cobos et al., 2005a; Mao et al., 2009, 2011; Wang et al., 2010).

The *Gsx2* and *Dlx1* and *-2* genes mediate their subcortical transcriptional programs in combination with the *Ascl1* (*Mash1*) bHLH gene. A feature of the *Dlx1/2* mutants is overexpression of *Ascl1*, *Gsx1*, and *Gsx2* (Yun et al., 2002; Long et al., 2009a,b). We hypothesized that some of the *Dlx1/2* mutant phenotype is caused by the increased levels of *Ascl1*, *Gsx1*, and *Gsx2* and therefore set out to make compound mutants that reduce *Ascl1*, *Gsx1*, or *Gsx2* dosage in *Dlx1/2* mutants. *Dlx1/2*;*Ascl1* compound mutants do not exhibit a rescue of *Dlx1/2* mutant properties; rather, their phenotype is much more severe than that of the individual mutants, because *Dlx1/2* and *Ascl1* regulate parallel pathways of subcortical differentiation (Long et al., 2009a,b).

Here we explored the effect of reducing *Gsx1* or *Gsx2* expression in the *Dlx1/2* mutants by making compound mutants. We performed our phenotypic analysis on the CGE, LGE, MGE, and septum and focused on the expression of transcription factors that are abnormally expressed in the *Dlx1/2* mutants (Long et al., 2009a,b).

We also focused on *GAD1* expression, given its fundamental importance in defining the GABAergic phenotype.

Eliminating *Gsx2* function from the *Dlx1/2* mutants rescued the increased expression of *Hes5* (Notch signaling indicator), *Olig2* (oligodendrogenesis indicator), and *Gbx1* (unknown function). In addition, *Dlx1/2*;*Gsx2* mutants, like *Dlx1/2*;*Ascl1* mutants, showed an exacerbation of the *Gsx2* and *Dlx1/2* mutant phenotypes, including *GAD1* expression, particularly in the LGE, CGE, and septum. On the other hand, eliminating *Gsx1* function from the *Dlx1/2* mutants (*Dlx1/2*;*Gsx1* mutants) did not severely exacerbate their phenotype; rather, the mutants exhibited a partial rescue of MGE properties and MGE interneuron migration to the cortex. Thus, despite their partially redundant properties, *Gsx1* and *Gsx2* have distinct interactions with the *Dlx1/2* mutants. We present a model of the transcriptional network that regulates early steps of subcortical development.

## MATERIALS AND METHODS

### Animals

Mice were maintained under standard conditions with food and water ad libitum. All experimental procedures were approved by the Committee on Animal Health and Care at the University of California, San Francisco (UCSF). Mouse colonies were maintained at UCSF, in accordance with National Institutes of Health and UCSF guidelines. Mouse strains with a null allele of *Dlx1*, *Dlx2*, *Gsx1*, and *Gsx2* were used in this study (Anderson et al., 1997b; Casarosa et al., 1999). These strains were maintained on a CD-1 background. For staging of embryos, midday of the vaginal plug was calculated as embryonic day 0.5 (E0.5). PCR genotyping was performed as described elsewhere (Anderson et al., 1997b; Casarosa et al., 1999). *Gsx1* and *-2* genotyping was performed as described by Yun et al. (2003) and Wang et al. (2009).

### Tissue preparation, in situ hybridization, and immunofluorescence

Preparation of sectioned embryos, immunofluorescence, and in situ hybridization were performed using digoxigenin riboprobes on 20- $\mu$ m frozen sections cut on a cryostat using methods described by Long et al. (2007, 2009a,b). We used a rabbit polyclonal anti-GSX2 antibody (Toresson et al., 2000), a guinea pig polyclonal anti-DLX2 (Kuwajima et al., 2006), and a mouse monoclonal anti-MASH1 (BD Pharmingen, San Jose, CA). Riboprobes have been described by Long et al. (2009a,b), except for *Ngn2* (1.5-kb mouse full coding sequence from Francois Guillemot).

**TABLE 1.**  
Antibody Characterization

Antigen	Immunogen	Species; manufacturer	Dilution
ASCL1 (MASH)	Full-length ASCL1	Mouse monoclonal; BD Pharmingen; catalog No. 556604	1/500
DLX2	N-terminal amino acids 1–154	Guinea pig; Kuwajima et al. (2006)	1/3,000
GSX2	10-mer peptide (ANEDKEISPL) from the C-terminal of the protein	Rabbit; Pei et al. (2011)	1/500

### Antibody specificity characterization

DLX2 immunoreactivity closely matches endogenous Dlx2 RNA expression and disappears in the brain of Dlx1/2<sup>-/-</sup> mutants (Long et al., 2007). In Dlx1/2 mutants, a deletion removes most of the coding exons for both Dlx1 and Dlx2 but does not remove exon 1, so an N-terminal truncated protein could be produced. Because the guinea pig anti-DLX2 antibody was made to the N-terminal amino acids (1–154; Kuwajima et al., 2006), we conclude that very little of this protein is present in the Dlx1/2<sup>-/-</sup> mutant brain (Table 1).

GSX2 immunoreactivity closely matches endogenous Gsx2 RNA expression and disappears in the brain of Gsx2<sup>-/-</sup> mutants (Toresson et al., 2000). Moreover, it does not recognize GSX1 even when Gsx1 is overexpressed (Pei et al., 2011).

ASCL1 antibody specificity was tested using lysate from rat embryonic brain by Western blot; the antibody specifically recognized a 34-kDa protein (information provided by the manufacturer; BD Pharmingen product 556004). Furthermore, its immunoreactivity in the sections from the embryonic mouse brain closely resembles the expression of ASCL1 RNA, as detected here by in situ hybridization.

### Microscopy

Images of in situ hybridization results were captured with a Zeiss AxioCam MR (Zeiss, Thornwood, NY) and saved as TIFF files. Images of immunofluorescence were captured with a Zeiss LSM 510 confocal microscope. The images were then processed in Adobe Photoshop CS3 to optimize the contrast and brightness to illustrate best the gene expression patterns.

### Analysis of the number of DLX2-, GSX2-, and ASCL1-expressing cells

DLX2-, GSX2-, and MASH1-expressing cells (nuclei) were visualized on 20- $\mu$ m coronal forebrain sections from E10.5 and E12.5 wild-type mice by immunofluorescence confocal microscopy. The images were imported into Adobe Photoshop CS3, and a rectangle encompassing the VZ and SVZ domains was placed orthogonal to the ventricular surface (see Fig. 1). The labeled cells in the

VZ, SVZ1, and SVZ2 within each rectangle were manually counted from one section by using the Photoshop counting tool. We calculated the mean size of the stained part of the cell (nucleus) separately for each population of neurons we counted, and at each age, and then corrected the profile counts separately for each population using the Abercrombie equation with the mean nuclear diameter for that population (Guillery, 2002). The average size of the staining was as follows at E10.5: Dlx2, 4.3  $\pm$  0.2  $\mu$ m (SEM); Gsx2, 4.2  $\pm$  0.2  $\mu$ m; Ascl1, 4.0  $\pm$  0.2  $\mu$ m; and, at E12.5: Dlx2, 4.3  $\pm$  0.2  $\mu$ m (SEM); Gsx2, 4.5  $\pm$  0.2  $\mu$ m; Ascl1, 4.0  $\pm$  0.2  $\mu$ m. For Table 2, we used the following scoring system to describe the number of positive nuclei/section: 1+ = 1–9, 2+ = 10–29, 3+ = 30–59, 4+ =  $\geq$ 60.

### Qualitative analysis of gene expression changes

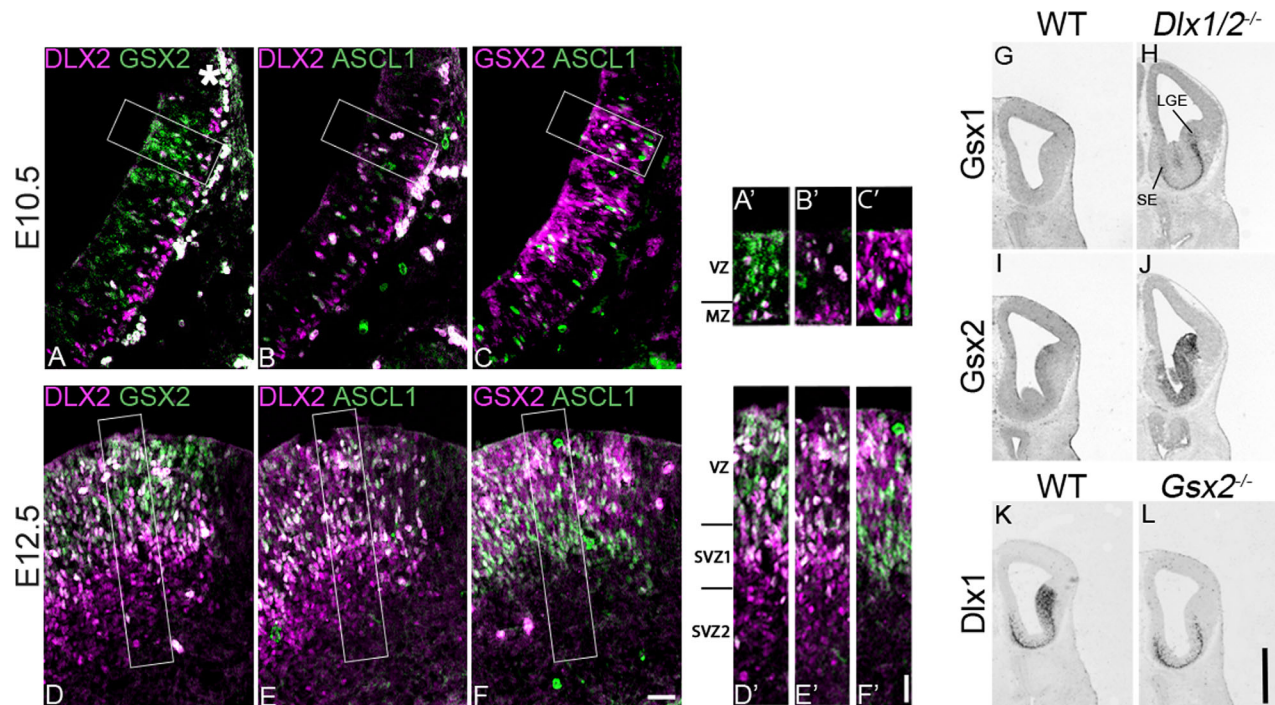
In describing the gene expression changes between control and mutant brains, in the text and in Figures 16 and 17, we made our best judgment assessments, independently by two or three people during at least two separate rating sessions. Similar approaches and figures were used by Long et al. (2009a,b) in describing gene expression changes in the Dlx1/2<sup>-/-</sup>, Ascl1<sup>-/-</sup>, and Dlx1/2;Ascl1<sup>-/-</sup> mutants, so we have used a related system in this paper to allow comparison between these mutants. In Figures 16 and 17, we used a color-coded scoring system in which black indicates that expression was not analyzed (if no squares are listed, this also means that this analysis was not performed); gray indicates that expression was not clearly changed in the mutant; white indicates no detectable expression; red indicates severe reduction in expression; orange indicates moderate/mild reduction in expression; green indicates ectopic expression; and blue indicates increased expression. If the box is subdivided diagonally, the top part corresponds to the dorsal region, the bottom to the ventral region.

## RESULTS

### Subpallial expression of DLX2, GSX2, and ASCL1 proteins

To compare DLX2, GSX2, and ASCL1 protein expression at the cellular level in the developing subpallium, we





**Figure 1.** LGE expression of ASCL1, DLX2, and GSX2 proteins using two-color immunofluorescence, at E10.5 (A–C) and E12.5 (D–F). Cells (nuclei) expressing both proteins are yellow; green and red correspond to specific transcription factors defined at the top of each panel. The boxed areas in each panel are shown at higher magnification in A'–F'. E12.5 data showing previously published information about the relationship between Gsx and Dlx expression in *Gsx2* and *Dlx1/2* mutants. *Dlx1/2* mutants overexpress *Gsx1* (G,H) and *Gsx2* (I,J). *Gsx2* mutants express less *Dlx1*, especially in the dorsal LGE (K,L). LGE, lateral ganglionic eminence; MZ, mantle zone; SE, septum; SVZ1 and SVZ2, subventricular zones 1 and 2. Scale bars = 20  $\mu$ m in F (applies to A–F); 20  $\mu$ m in F' (applies to A'–F'); 500  $\mu$ m in L (applies to G–L).

used double immunofluorescence at E10.5, E12.5, and E15.5 (Fig. 1A–F, and not shown). We focused on expression in the rostral telencephalon in the region of the LGE and septum. These analyses complement previous studies that examined expression of GSX2 (Corbin et al., 2000; Yun et al., 2003; Wang et al., 2009), DLX2 (Porteus et al., 1994; Eisenstat et al., 1999; Yun et al., 2002), DLX2 and ASCL1 (Porteus et al., 1994; Yun et al., 2002), DLX2 and OLIG2, and ASCL1 and OLIG2 (Petryniak et al., 2007).

At E10.5, most VZ progenitors in the dorsal LGE (dLGE) strongly expressed GSX2, with weaker expression spreading ventrally through the ventral LGE (vLGE) and the MGE (Fig. 1A–C, and not shown). ASCL1<sup>+</sup> cells were scattered throughout the VZ and mantle zone (MZ) of the LGE and MGE. DLX2<sup>+</sup> cells in the VZ were most concentrated in the dLGE, whereas the thin MZ had many DLX2<sup>+</sup> cells. In the VZ (of the LGE), most ASCL1<sup>+</sup> and DLX2<sup>+</sup> cells coexpress GSX2, whereas, in the MZ, most ASCL1<sup>+</sup> and DLX2<sup>+</sup> cells were GSX2<sup>-</sup> (Table 1).

Expression at E12.5 and E15.5 showed similar results (Fig. 1D–F, and not shown). The main difference was the formation of the SVZ. Previously, we presented evidence that the SVZ consists of two layers, SVZ1 (adjacent to the

**TABLE 2.**

Cells Expressing *Ascl1*, *Dlx2*, and *Gsx2* in the E10.5 and E12.5 Dorsal LGE<sup>1</sup>

	E10.5		E12.5		
	VZ	MZ	VZ	SVZ1	SVZ2
<i>Dlx2</i>	2+	1+	4+	3+	3+
<i>Gsx2</i>	2+	1+	4+	3+	1+
<i>Dlx2</i> / <i>Gsx2</i>	2+	1+	4+	3+	1+
<i>Dlx2</i>	2+	1+	4+	3+	3+
<i>Ascl1</i>	2+	1+	3+	3+	1+
<i>Dlx2</i> / <i>Ascl1</i>	1+	1+	3+	3+	1+
<i>Gsx2</i>	2+	1+	–	–	1+
<i>Ascl1</i>	1+	1+	4+	3+	1+
<i>Gsx2</i> / <i>Ascl1</i>	1+	1+	3+	3+	1+

<sup>1</sup>See data in Figure 1. A dash indicates that *Gsx2*<sup>+</sup> cell density was too high to count with accuracy but clearly was greater than *Ascl1* density. The scoring system for positive nuclei/section is 1+ = 1–9, 2+ = 10–29, 3+ = 30–59, 4+ =  $\geq$ 60.

VZ) and SVZ2 (Yun et al., 2002; Petryniak et al., 2007). DLX2 was robustly expressed in nearly all cells in SVZ1 and SVZ2. The intensity of GSX2 expression decreased as cells moved from the VZ to SVZ1, although most cells continued to express detectable GSX2 and coexpressed DLX2 and ASCL1 (Table 1). However, in SVZ2, GSX2 and



ASCL1 expression was at background levels, except in occasional cells.

Analysis of GSX1 protein expression was hampered by the lack of a specific antibody. However, previous analyses showed that expression of Gsx1 RNA, and EGFP from a BAC transgenic, is largely complementary to that of Gsx2 at E12.5 and later stages. Whereas Gsx2 was highly expressed in the VZ, Gsx1 expression began in SVZ1. Furthermore, although their expression overlapped along the dorsoventral axis of the subpallium, Gsx2 was most strongly expressed in the LGE, CGE, and septum, and Gsx1 was most strongly expressed in the MGE (Toresson et al., 2000; Yun et al., 2001; Pei et al., 2011). Overall, GSX2 expression in the VZ was temporally upstream of DLX2 expression; as progenitors mature to the SVZ1 state, there generally was coexpression of GSX2, ASCL1, and DLX2. We used analysis of *Gsx2*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup> (*Gsx2*;*Dlx1/2*) compound mutants to assess the effects of losing expression of these transcription factors in the same progenitor cells.

### Combined functions of Gsx2 and Dlx1/2 in defining regional identity of LGE and CGE progenitor cells

Previous studies showed that Gsx2 promoted the expression of the Dlx genes (Corbin et al., 2000; Toresson et al., 2000, 2001; Yun et al., 2001, 2003), whereas Dlx1/2 repressed Gsx1 and Gsx2 expression (Fig. 1G–L; Yun et al., 2002; Long et al., 2009a). Both Gsx2 and Dlx1/2 promoted LGE (dLGE) identity. Loss of Gsx2 resulted in transformation of the dLGE toward a ventral pallial fate (Corbin et al., 2000; Toresson et al., 2000; Toresson and Campbell, 2001; Yun et al., 2001, 2003; Waclaw et al., 2004); loss of Dlx1/2 transformed neurons in the rostradorsal striatal region toward mixed pallial/subpallial properties (Long et al., 2009a).

Here we further investigated Gsx2 function, with the goal of understanding the ramifications of its upregulation in the Dlx1/2 mutant SVZ (Long et al., 2009a,b). We studied the phenotype of the *Gsx2*;*Dlx1/2* compound mutants to determine 1) the combined functions of Dlx1/2 and Gsx2 and 2) whether some of the Dlx1/2 mutant phenotype was reversed by removing expression of Gsx2.

At E12.5, Gsx2 mutants lose expression of VZ progenitor regulators (Ascl1, Dlx1, and Vax1) in the dLGE and likewise have reduced vLGE expression of these transcription factors (Fig. 2B,F,J). Loss of Dlx1/2 has modest effects on the LGE VZ properties at E12.5 (Fig. 2C,G,K,O), but combined loss of Dlx1/2 and Gsx2 function (*Gsx2*;*Dlx1/2* compound mutants) greatly reduced LGE VZ properties, judged from the accentuated reduction of Ascl1, Dlx1, and Vax1 expression as well as the more ventral expansion of Ngn2 (cortical) expression (Fig. 2D,H,L,P). SVZ properties

of the LGE are also greatly reduced in the triple mutant, judged from expression of Arx, Dlx1, Gad1, and Vax1 (Figs. 2, 5). The LGE generates the striatum, so we assessed expression of LGE MZ markers in the *Gsx2*;*Dlx1/2* compound mutants. Expression of Gad1 was not detected, but expression of Ebf1, FoxP4, and Islet1 were preserved, albeit reduced (see Fig. 5H,L,P,T).

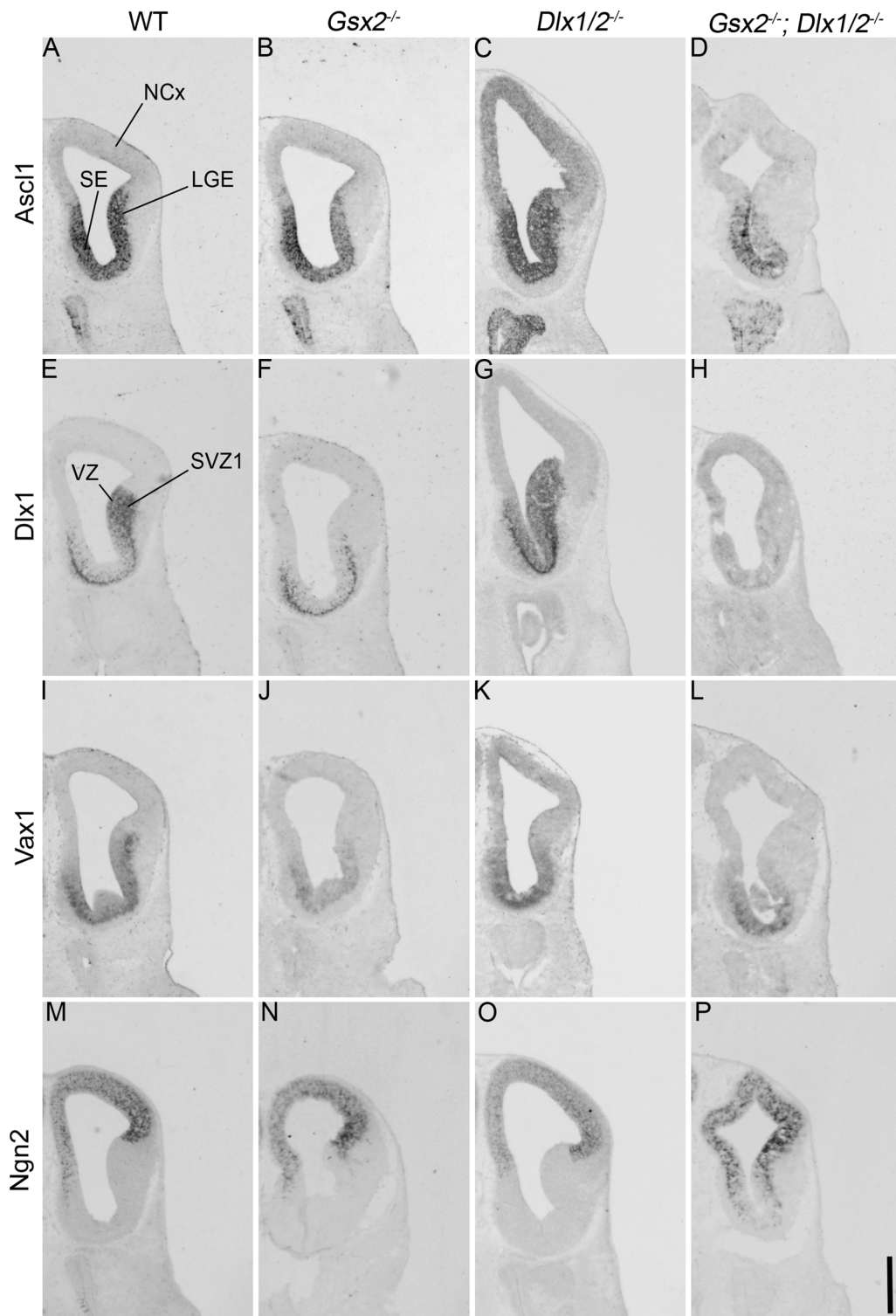
On the other hand, by E15.5, the LGE of *Gsx2*;*Dlx1/2* compound mutant largely recovered its morphology, progenitor cell properties, and expression of some striatal markers (e.g., FoxP4; Figs. 5, 12), presumably because of the Gsx1-mediated rescue (see Toresson and Campbell, 2001; Yun et al., 2003). However, despite the recovery of some LGE/striatal properties, expressions of Arx and Gad1 were less than in the single mutants (Figs. 5D,P, 12D,L); thus, together, Gsx2 and Dlx1/2 have a central role in promoting expression of Arx and Gad1 in striatal progenitors and neurons.

The CGE is largely a caudal extension of the LGE, although the neuronal output of these two progenitor zones differs (their principal derivatives include: LGE, striatum; CGE, cortical interneurons; Flames et al., 2007; Waclaw et al., 2009; Fishell and Rudy, 2011). Given the severe LGE defects in the *Gsx2*;*Dlx1/2* compound mutants, we examined the CGE phenotype at E12.5 and E15.5 (Figs. 6, 7). Previous studies demonstrated CGE deficiencies in *Gsx2* and *Dlx1/2* mutants (Long et al., 2009b; Xu et al., 2010), whose nature was confirmed here by the reduced CGE expression of Arx, Ascl1, Dlx1, Six3, and Sp9 (Figs. 6, 7). The CGE in the *Gsx2*;*Dlx1/2* compound mutants was severely hypoplastic based on morphology; on loss of Arx, Six3, and Sp9 expression; and on extension of FoxP4<sup>+</sup> pallial expression into the region (Figs. 6D,X,AB, 7D,X,AB). A CGE rudiment may persist based on residual Ascl1, Dlx1, and Gsx1 expression, although this could be from the caudal-most MGE (Figs. 6H,L,P, 7H,L,P). At E15.5, unlike the case in the LGE, we did not detect a recovery of CGE properties in the *Gsx2*;*Dlx1/2* compound mutants (there was a modest recovery in the *Gsx2* mutant; Fig. 7).

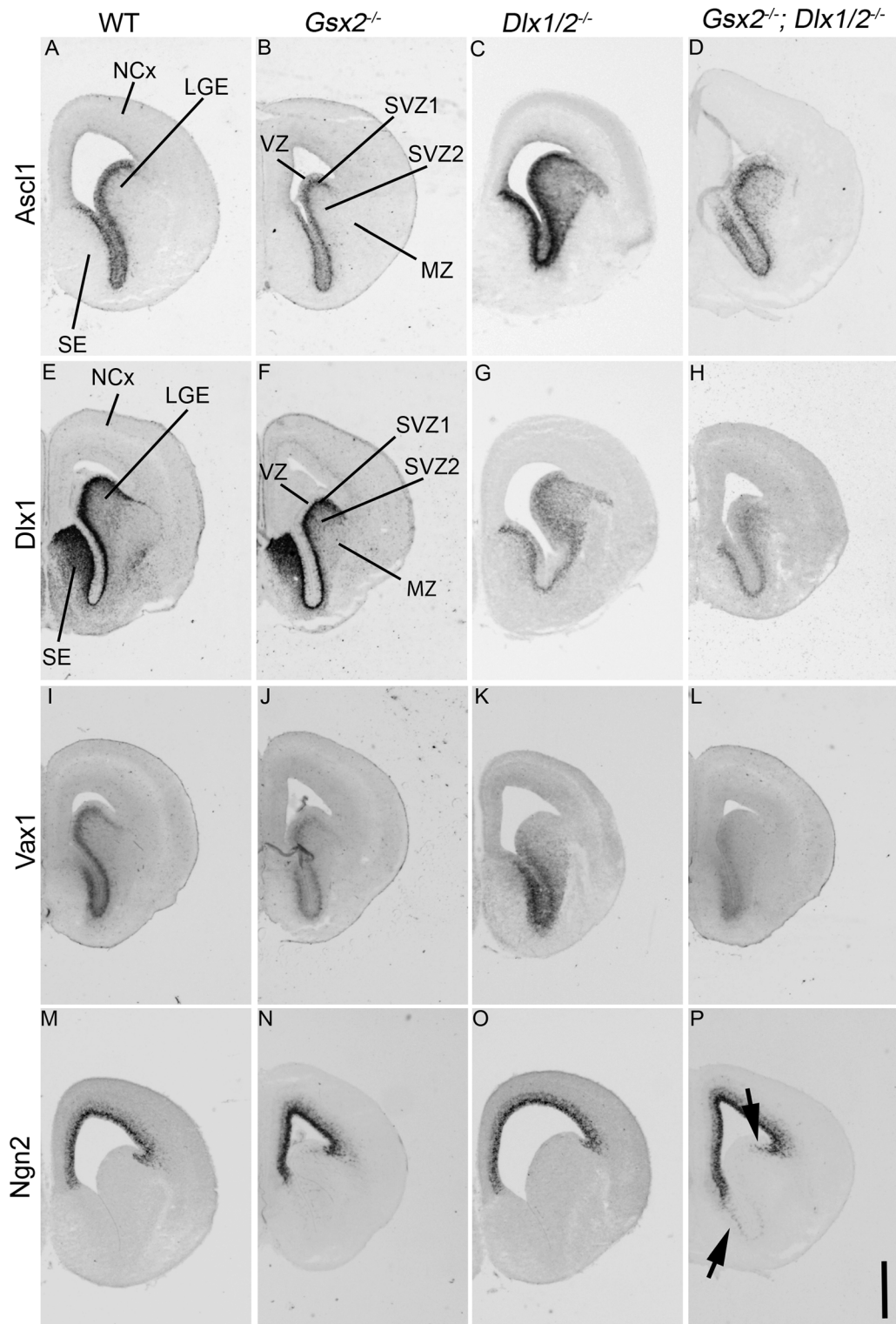
Overall, at E12.5 the *Gsx2*;*Dlx1/2* compound mutants had a severe defect in regional specification of the LGE and CGE that was more severe than that in either the *Dlx1/2* or the *Gsx2* mutants. By E15.5, although there was partial recovery of the LGE phenotype, the CGE phenotype was not rescued. Furthermore, together, Gsx2 and Dlx1/2 were essential for expression of Gad1 in LGE- and CGE-derived neurons.

### Gsx2 promotes and Dlx1/2 represses the Notch signaling pathway and oligodendrocyte progenitors in subpallial SVZ cells

Previously we showed that *Dlx1/2* mutants have elevated levels of Notch signaling in their SVZ, based on

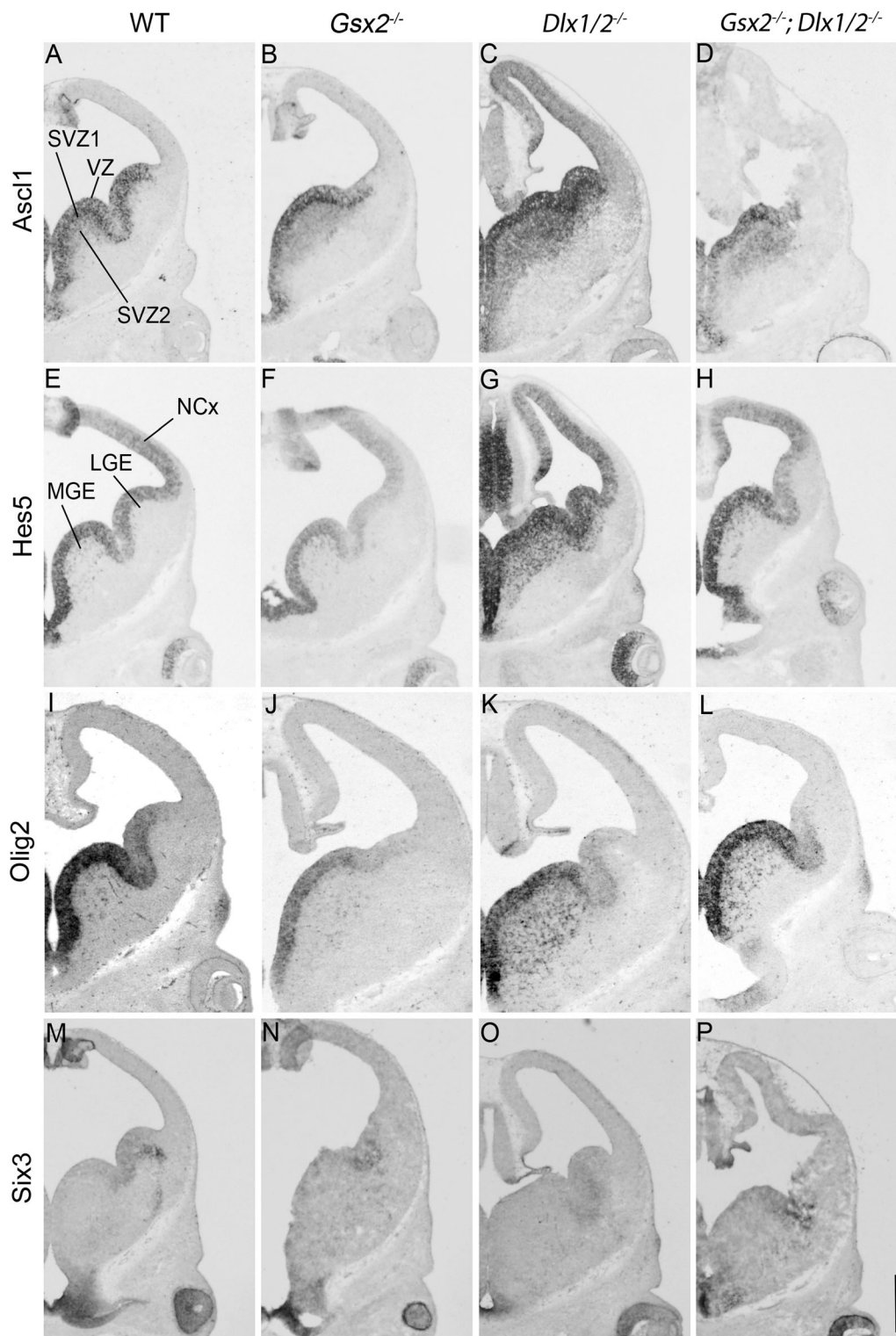


**Figure 2.** A–P: Combined functions of *Gsx2* and *Dlx1/2* define regional identity of LGE progenitor cells. In situ hybridization analysis of *Ascl1*, *Dlx1*, *Vax1*, and *Ngn2* expression at E12.5 in the rostral telencephalon, highlighting the septum and LGE, in wild-type (WT), *Gsx2*<sup>-/-</sup>, *Dlx1/2*<sup>-/-</sup>, and *Gsx2*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup>. Note that the LGE and septum of the mutant have lost subpallial properties and show expression of *Ngn2* (pallial marker). See Figure 3 for E15.5 data. Hemisections of the telencephalon are shown. LGE, lateral ganglionic eminence; NCx, neocortex; SE, septum; SVZ1, subventricular zone 1; VZ, ventricular zone. Scale bar = 500  $\mu$ m.

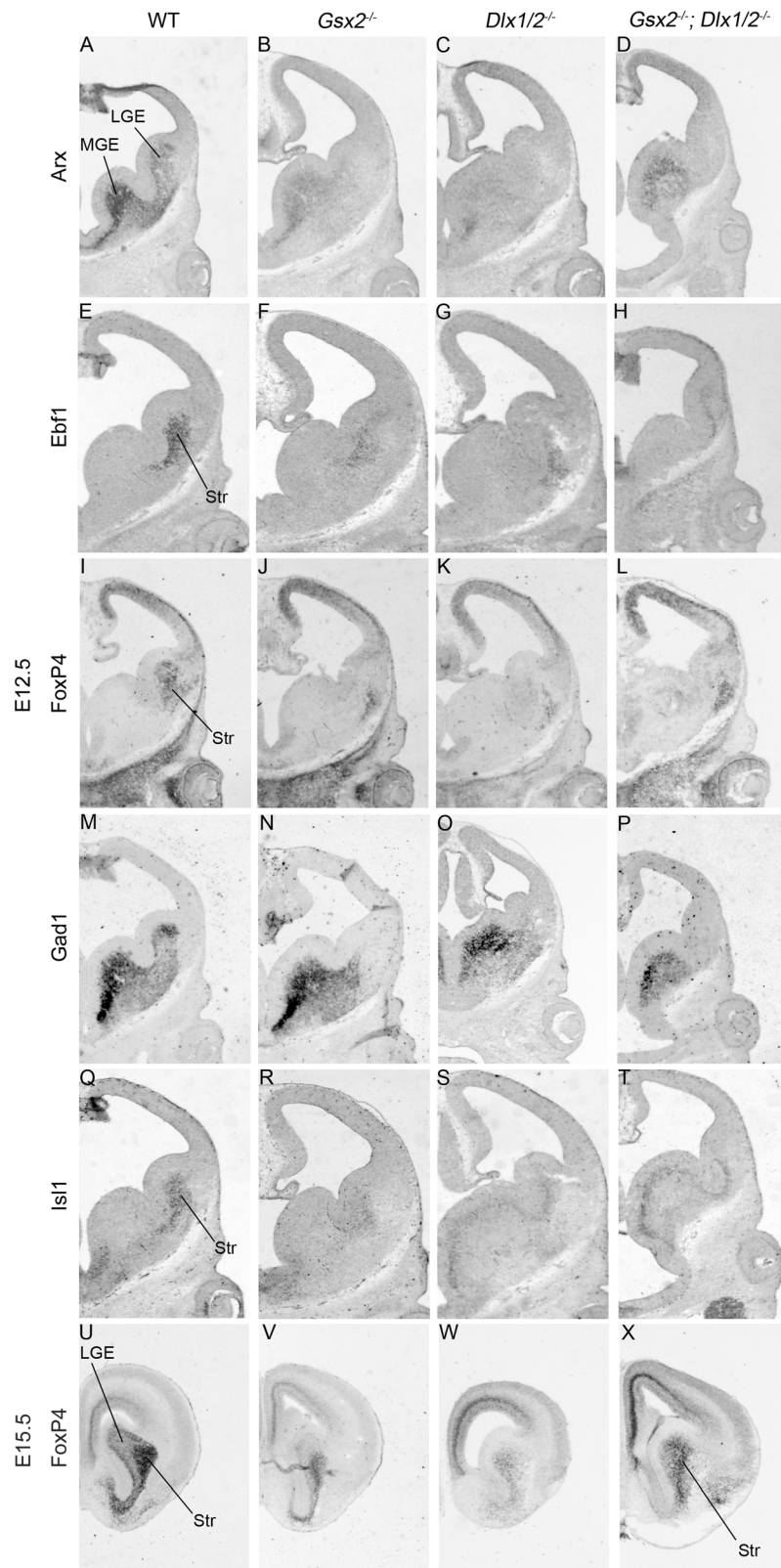


**Figure 3.** A–P: Combined functions of Gsx2 and Dlx1/2 define regional identity of LGE progenitor cells. In situ hybridization analysis of *Ascl1*, *Dlx1*, *Vax1*, *Ngn2* expression at E15.5 in the rostral telencephalon, highlighting the septum and LGE, in wild-type (WT), *Gsx2*<sup>-/-</sup>, *Dlx1/2*<sup>-/-</sup>, and *Gsx2*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup>. Note that by this age there is nearly full recovery of the wild-type phenotype in *Gsx2*<sup>-/-</sup>, whereas in *Gsx2*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup> there remains ectopic *Ngn2* expression in the septum and the dorsal LGE (arrows in P), as reduced expression of *Vax1* and *Dlx1* in the dLGE. Hemisections of the telencephalon are shown. LGE, lateral ganglionic eminence; NCx, neocortex; MZ, mantle zone; SE, septum; SVZ1, subventricular zone 1; VZ, ventricular zone. Scale bar = 1 mm.



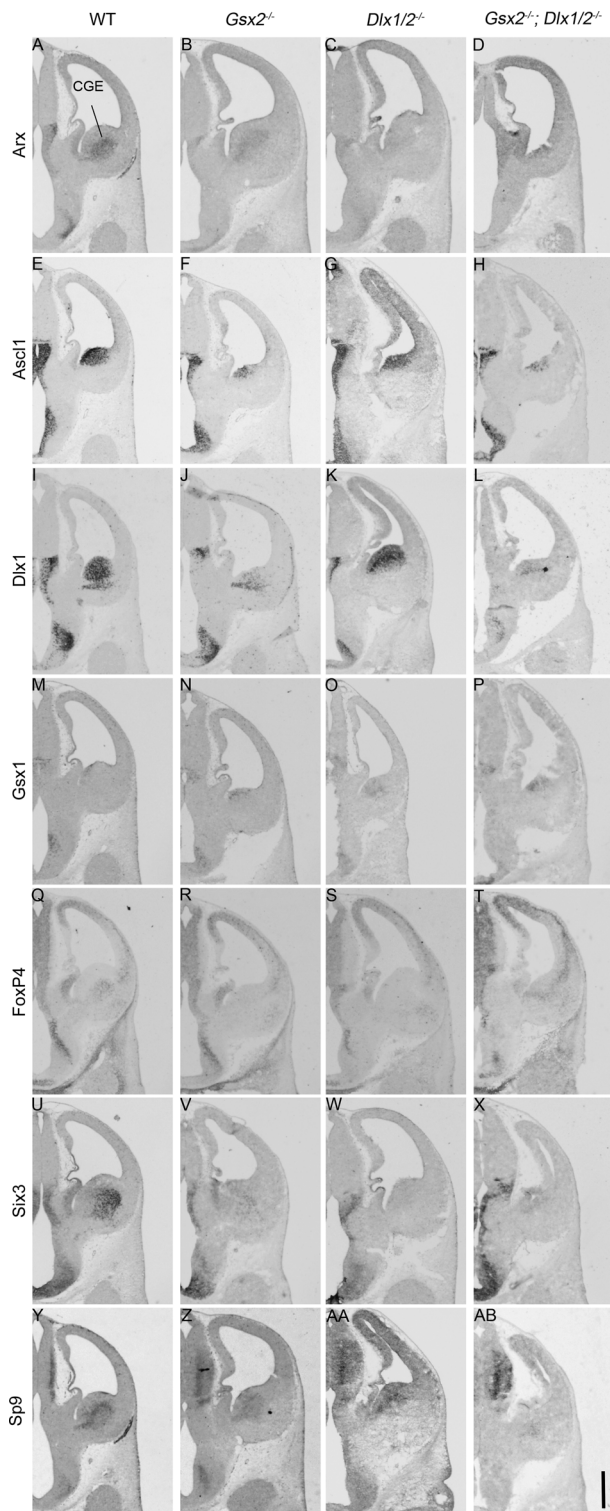


**Figure 4.** A–P: *Gsx2* promotes and *Dlx1/2* represses the Notch signaling pathway and oligodendrocyte progenitors in subpallial SVZ cells. In situ hybridization analysis of *Ascl1*, *Hes5*, *Olig2*, and *Six3* expression at E12.5 in the middle telencephalon, highlighting the LGE, along with MGE in wild-type (WT), *Gsx2*<sup>-/-</sup>, *Dlx1/2*<sup>-/-</sup>, and *Gsx2*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup>. Note that *Ascl1*, *Hes5*, and *Olig2* MGE expression in the *Gsx2*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup> is restored toward WT levels (compare with the phenotype of *Dlx1/2*<sup>-/-</sup>). See Figure 12 for E15.5 data. Hemisections of the telencephalon are shown. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; SE, septum; SVZ1 and SVZ2, subventricular zones 1 and 2; VZ, ventricular zone. Scale bar = 500  $\mu$ m.

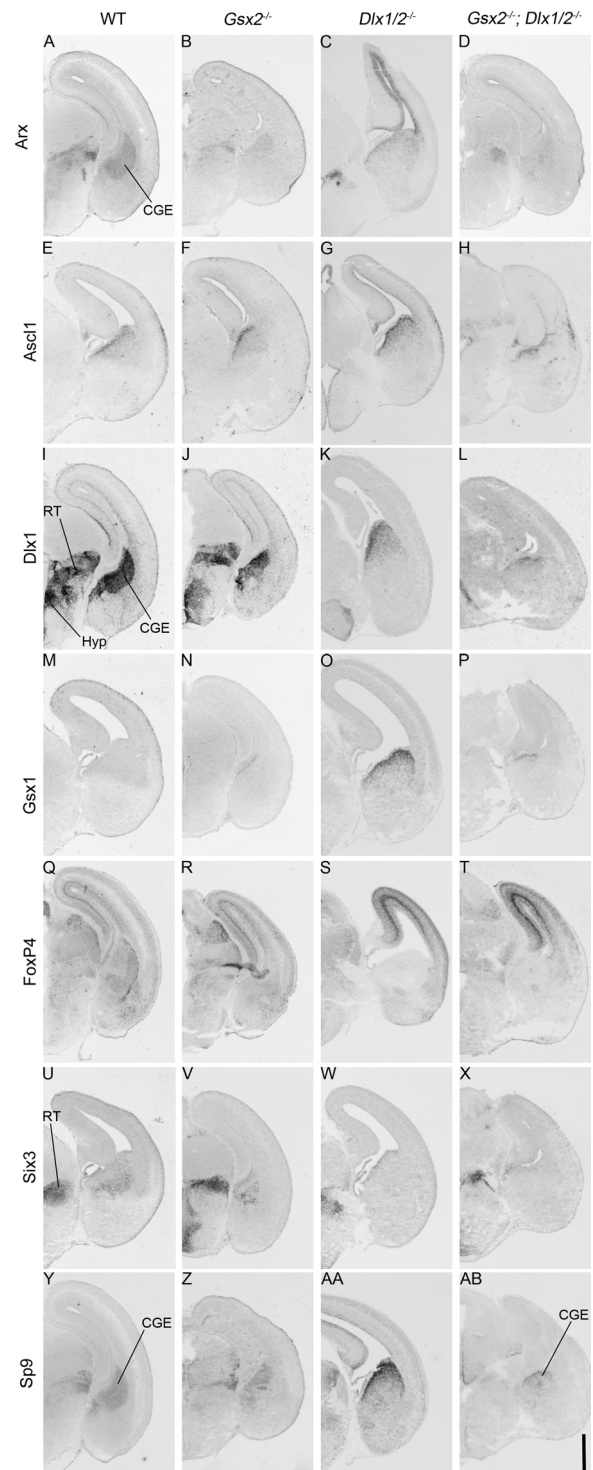


**Figure 5.** A–X: *Gsx2* and *Dlx1/2* have central roles in driving expression of *Arx*, *Ebf1*, *Gad1*, and *Isl1* in striatal progenitors and neurons. In situ hybridization analysis of *Arx*, *Ebf1*, *Foxp4*, *Gad1*, and *Isl1* expression at E12.5 (and *Foxp4* at E15.5) in the middle telencephalon, highlighting the LGE (striatum) and MGE in wild-type (WT), *Gsx2*<sup>-/-</sup>, *Dlx1/2*<sup>-/-</sup>, and *Gsx2*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup>. Note that LGE/striatal expression of *Arx*, *Ebf1*, *Gad1*, and *Isl1* is greatly decreased in all of the mutants, whereas striatal *Foxp4* expression is relatively well preserved at E12.5 and E15.5. See Figure 12 for E15.5 data. Hemisections of the telencephalon are shown. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str; striatum. Scale bars = 500  $\mu$ m in T (applies to A–T); 1 mm in X (applies to U–X).





**Figure 6.** A–AB: Combined functions of *Gsx2* and *Dlx1/2* define regional identity of CGE progenitor cells. In situ hybridization analysis of *Arx*, *Ascl1*, *Dlx1*, *Gsx1*, *FoxP4*, *Six3*, and *Sp9* expression at E12.5 in the caudal telencephalon, highlighting the CGE in wild-type (WT), *Gsx2*<sup>-/-</sup>, *Dlx1/2*<sup>-/-</sup>, and *Gsx2*<sup>-/-</sup>; *Dlx1/2*<sup>-/-</sup> mutant. Note that the CGE of the *Gsx2*<sup>-/-</sup>; *Dlx1/2*<sup>-/-</sup> mutant has lost most of its subpallial properties. See Figure 10 for E15.5 data. Hemisections of the telencephalon are shown. CGE, caudal ganglionic eminence. Scale bar = 500  $\mu$ m.



**Figure 7.** A–AB: Combined functions of *Gsx2* and *Dlx1/2* define regional identity and differentiation of CGE progenitor cells. In situ hybridization analysis of *Arx*, *Ascl1*, *Dlx1*, *Gsx1*, *FoxP4*, *Six3*, and *Sp9* expression at E15.5 in the caudal telencephalon, highlighting the CGE in wild-type (WT), *Gsx2*<sup>-/-</sup>, *Dlx1/2*<sup>-/-</sup>, and *Gsx2*<sup>-/-</sup>; *Dlx1/2*<sup>-/-</sup> mutant. Note that the CGE of the *Gsx2*<sup>-/-</sup>; *Dlx1/2*<sup>-/-</sup> mutant has lost most of its subpallial properties, except for residual *Ascl1*, *Dlx1*, *Gsx1*, and *Sp9* expression in progenitor cells. Hemisections of the telencephalon are shown. CGE, caudal ganglionic eminence; Hyp, hypothalamus; RT, reticular thalamus. Scale bar = 1 mm.



increased expression of *Ascl1*, *Delta1*, and *Hes5* (Yun et al., 2002; Long et al., 2009a,b). The *Gsx2* mutant had reduced expression of *Ascl1* and *Hes5* (Fig. 4B,F). Given that *Dlx1/2* mutants have elevated *Gsx2* expression (Yun et al., 2002; Long et al., 2009a), we tested whether *Gsx2;Dlx1/2* compound mutants have normalized Notch signaling. To do this, we studied the MGE at E12.5, because the LGE and CGE in the triple mutant are nearly eliminated. Indeed, *Ascl1* and *Hes5* expression was restored toward WT levels (Fig. 4D,H). In addition, we previously showed that *Dlx1/2* mutants have elevated levels of oligodendrogenesis in their MGE because they overexpress *Olig2* (Petryniak et al., 2007; Fig. 4K). On the contrary, here we found that *Gsx2* mutants had reduced *Olig2* expression (Fig. 4J). *Gsx2;Dlx1/2* compound mutants have normalized level of *Olig2* expression (Fig. 4L).

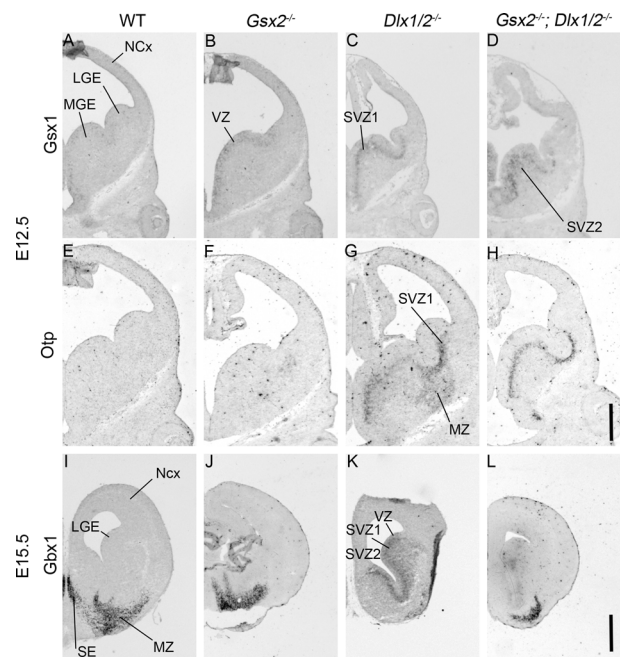
Therefore, major features of subpallial progenitors were disrupted in *Gsx2;Dlx1/2* compound mutants (*Arx*, *Gad1*, *Gsx1*, *Pbx1*, *Otp*, *Sp8*, *Sp9*, *Vax1* expression). However, some fundamental features (*Ascl1* and *Olig2* expression) were preserved, showing that part of the subpallial progenitor transcriptional program remains. Furthermore, removing *Gsx2* function in the *Dlx1/2* mutants rescued the elevated levels of *Olig2* and *Hes5* expression (Notch signaling). Next, we investigated whether removing *Gsx2* function in the *Dlx1/2* mutants rescued abnormal expression of *Gsx1*, *Gbx1*, and *Otp*.

### Role of *Gsx2* in *Dlx1/2*-mediated repression of *Gsx1*, *Gbx1*, and *Otp* expression in subpallial progenitors

*Dlx1/2* mutants overexpressed *Gsx1*, and ectopically expressed *Gbx1* and *Otp*, in subpallial progenitors (Fig. 1H; Long et al., 2009a). To assess whether *Gsx2* overexpression in the *Dlx1/2* mutant mediated the abnormal *Gsx1*, *Gbx1*, and *Otp* expression, we studied the *Gsx2;Dlx1/2* compound mutants. *Gsx1* overexpression and *Otp* ectopic expression were not rescued in the triple mutant (Fig. 8D,H). On the other hand, *Gbx1* ectopic expression was rescued (Fig. 8L). Thus, ectopic expression of *Gbx1*, like overexpression of *Ascl1*, *Hes5*, and *Olig2* (Fig. 4), was rescued by removing *Gsx2* from the *Dlx1/2* mutant.

### Combined functions of *Gsx2* and *Dlx1/2* are required to maintain *Arx* and *Gad1* expression in the MGE

Specification of MGE progenitors (based on expression of *Nkx2.1* and *Lhx6*), unlike the LGE and CGE, was not strongly affected by loss of either *Gsx2* or *Dlx1/2* (Yun et al., 2003; Long et al., 2009b). To test whether this

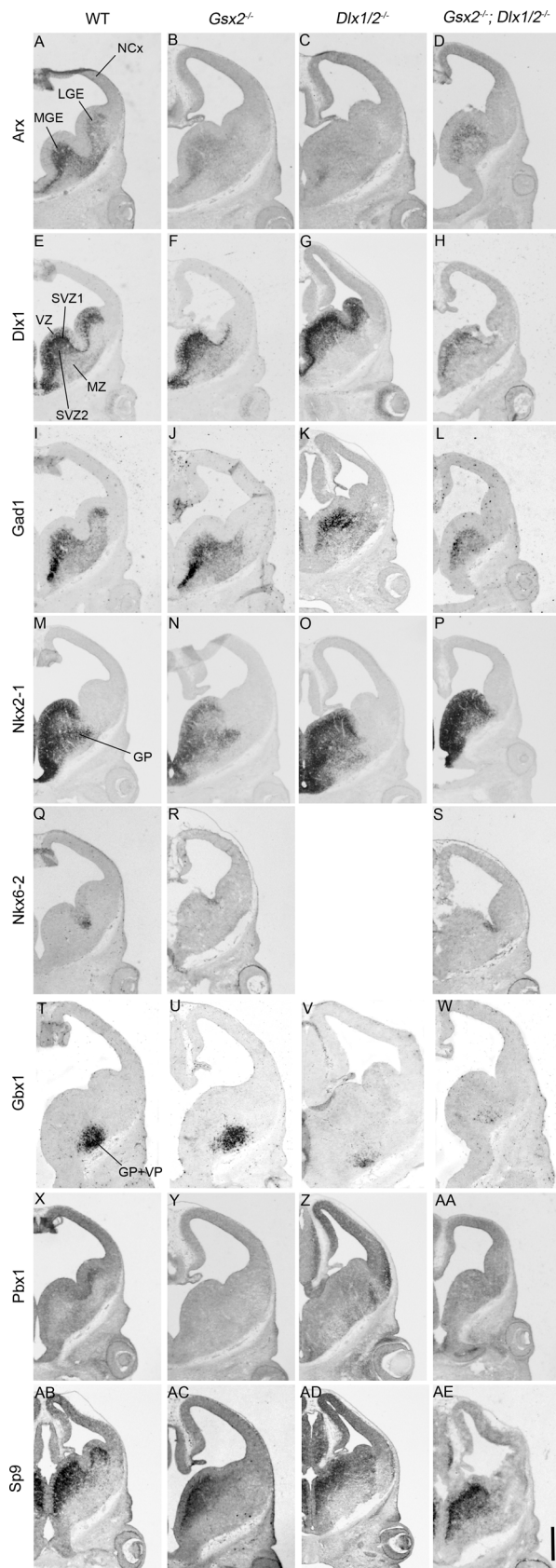


**Figure 8.** A–L: *Gsx2* is required for *Dlx1/2*-mediated repression of *Gbx1*, but not of *Gsx1* and *Otp* expression, in subpallial progenitors. In situ hybridization analysis of *Gsx1* and *Otp* expression at E12.5 and *Gbx1* at E15.5, in the middle telencephalon, highlighting the LGE and MGE in wild-type (WT), *Gsx2*<sup>-/-</sup>, *Dlx1/2*<sup>-/-</sup>, and *Gsx2*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup>. Abnormal expression of *Gbx1* in the *Dlx1/2*<sup>-/-</sup> LGE is reversed in *Gsx2*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup>, whereas the *Gsx1* and *Otp* phenotypes are not reversed. See Figure 7 for E15.5 *Gsx1* data. Hemisections of the telencephalon are shown. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MZ, mantle zone; NCx, neocortex; SE, septum; SVZ1 and SVZ2, subventricular zones 1 and 2; VZ, ventricular zone. Scale bars = 500 μm in H (applies to A–H); 1 mm in L (applies to I–L).

could be due to compensation by *Gsx2* and *Dlx1/2*, we studied the *Gsx2;Dlx1/2* compound mutants. However, as in the individual mutants, the triple mutant continued to show relative normal indices of MGE regional identity, including VZ expression of *Nkx2.1*, *Nkx6.2*, and *Olig2* (Figs. 4L, 9P,S).

In the *Dlx1/2* mutant, although MGE VZ specification did not appear to be altered, MGE SVZ properties were altered, including elevated *Ascl1*, *Hes5* and *Olig2* expression (described above; Fig. 4C,G,K) and reduced *Arx* and *Gad1* expression (Cobos et al., 2005b; Long et al., 2009b). At E12.5, the *Gsx2;Dlx1/2* compound mutant MGE SVZ had “normalized” *Ascl1*, *Hes5*, and *Olig2* expression and continued to express *Arx* and *Gad1* (Figs. 4D,H,L, 9D,L). However, by E15.5, *Arx* and *Gad1* expression in the *Gsx2;Dlx1/2* compound mutant was reduced compared with that in either the *Gsx2* or the *Dlx1/2* mutant (Fig. 10D,L), although other MGE SVZ properties were maintained (*Ascl1*, *Hes5*, *Lhx6*, *Nkx2.1*, *Olig2*, and *Sp9*; Fig. 10S,W,AE, and not shown).

MGE MZ properties were also defective in *Dlx1/2* mutants, including a small globus pallidus ( $Gbx1^+$ ,  $Lhx6^+$ ,  $Nkx2.1^+$ ; Long et al., 2009b; Fig. 10O,R,V). This defect



was similar in the *Gsx2*;*Dlx1/2* compound mutant, although there may be increased numbers of  $Lhx6^+$  and  $Nkx2.1^+$  cells in the MZ at E15.5 (Fig. 10S,W). Tangential migration of  $Lhx6^+$  cells to the cortex remained strongly blocked (Fig. 10S); this should be compared with a partial rescue of this phenotype in the *Gsx1*;*Dlx1/2* compound mutant (discussed below; see Fig. 14).

### Transcription factor expression in septal progenitor and mantle cells at E12.5 and E15.5 in *Gsx2*, *Dlx1/2*, and *Gsx2*;*Dlx1/2* mutants

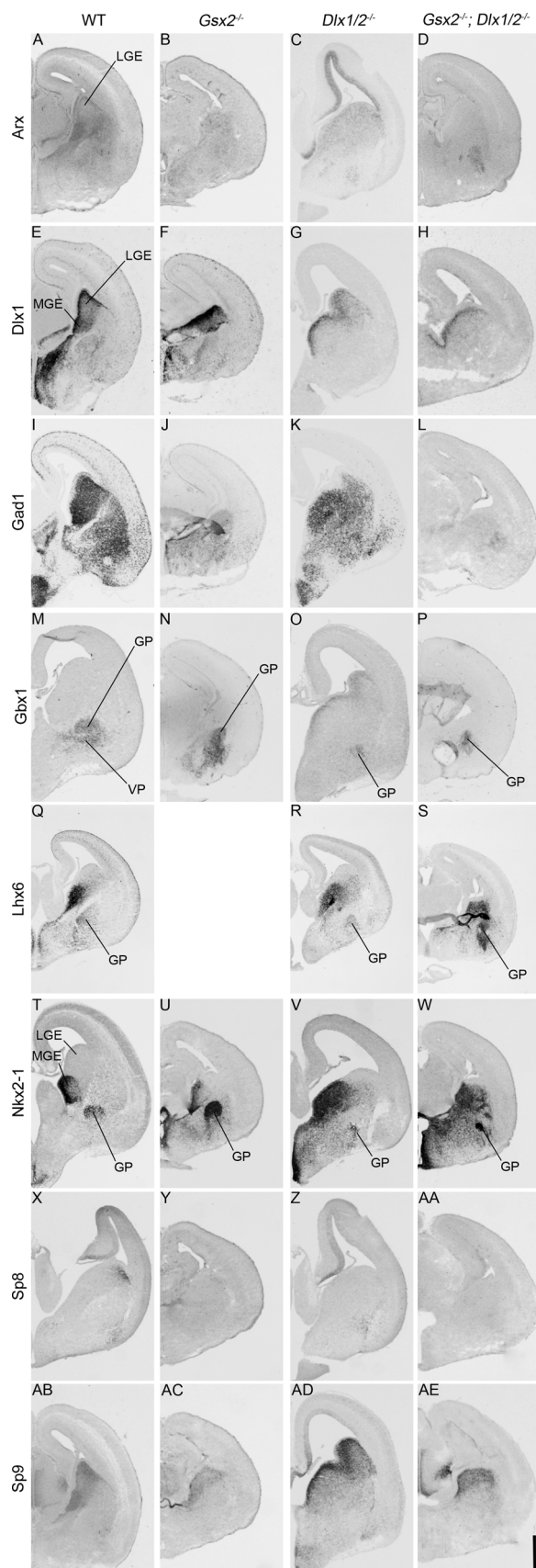
*Gsx2* function in septal development has previously not been reported. At E12.5, *Gsx2* mutants showed reduced expression of *Arx*, *Hes5*, *Islet1*, *Olig2*, and *Vax1*, whereas *Ascl1*, *Dlx1*, *Foxp4*, *GAD1*, *Ngn2*, and *Six3* expression did not appear modified (Figs. 2, 11; not shown). *Pbx1* and *Sp9* septal expression was just beginning, so it was difficult to discern a phenotype (not shown).

By E15.5 in the *Gsx2* mutant, *Arx* expression appeared to be restored (as in the LGE), and *Gsx1* was increased (Fig. 12B,N). However, expression was reduced for *Ascl1* (thinner VZ), *FoxP4*, *Islet1*, *Olig2* (ventral VZ), *Sp8* (SVZ and MZ), *Sp9* (MZ), and *Vax1* (Figs. 3, 12, and not shown). Expression appeared normal for *Dlx1*, *GAD1*, *Gbx1*, *Ngn2*, and *Pbx1* (Fig. 12, and not shown). Expression of *Six3* may be increased (Fig. 12AH).

*Dlx1/2* mutants have septal defects (Long et al., 2009a); here we extended these observations at E12.5 and E15.5. At E12.5, there was reduced expression of *Arx*, *Olig2*, and *Six3* (Fig. 11C,AE,AI); increased expression of *Ascl1*, *Gsx2*, *Islet1*, and *Sp9* (Fig. 11G,S,W, and not shown); no obvious change in expression of *Dlx1*, *FoxP4*, *GAD1*, *Pbx1*, and *Vax1* (Fig. 11K,O,AM, and not shown). At E15.5, there was reduced expression of *Dlx1*, *Gbx1* (MZ) *Olig2* (slight), *Pbx1*, and *Six3* (Fig. 12G,AE,AI, and not shown); increased expression of *Ascl1*, *Gsx1*,

**Figure 9.** A–AC: Relatively mild MGE phenotypes in the *Gsx2*<sup>-/-</sup>, *Dlx1/2*<sup>-/-</sup>, and *Gsx2*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup> mutants. In situ hybridization analysis of *Arx*, *Dlx1*, *Gad1*, *Nkx2-1*, *Nkx6-2*, *Gbx1*, *FoxP4*, *Pbx1*, and *Sp9* expression at E12.5 in the middle telencephalon, highlighting the LGE and MGE in wild-type (WT), *Gsx2*<sup>-/-</sup>, *Dlx1/2*<sup>-/-</sup>, and *Gsx2*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup>. Although MGE differentiation is abnormal in the *Dlx1/2* mutant (e.g., small globus pallidus, reduced *Arx* and *Gad1* expression), the phenotype is not strongly altered by removing *Gsx2*. See Figure 10 for E15.5 data. Hemisections of the telencephalon are shown. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MZ, mantle zone; NCx, neocortex; GP, globus pallidus; SVZ1 and SVZ2, subventricular zones 1 and 2; VP, ventral pallidum; VZ, ventricular zone. Scale bar = 500  $\mu$ m.





Gsx2, Islet1, and Sp9 (Fig. 12O,W, and not shown); no obvious change in expression of GAD1, Arx, FoxP4, and Vax1 (Fig. 12C,K,AM, and not shown); and ectopic expression of Otp (not shown).

The Gsx2;Dlx1/2 compound mutant septum showed complex and time-dependent phenotypes. At E12.5, the phenotype was more severe for *Ascl1* (reduced), *Dlx1* (reduced), *GAD1* (reduced), *Gsx1* (increased), *Ngn2* (increased), and *Vax1* (reduced; Figs. 11H,L,P,T,AB,AN, 16). For other genes, the compound mutant phenocopied the *Dlx1/2* mutant: *Arx* (decreased), *Islet1* (increased), *Six3* (decreased), *Sp9* (increased); or it phenocopied the *Gsx2* mutant: *Arx* (decreased), *Ngn2* (ectopic, but more severe), *Vax1* (decreased; Fig. 11D,X,AB,AJ,AN, and not shown). There may be partial rescue of the *Olig2* expression in the compound mutant (Fig. 11AF).

At E15.5, the Gsx2;Dlx1/2 compound mutant's septum showed a more severe reduction of *GAD1*, *Arx*, *FoxP4*, *Pbx1*, *Sp8*, *Sp9*, and *Vax1* (Fig. 12D,L,AN, and not shown). For other genes, the compound mutant phenocopied the *Dlx1/2* mutant: *Dlx1* (reduced), *Gbx1* (reduced), *Gsx1* (increased), *Islet1* (increased), *Otp* (increased), *Sp9* (increased; Fig. 12H,P,X, and not shown); or phenocopied the *Gsx2* mutant: *Six3* (increased), *Vax1* (reduced; Fig. 12AJ,AN). There was an intermediate phenotype for *Islet1*: increased in the SVZ (like *Dlx1/2*) and decreased in the MZ (like *Gsx2*). *Ngn2* was not ectopically expressed (Fig. 12AB). *Ascl1* and *Hes5* expression appeared intermediate between the *Gsx2* mutant and the *Dlx1/2* mutant phenotypes (Fig. 12T, and not shown). *Nkx2.1* (medial septum MZ) and *Olig2* (VZ) expression was not clearly changed in the compound mutant (Fig. 12AF, and not shown).

### Analysis of Gsx1, Dlx1/2, and Gsx1;Dlx1/2 mutants: partial rescue of the MGE and tangential migration of Lhx6<sup>+</sup> cells to the cortex

Previous studies have failed to identify strong molecular or cellular defects in *Gsx1* mutant basal ganglia, aside

**Figure 10.** A–AE: Relatively mild MGE phenotypes in the *Gsx2*<sup>-/-</sup>, *Dlx1/2*<sup>-/-</sup>, and *Gsx2*<sup>-/-</sup>; *Dlx1/2*<sup>-/-</sup> mutants. In situ hybridization analysis of *Arx*, *Dlx1*, *Gad1*, *Lhx6*, *Nkx2-1*, *Nkx6-2*, *Gbx1*, *FoxP4*, *Pbx1*, and *Sp9* expression at E15.5 in the middle telencephalon, highlighting the LGE and MGE in wild-type (WT), *Gsx2*<sup>-/-</sup>, *Dlx1/2*<sup>-/-</sup>, and *Gsx2*<sup>-/-</sup>; *Dlx1/2*<sup>-/-</sup>. Although MGE differentiation is abnormal in the *Dlx1/2* mutant (e.g., small globus pallidus, reduced *Arx* and *Gad1* expression), the phenotype is not strongly altered by removing *Gsx2*. Expression of *Lhx6* in the *Gsx2*<sup>-/-</sup> is not shown; previously, we found it to be normal at E11.5 and E18.5 (Yun et al., 2003). Hemisections of the telencephalon are shown. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; GP, globus pallidus; VP, ventral pallidum. Scale bar = 1 mm.





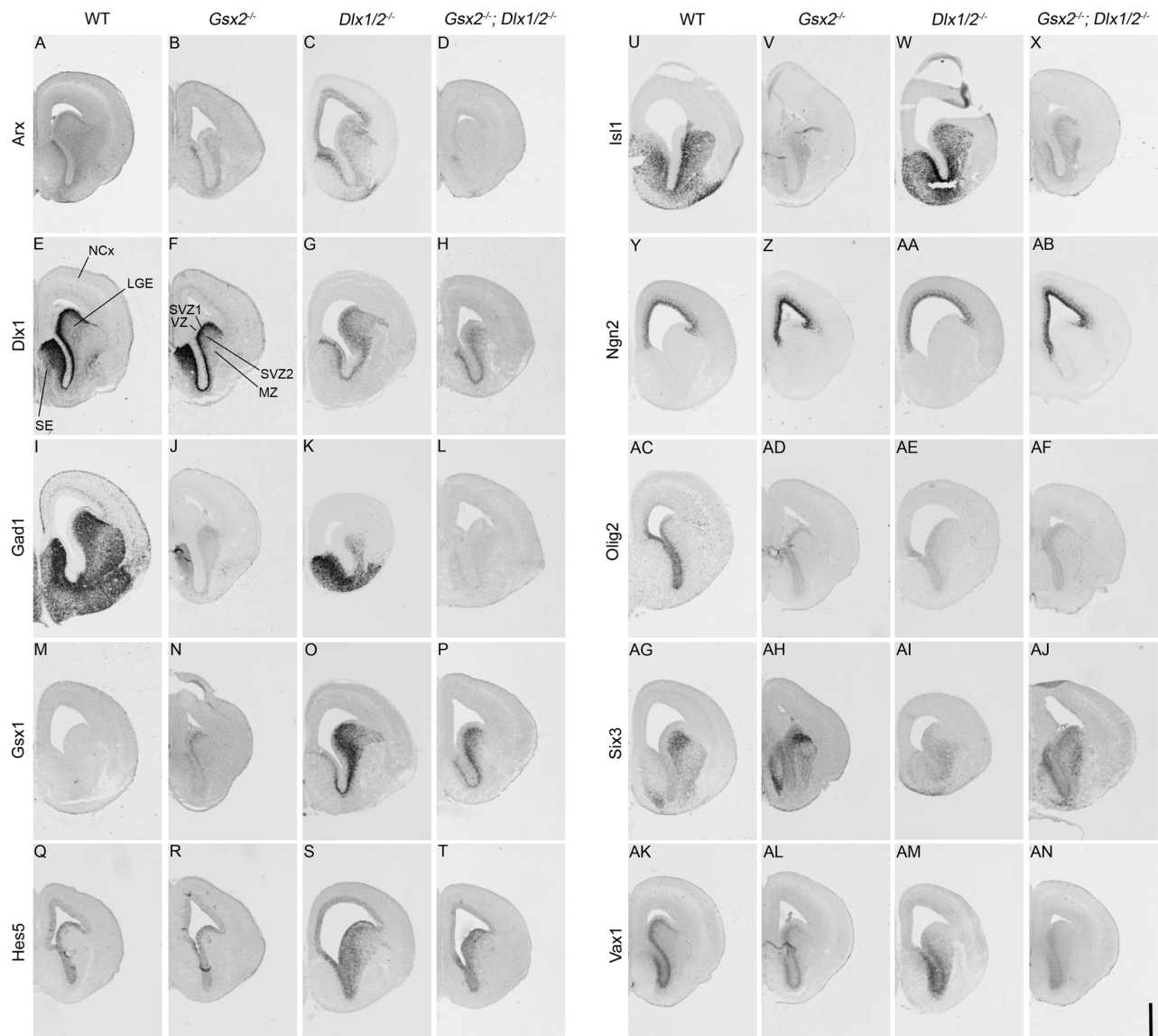
**Figure 11.** A–AN: *Dlx1/2* and *Gsx2* are critical for septal development. In situ hybridization analysis of *Arx*, *Ascl1*, *Dlx1*, *Gad1*, *Gsx1*, *Isl1*, *Ngn2*, *Olig2*, *Six3*, and *Vax1* at E12.5 in the rostral telencephalon, highlighting the LGE and septum in wild-type (WT), *Gsx2*<sup>-/-</sup>, *Dlx1/2*<sup>-/-</sup>, and *Gsx2*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup>. Each single mutant shows gene expression defects, which are amplified in *Gsx2*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup>. See Figure 12 for E15.5 data. Hemisections of the telencephalon are shown. LGE, lateral ganglionic eminence; NCx, neocortex; SE, septum. Scale bar = 500  $\mu$ m.

from ectopic *Dbx1* expression (Toresson and Campbell, 2001; Yun et al., 2003). Here we investigated *Gsx1* function because its expression is increased in the *Dlx1/2* mutants (Long et al., 2009a,b). We studied the phenotype of the *Gsx1*;*Dlx1/2* compound mutants to determine whether some of the *Dlx1/2* phenotype was caused by overexpression of *Gsx1*. Analysis was performed at E15.5.

First, we identified some subtle phenotypes in the *Gsx1* mutant. The septum had the most obvious phenotypes, with reduced expression of *Dlx1* and *Dlx2* in the VZ; reduced *Hes5* in the SVZ; and reduced *Dlx2*, *Gbx1*, *Lhx6*, and *Nkx2.1* in the MZ (particularly medial septum);

*Gbx1* expression was increased in the SVZ (Fig. 13R). The LGE showed reduced expression of *Dlx1* and *Dlx2* in the VZ (ventral more severe than dorsal; Fig. 13F,J) and reduced *Gad1* expression in the SVZ (Fig. 13N); *Gbx1* expression was increased in the VZ (Fig. 13R).

Next, we assessed whether removing *Gsx1* expression in the *Dlx1/2* mutants rescued any of the *Dlx1/2* mutant phenotypes. Remarkably, there was partial reduction of tangential migration of *Lhx6*<sup>+</sup> cells into the cortex of *Gsx1*;*Dlx1/2* compound mutants (Fig. 14AF). Associated with this was an increase in *Lhx6* expression in the progenitor zones of the MGE to a level similar to the wild type (Fig. 14AF). Likewise, in the *Gsx1*;*Dlx1/2* compound



**Figure 12.** A–AN: *Gsx2* and *Dlx1/2* have central roles in maintaining expression of *Gad1* and other features of septal and striatal development. In situ hybridization analysis of *Arx*, *Dlx1*, *Gad1*, *Gsx1*, *Isl1*, *Ngn2*, *Olig2*, *Six3*, and *Vax1* expression at E15.5 in the rostral telencephalon, highlighting the LGE (striatum) and septum in wild-type (WT), *Gsx2*<sup>-/-</sup>, *Dlx1/2*<sup>-/-</sup>, and *Gsx2*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup>. Note the loss of *Arx*, *Gad1*, and *Isl1* expression in the LGE/striatum and septum. Hemisections of the telencephalon are shown. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; Str, striatum. Scale bar = 1 mm.

mutant MGE, *Hes5* expression in the SVZ was reduced toward wild-type levels, and *GAD1* expression was increased toward wild-type levels (Fig. 14P,AB). This provides evidence that elevated *Gsx1* expression in the *Dlx1/2* mutant contributes to MGE differentiation and migration defects.

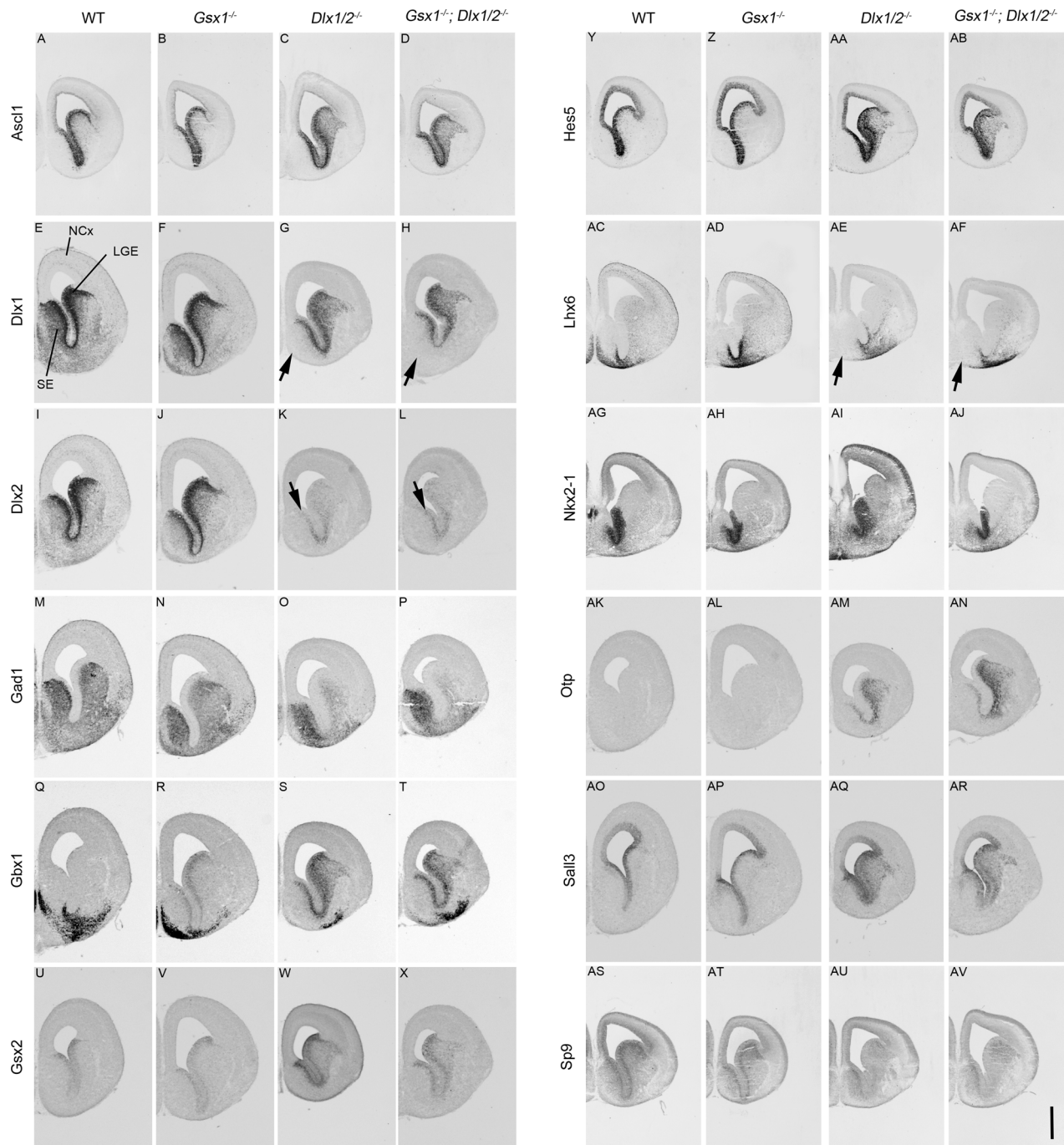
In addition to the partial rescue of the MGE phenotypes, there was subtle rescue of septal and CGE phenotypes, including expression of *Ascl1* (VZ of septum and CGE; Figs. 13D, 15D), *Dlx1* (VZ of septum, MGE and CGE; Figs. 13H, 14H, 15H), *Hes5* (SVZ of MGE; Fig. 14AB), and *Sall3* (VZ of septum; Fig. 13AR). By contrast with partial

rescue in the MGE, septum, and CGE, some phenotypes were worsened in the triple mutants, including expression of *Ascl1* (VZ of vLGE; Fig. 13D), *Hes5* (SVZ of septum; Fig. 13AB), and *Sall3* (VZ of vLGE; Fig. 13AV). Finally, we did not observe any clear rescue of LGE phenotypes (Long et al., 2009a; Fig. 13).

## DISCUSSION

Elucidating transcriptional networks will be required to understand the mechanisms that control brain development. Here we have focused on the roles of the *Gsx1* and *-2* and *Dlx1* and *-2* genes in regulating the expression of



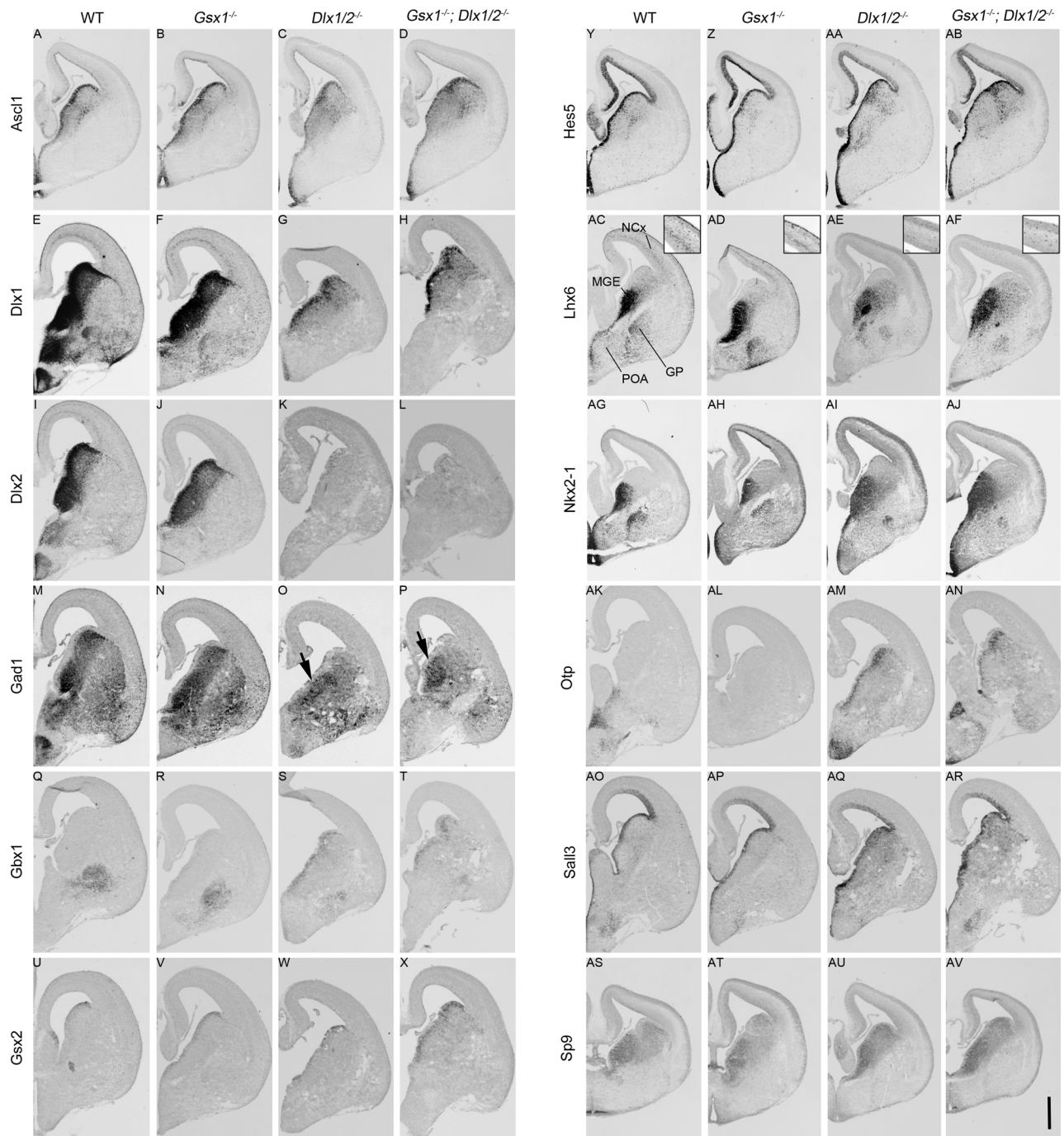


**Figure 13.** A–AV: *Gsx1*, in combination with *Dlx1/2*, regulates septal development. In situ hybridization analysis of *Ascl1*, *Dlx1*, *Dlx2*, *Gad1*, *Gbx1*, *Gsx2*, *Hes5*, *Lhx6*, *Nkx2-1*, *Otp*, *Sall3*, and *Sp9* at E15.5 in the rostral telencephalon, highlighting the LGE and septum in wild-type (WT), *Gsx1*<sup>-/-</sup>, *Dlx1/2*<sup>-/-</sup>, and *Gsx1*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup>. The *Gsx1* septum had more obvious phenotypes than the LGE (see Results); arrows point to reduced *Dlx1*, *Dlx2*, and *Lhx6* expression in the septum (G,H,K,L,AE,AF). Hemisections of the telencephalon are shown. LGE, lateral ganglionic eminence; NCx, neocortex; SE, septum. Scale bar = 1 mm.

23 transcription factors during patterning and differentiation of the mouse subcortical telencephalon. Based on these results, and in previous publications on basal ganglia phenotypes in *Dlx1* and -2 (Anderson et al., 1997b; Petryniak et al., 2007; Long et al., 2009a,b), *Gsx1* and -2

(Corbin et al., 2000; Toresson et al., 2000; Toresson and Campbell, 2001; Yun et al., 2001, 2003; Waclaw et al., 2004), *Ascl1* (Mash1; Casarosa et al., 1999; Horton et al., 1999; Yun et al., 2002; Castro et al., 2006; Long et al., 2009a,b), and *Olig2* (Petryniak et al., 2007) mouse

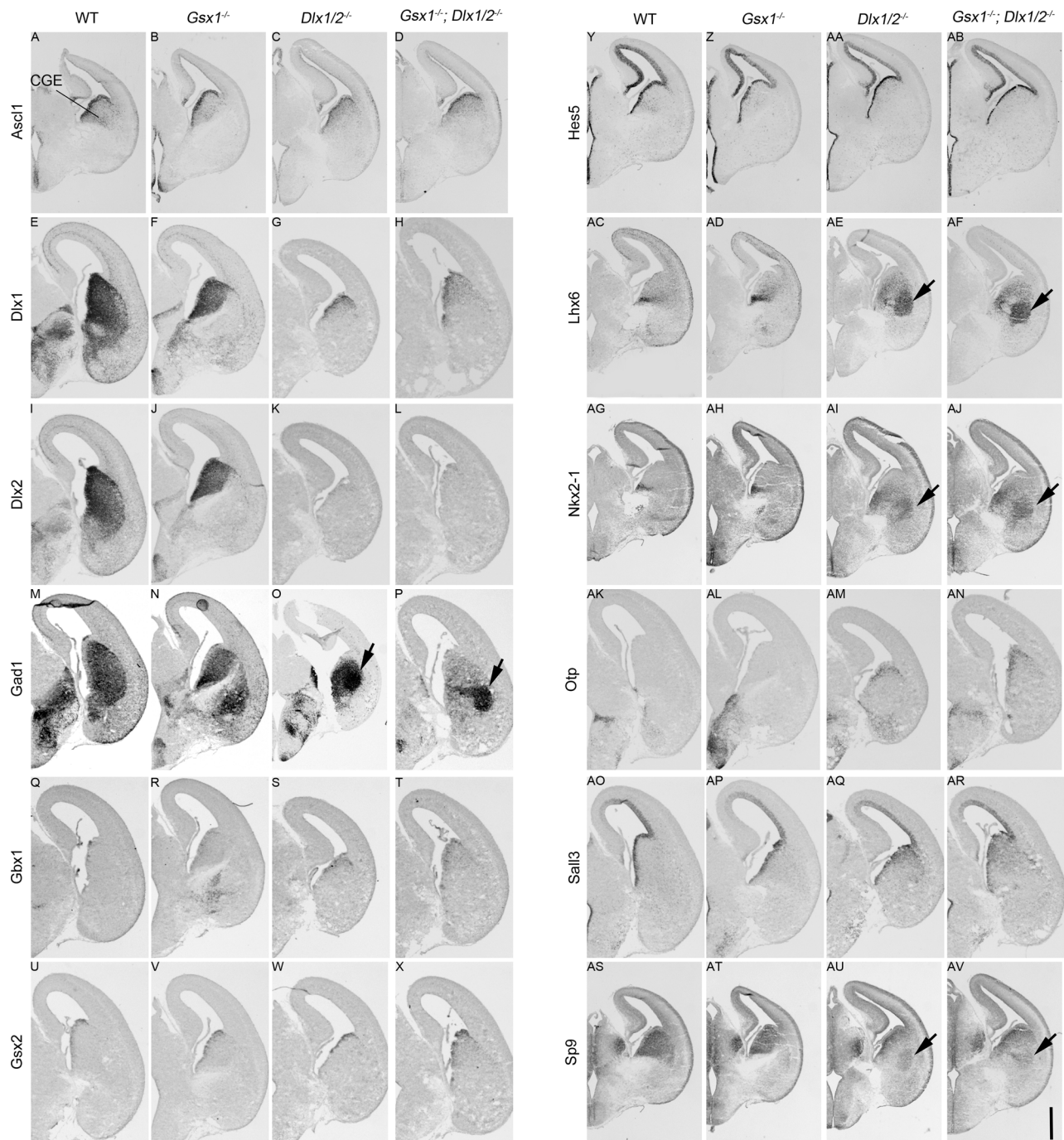




**Figure 14.** A–AV: Removal of Gsx1 partially rescues MGE differentiation in Dlx1/2 mutants. In situ hybridization analysis of *Ascl1*, *Dlx1*, *Dlx2*, *Gad1*, *Gbx1*, *Gsx2*, *Hes5*, *Lhx6*, *Nkx2-1*, *Otp*, *Sall3*, and *Sp9* at E15.5 in the middle telencephalon, highlighting the LGE and MGE in wild-type (WT), *Gsx1*<sup>-/-</sup>, *Dlx1/2*<sup>-/-</sup>, and *Gsx1*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup>. Note the partial rescue of *Lhx6*<sup>+</sup> cells in the neocortex (higher magnifications in AC–AF) and the increased expression of *Gad1* and *Lhx6* in the SVZ of the MGE. Hemisections of the telencephalon are shown. GP, globus pallidus; MGE, medial ganglionic eminence; NCx, neocortex; POA, preoptic area. Scale bar = 1 mm.

mutants, we have generated a provisional model of the genetic hierarchy of transcription factor genes in the LGE/dCGE (Fig. 18); a definitive model will require additional data, including demonstration of direct transcription regulation at each step. Here we address the basis

for this model. We have summarized the gene expression changes in Figures 16 and 17; for comparison see Long et al. (2009a,b) for summaries of gene expression changes in the *Dlx1/2*, *Ascl1*, and *Dlx1/2*;*Ascl1* mutants, using the same schemata.



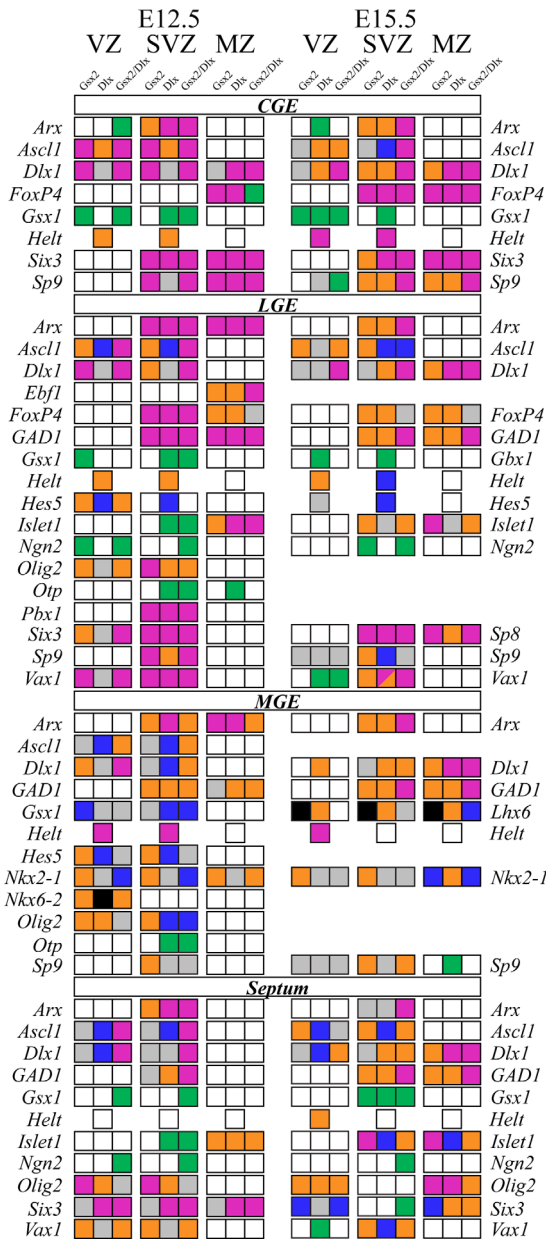
**Figure 15.** A–AN: Although loss of *Gsx1* partially rescues the *Dlx1/2*<sup>-/-</sup> MGE, *Lhx6*<sup>+</sup>, *Nkx2-1*<sup>+</sup>, *Gad1*<sup>+</sup>, and *Sp9*<sup>+</sup> cells continue to migrate ectopically in the CGE (arrows in O,P,AE,AF,AI,AJ,AU,AV). In situ hybridization analysis of *Ascl1*, *Dlx1*, *Dlx2*, *Gad1*, *Gbx1*, *Gsx2*, *Hes5*, *Lhx6*, *Nkx2-1*, *Otp*, *Sall3*, and *Sp9* at E15.5 in the caudal telencephalon, highlighting the CGE in wild-type (WT), *Gsx1*<sup>-/-</sup>, *Dlx1/2*<sup>-/-</sup>, and *Gsx1*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup>. Hemisections of the telencephalon are shown. CGE, caudal ganglionic eminence. Scale bar = 1 mm.

### GSX2, ASCL1 (MASH1), and DLX2 expressions define their temporal hierarchy in the LGE

ASCL1 and DLX2 proteins are strongly expressed throughout the subpallium, but GSX2 expression is most easily detected in the LGE, septum, and CGE, although

GSX2 is also expressed in the MGE. Here we were focused on LGE expression at E10.5–E15.5 (Fig. 1, and not shown). Double-immunofluorescence analysis of GSX2, ASCL1, and DLX2 protein expression in the LGE provides evidence for a temporal hierarchy of their expression. At E10.5, the most immature cells (VZ cells) express only





**Figure 16.** Expression of transcription factors in the ventricular zone (VZ), subventricular zone (SVZ), and mantle zone (MZ) of the LGE, MGE, and CGE in the *Gsx2*<sup>-/-</sup> (*Gsx2*), *Dlx1/2*<sup>-/-</sup> (*Dlx*), and *Gsx2*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup> (*Gsx2/Dlx*) mutants at E12.5 and E15.5. This figure depicts, as discrete boxes, the VZ, SVZ, and MZ of the CGE, LGE, MGE, and septum. The genes are listed alphabetically. The effect of each mutation on transcription factor expression in each box is indicated using a color code. Black indicates that expression was not analyzed (if no squares are listed, this also means that this analysis was not performed). Gray indicates that expression was not clearly changed in the mutant. White indicates no detectable expression. Red indicates severe reduction in expression. Orange indicates moderate/mild reduction in expression. Green indicates ectopic expression. Blue indicates increased expression. If the box is subdivided diagonally, the top part corresponds to the dorsal region, the bottom to the ventral region.

GSX2. As the VZ cells mature, scattered cells express ASCL1 and DLX2, most of which coexpress GSX2. By E12.5, many LGE progenitors (VZ + SVZ) coexpress GSX2, ASCL1, and DLX2 (Table 1). Coexpression is strongest in SVZ1, the part of the SVZ adjacent to the VZ.

The *Dlx1/2*;*Ascl1* mutant phenotype of subpallial progenitors and neurons showed much more severe defects than either the *Dlx1/2* or the *Ascl1* mutants (Long et al., 2009a,b). Likewise, *Gsx2* and *Ascl1* double mutants showed more severe defects than the single mutants (Wang et al., 2009). Here we demonstrated a functional interaction between *Gsx1* and *Gsx2* with *Dlx1/2* and provided evidence for the functional hierarchy of *Gsx2*, *Gsx1*, *Ascl1*, and *Dlx1/2*. We suggest that these phenotypes are due in large part to cell autonomous defects, particularly in the SVZ1, where GSX2, ASCL1, and DLX2 are coexpressed.

### **Gsx2 homeodomain: top of the hierarchy of dLGE/dCGE identity**

We propose that *Gsx2* promotes the identity of primary progenitors in the VZ of the dLGE and dCGE. *Gsx2* null mutants fail to specify dorsal parts of the LGE and CGE, showing reduced expression of other transcription factors that mark the VZ of these regions (*Ascl1*, *Dlx2*, *Olig2*). Our loss-of-function analysis is consistent with ectopic expression experiments in which cortical misexpression of *Gsx2* induces *Ascl1* and *Dlx1/2* (Waclaw et al., 2009). Therefore, we hypothesize that *Gsx2* promotes the expression of *Ascl1*, *Dlx2*, and *Olig2*, from which emanate three major pathways (Fig. 18): 1) neural differentiation driven by *Dlx1* and -2; 2) lateral inhibition to promote the maintenance of multipotent progenitors driven by *Ascl1* promoting *Delta* expression, which in turn increases Notch signaling and *Hes5* expression; and 3) progenitor cell maintenance through *Hes5* and competence to produce oligodendrocytes through *Olig2*.

### **Gsx1 homeodomain is upregulated in the absence of Gsx2 and Dlx1/2 and contributes to MGE phenotypes in the Dlx1/2 mutants**

Previous studies showed that *Gsx1* mutants have a very mild telencephalic phenotype. They have increased *Gsx2* expression (Pei et al., 2011) and ectopic expression of *Dbx1*, a marker of the ventral cortex and preoptic area; the ramifications of this are not known. *Gsx2* mutants are partially rescued by *Gsx1*, providing evidence that *Gsx1* can compensate for *Gsx2*. Combined removal of *Gsx1* and -2 leads to misspecification of the dorsal and ventral LGE (Toresson and Campbell, 2001; Yun et al., 2003). Overexpression of *Gsx1* throughout the telencephalon



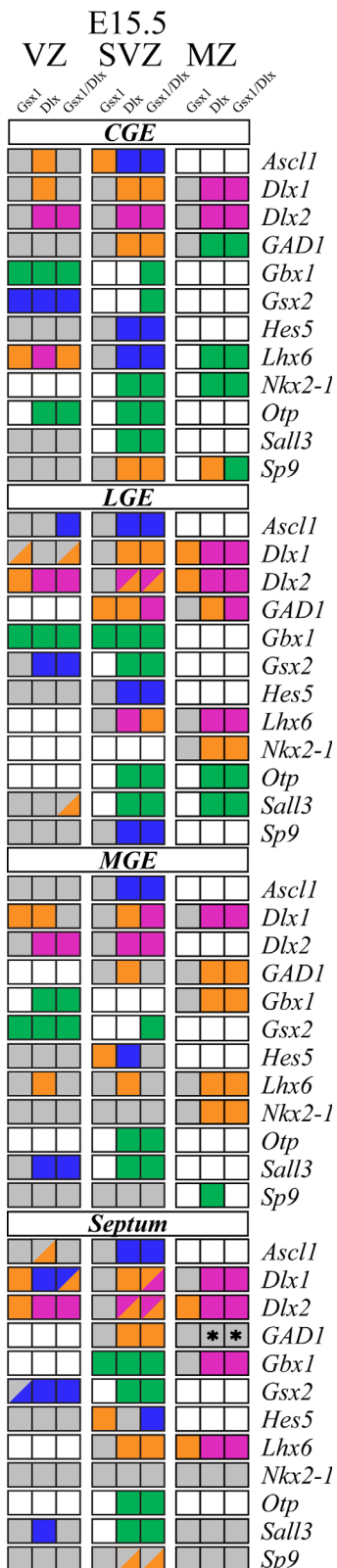
(tetO-Gsx1-IRES-EGFP;Foxg1tTA/+ mice) induced *Ascl1* and *Dlx1/2*. This result is similar to the phenotype of *Gsx2* overexpression, confirming that *Gsx1* and *Gsx2* share properties (Pei et al., 2011). On the other hand, misexpressions of *Gsx1* and *Gsx2* show opposite effects

on the switch between proliferation and differentiation: *Gsx1* lengthens the cell cycle and promotes neurogenesis (and represses *Gsx2* expression), whereas *Gsx2* maintains the progenitor state (Waclaw et al., 2009; Pei et al., 2011). Thus, *Gsx1* and *Gsx2* share functions in promoting subpallial identity and appear to have opposite functions in regulating the switch between progenitor and neuronal fates.

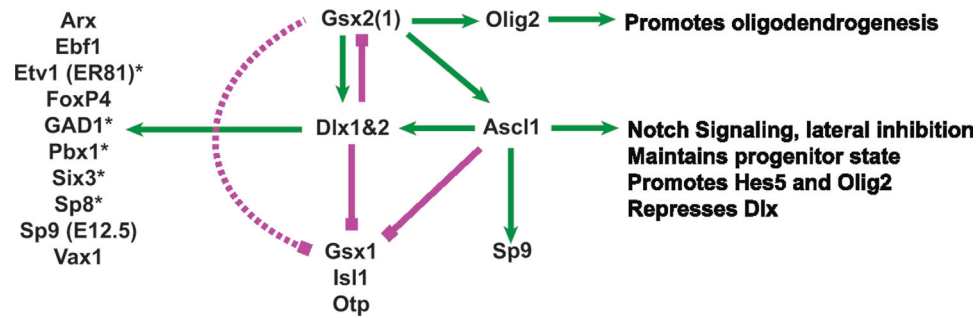
*Gsx2* and *Dlx1* and -2 are negative regulators of *Gsx1* (Fig. 1G–L; Toresson et al., 2000; Yun et al., 2001; Long et al., 2009a,b). *Dlx1* and -2 repression of *Gsx1* was explored here by making *Gsx1*;*Dlx1/2* mutants. We found that loss of *Gsx1* partially rescued MGE phenotypes in the *Dlx1/2* mutant, including interneuron migration to the cortex (see below). As noted above, overexpression of *Gsx1* promoted neurogenesis and repressed proliferation (Pei et al., 2011); therefore, we had anticipated that removing *Gsx1* in the *Dlx1/2* mutants might further block their differentiation. On the contrary, the MGE of the *Gsx1*;*Dlx1/2* mutants had a subtle reduction of *Hes5* expression (indicator of Notch signaling) compared with *Dlx1/2* mutants and had increased *GAD1* and *Lhx6* expression (indicators of differentiation; Fig. 14). Thus, in the *Dlx1/2* mutant MGE, removing *Gsx1* reduced *Hes5* (Notch signaling), which is similar to the effect of removing *Gsx2* in the *Dlx1/2* mutant (Fig. 4).

### Two functions of *Ascl1* (*Mash1*) bHLH: promoting the subcortical progenitor state through Notch signaling and promoting subcortical differentiation with *Gsx2* and *Dlx1/2*

Previous studies demonstrated that *Ascl1* promotes the subcortical progenitor state through cell autonomously increasing *Delta1* expression and cell nonautonomously (through lateral inhibition) increasing Notch



**Figure 17.** Expression of transcription factors in the ventricular zone (VZ), subventricular zone (SVZ), and mantle zone (MZ) of the LGE, MGE, and CGE in the *Gsx1*<sup>-/-</sup> (*Gsx1*), *Dlx1/2*<sup>-/-</sup> (*Dlx*), and *Gsx1*<sup>-/-</sup>;*Dlx1/2*<sup>-/-</sup> (*Gsx1/Dlx*) mutants at E15.5. This figure depicts, as discrete boxes, the VZ, SVZ, and MZ of the CGE, LGE, MGE, and septum. The genes are listed alphabetically. The effect of each mutation on transcription factor expression in each box is indicated using a color code. Gray indicates that expression was not clearly changed in the mutant. White indicates no detectable expression. Red indicates severe reduction in expression. Orange indicates moderate/mild reduction in expression. Green indicates ectopic expression. Blue indicates increased expression. If no squares are listed, this means that this analysis was not performed. If the box is subdivided diagonally, the top part correspond to the dorsal region, the bottom to the ventral region. Asterisk indicates that diagonal band *GAD1* expression was absent.



**Figure 18.** Model of transcription factor network interactions in the developing LGE based largely on loss-of-function analyses (see Discussion). Green arrows indicate activation; magenta squares indicate inhibition. Genes activated by Dlx1 and -2 that have asterisks correspond to genes whose expression is most strongly reduced in the  $Dlx1/2^{-/-}$  mutant. At the top of the hierarchy is Gsx2; Gsx2(1) indicates that Gsx1 can compensate for loss of Gsx2. For comparison see Long et al. (2009a,b) for summaries of gene expression changes in the  $Dlx1/2$ ,  $Ascl1$ , and  $Dlx1/2;Ascl1$  mutants, using the same schemata.

signaling and repressing Dlx expression (Casarosa et al., 1999; Horton et al., 1999; Yun et al., 2002; Castro et al., 2006); this has the effect of repressing neurogenesis and promoting gliogenesis, including oligodendrogenesis (Paras et al., 2007; Petryniak et al. 2007).  $Ascl1$  mutants continue to express Gsx2 at roughly normal levels at E12.5 (Wang et al., 2009) and E15.5 (Long and Rubenstein, unpublished).

$Ascl1;Gsx2$  compound mutants have a severe reduction in LGE differentiation (Wang et al., 2009), despite continued expression of Gsx1. Thus, Gsx2 and  $Ascl1$  together contribute to specifying the LGE developmental program.

Analysis of  $Ascl1;Dlx1/2$  individual and compound mutants provided evidence for distinct  $Ascl1$ - and  $Dlx1/2$ -dependent pathways of LGE/dCGE development; we have proposed that the  $Ascl1$  pathway operates through promoting the expression of Hes5, Olig2, and Sp9; the Dlx pathway components are described below (Long et al., 2007, 2009a,b).

Gsx2 also is a positive regulator of  $Ascl1$ , Hes5, and Sp9 (which could occur through  $Ascl1$ ; Figs. 2, 6, 9; Wang et al., 2009). Thus, Gsx2 and  $Ascl1$  share common regulatory functions for promoting Notch signaling (based on Hes5 expression) and Sp9 expression that distinguish them from  $Dlx1/2$  function.

$Ascl1;Dlx1/2$  compound mutants have greatly reduced subcortical differentiation but continue to express limited aspects of subcortical identity, based on expression of GAD1 and truncated  $Ascl1$  and  $Dlx1$  RNAs; we have postulated that subcortical identity is maintained in these mutants through the function of a few key transcription factors, including Gsx1 and -2 and  $Isl1$  (Long et al., 2009a,b). Thus, to evaluate the core functions of Gsx1 and -2, we generated the  $Gsx2;Dlx1/2$  mutants and  $Gsx1;Dlx1/2$  mutants.

### Elimination of Gsx2 partially rescues subpallial Notch signaling and oligodendrogenesis deficits in $Dlx1/2$ mutants

The Dlx genes promote LGE/dCGE development through controlling the expression of multiple transcription factors (Figs. 2, 4–6, 11; Long et al., 2009a,b). Generally, they repress the expression of transcription factors that promote the progenitor and/or glia cell state, including  $Ascl1$ , Gsx1 and -2, Hes5, and Olig2. The block in subcortical neural differentiation in  $Dlx1$  and -2 mutants may be due, in part, to persistent expression of transcription factors that promote progenitor cell properties. For instance, in the  $Dlx1$  and -2 mutants, there is overexpression of Olig2 that is linked to their overproduction of oligodendrocytes (Petryniak et al., 2007). This phenotype is reversed in  $Ascl1;Dlx1/2$  compound mutants (Petryniak et al., 2007).

Compound  $Gsx2;Dlx1/2$  mutants also show a recovery of specific aspects of the  $Dlx1/2$  mutant phenotype. In the  $Gsx2;Dlx1/2$  mutants, there was rescue of progenitor zone overexpression of several transcription factors, including  $Ascl1$ , Hes5, Olig2, and Gbx1 (Figs. 2–7, 11). The reduction in  $Ascl1$  and Hes5 expression at E12.5 and E15.5 provides evidence that the increase in Notch signaling present in the  $Dlx1/2$  mutants is mediated by Gsx2 expression. The reduction in Olig2 expression provides evidence that the  $Ascl1$ -mediated increase in oligodendrogenesis present in the  $Dlx1/2$  mutants (Petryniak et al., 2007) is mediated via Gsx2 promoting  $Ascl1$  expression.

### Elimination of Gsx2 exacerbates LGE/dCGE specification and differentiation defects in $Dlx1/2$ mutants

Although certain features of the subpallial progenitors are partially rescued in the  $Gsx2;Dlx1/2$  compound

mutants, other features are worsened compared with the Gsx2 and Dlx1/2 mutants, including regional specification/patterning and neural differentiation. The LGE, CGE, and septum show regional patterning defects, including ectopic expression of pallial markers (Ngn2), loss of subpallial markers (Ascl1, Dlx1, Vax1), and frank hypoplasia, especially of the CGE (Figs. 2, 4, 6, 11). We postulate that reducing Ascl1 levels in the Gsx2;Dlx1/2 compound mutants is an important mechanism that contributes to their these phenotypes.

Many of the Gsx2;Dlx1/2 compound mutant's phenotypes are partially rescued in the LGE and septum by E15.5 (Figs. 3, 12), whereas the CGE continues to be extremely hypoplastic at this stage (Figs. 7, 10). Thus, as in the Gsx2 mutant, there is a time-dependent aspect to the LGE patterning defect (Corbin et al., 2000; Toresson et al., 2000; Yun et al., 2001); previous work demonstrated that upregulation of Gsx1 contributes to this temporal rescue (Toresson and Campbell, 2001; Yun et al., 2003).

In addition to exacerbating patterning defects, removal of Gsx2 function in the Dlx1/2 mutants increases their neural differentiation defects. Dlx1/2 promotes the expression of transcription factors that direct specific pathways of neural differentiation, including Arx, Dlx5 and -6, EBF, Pbx1, Six3, Sp8, and Vax1, as well as expression of key features of the GABAergic state, including GAD1 expression (Long et al., 2007, 2009a,b).

Gsx2;Dlx1/2 compound mutants have further reductions in the expression of critical regulators of subpallial neuronal development, including Arx, GAD1, and Islet1 (Figs. 5, 9, 10, 12). Notably, although GAD1 expression is maintained in the septum and MGE of the Dlx1/2 mutants, its expression is nearly lost throughout the subpallium of the E15.5 Gsx2;Dlx1/2 compound mutants (Figs. 10, 12). GAD1 expression is present, albeit reduced, at E12.5 (Figs. 5, 9). Thus, together, Dlx1/2 and Gsx2 are essential for promoting and maintaining expression of GAD1, a central feature of forebrain GABAergic neurons.

### Elimination of Gsx1 partially rescues Dlx1/2 mutant MGE properties, including interneuron migration

Whereas Gsx, Mash, and Dlx participate in MGE differentiation, regional and cellular fate specification in this region also operates through a parallel/overlapping program mediated by the Nkx2.1 and the Lhx6/7(8) genes (Sussel et al., 1999; Liadis et al., 2007; Zhao et al., 2008; Flandin et al., 2011). This may explain why MGE properties are relatively better preserved than LGE/CGE/septal properties in the Gsx2;Dlx1/2 mutants. For instance, at

E12.5 Arx, GAD1, and Sp9 expression are maintained only in the MGE (Fig. 9). We suggest that the preserved expression of Ascl1 and Nkx2.1 plays a major role in maintaining the MGE developmental program in the Gsx2;Dlx1/2 mutants.

Gsx1 appears to be more important than Gsx2 in MGE development. Remarkably, the Gsx1;Dlx1/2 mutants, but not the Gsx2;Dlx1/2 mutants, show a partial rescue of the migration of Lhx6<sup>+</sup> cells to the cortex (Figs. 13–15). Loss of Gsx1 normalizes molecular properties of the Dlx1/2 mutant MGE, including increasing Lhx6 and GAD1 expression and reducing Hes5 expression (Notch signaling marker; Fig. 14). Thus, Gsx1 overexpression in the MGE may repress its differentiation, including its ability to produce interneurons that can migrate to the cortex. Alternatively, Gsx1 overexpression may alter the expression of factors that directly promote interneuron migration. In either case, overexpression of Gsx1 contributes to the block of interneuron migration of the Dlx1/2 mutants. It should be noted that, despite the partial rescue, most MGE-derived interneurons in the Gsx1;Dlx1/2 mutants remain in the subpallium, as in the Dlx1/2 mutant. Many of these cells appear to coalesce as an ectopia in the caudoventral subpallium, which expresses Gad1, Lhx6, Nkx2.1, and Sp9 (Fig. 15).

### Independent and combined functions of Gsx1 and Gsx2, with Dlx1/2, in septal development

Septal and LGE development share many similarities, but a key difference in the transcription programs of the LGE and the septum is that septal expression of Dlx5/6 is preserved in the Dlx1/2 mutant (Long et al., 2009a). Furthermore, the septum and the ventral LGE are particularly sensitive to loss of Ascl1 function (Long et al., 2009a).

Our previous and current analyses of the Ascl1 and Dlx1/2 mutants revealed, for instance, that Dlx1 and -2 are positive regulators of ER81, Gbx1, Pbx1, and Six3, whereas Ascl1 promotes expression of Arx, Hes5, Islet1, Olig2, Sp9, and Vax1 (Long et al., 2009a; Figs. 11, 12). Here we extend our analysis of the Dlx1/2 mutants; the results are summarized in Figures 16 and 17.

This is the first analysis of septal development in Gsx2 and Gsx1 mutants. We have provided evidence that Gsx2 is required for expression of Ascl1, FoxP4, Islet1, Olig2, Sp9, and Vax1 (Figs. 11, 12, 16, 17). We propose, based on these findings, that in septal progenitors Gsx2 lies upstream of both Ascl1 and Dlx1/2. Loss of either Gsx2 or Ascl1 leads to reduced expression of Islet1, Olig2, Sp9, and Vax1, whereas loss of Dlx1/2 leads to increased expression of these genes (Fig. 11). The septal



phenotype in the Gsx2;Dlx1/2 compound mutant was more severe than in either the Gsx2 or the Dlx1/2 mutant; the septum had reduced expression of supallial properties (Ascl1, Dlx1, and GAD1) and increased expression of the pallial marker (Ngn2), even through E15.5 (Figs. 11, 12). Thus, together, Gsx2 and Dlx1/2 are required for septal development. Gsx2 drives the Ascl1 program (Islet1, Olig2, Sp9, and Vax1), and Dlx1/2 drives a parallel program (ER81, Gbx1, Pbx1, and Six3).

Gsx1 also regulates septal development, particularly in the medial septum, where Dlx1, Gbx1, and Lhx6 expression are reduced (Fig. 13). The medial septal domain is generated from the MGE region (Flandin et al., 2010), consistent with Gsx1's MGE expression. Removing Gsx1 expression in the Dlx1/2 mutants showed a subtle rescue of septal and CGE phenotypes, including expression of Ascl1, Dlx1, and Sall3, showing that Gsx1 upregulation in the Dlx1/2 mutant septum contributes to its phenotypes (Figs. 13, 16, 17).

## CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to disclose.

## ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: JLRR. Acquisition of data: BW, PF, JEL, RRW. Analysis and interpretation of data: BW, PF, JEL, RP, RRW, KC, JLRR. Drafting of the manuscript: BW, PF, JEL, RRW, KC, JLRR. Critical revision of the manuscript for important intellectual content: BW, PF, JEL, RRW, KC, JLRR. Statistical analysis: RP. Obtained funding: KC, JLRR. Administrative, technical, and material support: BW, PF, JEL, RRW. Study supervision: KC, JLRR.

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