



## ORIGINAL ARTICLE

# *Dlx1/2* are Central and Essential Components in the Transcriptional Code for Generating Olfactory Bulb Interneurons

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## Abstract

Generation of olfactory bulb (OB) interneurons requires neural stem/progenitor cell specification, proliferation, differentiation, and young interneuron migration and maturation. Here, we show that the homeobox transcription factors *Dlx1/2* are central and essential components in the transcriptional code for generating OB interneurons. In *Dlx1/2* constitutive null mutants, the differentiation of *GSX2*<sup>+</sup> and *ASCL1*<sup>+</sup> neural stem/progenitor cells in the dorsal lateral ganglionic eminence is blocked, resulting in a failure of OB interneuron generation. In *Dlx1/2* conditional mutants (*hGFAP-Cre; Dlx1/2*<sup>F/-</sup> mice), *GSX2*<sup>+</sup> and *ASCL1*<sup>+</sup> neural stem/progenitor cells in the postnatal subventricular zone also fail to differentiate into OB interneurons. In contrast, overexpression of *Dlx1&2* in embryonic mouse cortex led to ectopic production of OB-like interneurons that expressed *Gad1*, *Sp8*, *Sp9*, *Arx*, *Pbx3*, *Etv1*, *Tshz1*, and *Prokr2*. *Pax6* mutants generate cortical ectopia with OB-like interneurons, but do not do so in compound *Pax6; Dlx1/2* mutants. We propose that *DLX1/2* promote OB interneuron development mainly through activating the expression of *Sp8/9*, which further promote *Tshz1* and *Prokr2* expression. Based on this study, in combination with earlier ones, we propose a transcriptional network for the process of OB interneuron development.

**Key words:** *Ascl1*, *Pax6*, *Dlx1*, *Dlx2*, *Gsx2*, interneuron, olfactory bulb, *Sp8*, *Sp9*

## Introduction

GABAergic interneurons in the olfactory bulb (OB) are not born within the OB; they are generated by progenitors located in different germinal zones of telencephalon, including the ganglionic eminences, septum, pallidum; this process continues postnatally in the subventricular zone (SVZ) and the rostral migratory stream (RMS) (Doetsch et al. 1999; Stenman et al. 2003; Kohwi et al. 2007; Long et al. 2007; Merkle et al. 2007; Ventura and Goldman 2007; Young et al. 2007; Xu et al. 2008; Fuentealba et al. 2015). The lateral ganglionic eminence (LGE) contains 2 distinct compartments: the dorsal LGE (dLGE) generates the interneurons for the OB (Yun et al. 2001; Stenman et al. 2003; Waclaw et al. 2006; Li et al. 2018) and the intercalated cells for the amygdala (Carney et al. 2009; Waclaw et al. 2010; Cocas et al. 2011; Kuerbitz et al. 2018), and the ventral LGE (vLGE) generates striatal medium spiny neurons (MSNs) (Deacon et al. 1994; Olsson et al. 1995, 1998; Anderson et al. 1997; Stenman et al. 2003; Zhang et al. 2016; Xu et al. 2018). The dLGE and vLGE may be further divided into pLGE1/2 and pLGE3/4 (Flames et al. 2007; Xu et al. 2018).

Multiple transcription factors play important roles in OB interneuron development. *Gsx2*, which is highly expressed in the ventricular zone (VZ) neural stem/progenitor cells of the dLGE, plays a critical role in OB interneuron development (Corbin et al. 2000; Toresson et al. 2000; Toresson and Campbell 2001; Yun et al. 2001, 2003; Wang et al. 2009, 2013, 2013). *Ascl1*, which is expressed by LGE progenitors, nonautonomously promotes the maintenance of adjacent neural progenitors through activating Notch signaling and lateral inhibition, and autonomously promotes neurogenesis (Casarosa et al. 1999; Long et al. 2009; Castro et al. 2011). *Dlx1/2*, which are expressed by neural progenitors and migrating OB neuroblasts (immature OB interneurons) (Porteus et al. 1994; Doetsch et al. 2002), promote neuronal differentiation and migration. *Dlx1/2*<sup>-/-</sup> null mutant mice lack virtually all GABAergic interneurons in the OB (Porteus et al. 1994; Bulfone et al. 1995; Anderson et al. 1997; Long et al. 2007), whereas *Dlx1*<sup>-/-</sup>, *Dlx2*<sup>-/-</sup>, or *Dlx5*<sup>-/-</sup> mutants have less severe OB phenotypes (Qiu et al. 1995; Long et al. 2003, 2007). The zinc finger transcription factors *Sp8/9*, which are expressed by dividing, nondividing neuroblasts and mature OB interneurons (Waclaw et al. 2006; Liu et al. 2009; Li et al. 2011), are essential for the neuronal migration and differentiation of OB interneurons through promoting expression of *prokineticin receptor 2* (*Prokr2*), a G protein-coupled receptor, and *teashirt zinc finger family member 1* (*Tshz1*) (Li et al. 2018). The zinc finger transcription factor *Tshz1* is mainly expressed by neuroblasts and mature interneurons, and is required for the differentiation and radial migration of neuroblasts within the OB (Ragancokova et al. 2014). *Prokineticin 2* (*Prok2*) and *Prokr2* signaling is required for the neuronal migration and differentiation from the SVZ through the RMS to their final layers in the OB (Ng et al. 2005; Matsumoto et al. 2006; Prosser et al. 2007). Loss of *Prok2-Prokr2* signaling results in loss of most OB interneurons. This signaling is involved in human Kallmann syndrome, which is characterized by congenital hypogonadotropic hypogonadism (due to gonadotropin-releasing hormone [GnRH] deficiency) and anosmia/hyposmia (due to OB interneuron defects) (Ng et al. 2005; Dode et al. 2006; Matsumoto et al. 2006; Pitteloud et al. 2007; Prosser et al. 2007; Sarfati et al. 2010; Martin et al. 2011). In *Sp8/9* double conditional knockout mice, neuroblasts in the dLGE and postnatal SVZ, RMS, and OB fail to express *Prokr2* and *Tshz1*; accordingly, few neuroblasts are able to reach the OB, and those that do fail to migrate tangentially and radially and then

undergo apoptotic cell death, resulting in loss of nearly all mature OB interneurons (Li et al. 2018).

Although *Dlx1/2*<sup>-/-</sup> mice lack virtually all OB interneurons, there are many gaps in our knowledge of how *Dlx1/2* regulate OB interneuron development. Here, by analyzing *Dlx1/2*<sup>-/-</sup> double mutant, *Pax6*<sup>Sey/Sey</sup> single mutant and *Dlx1/2*<sup>-/-</sup>; *Pax6*<sup>Sey/Sey</sup> triple mutant mice, we provide new evidence that, without *Dlx1/2* function, virtually all neural progenitors in the dLGE fail to differentiate into OB interneurons. Importantly, we show that *Dlx1/2* function is required postnatally for OB interneuron development using *hGFAP-Cre* in the *Dlx1/2*<sup>F/-</sup> conditional mutants, as *GSX2*<sup>+</sup> and *ASCL1*<sup>+</sup> neural stem/progenitor cells in the postnatal SVZ fail to differentiate into immature OB interneurons. When *Dlx1/2* genes were overexpressed in the E14.5 mouse cortex by in utero electroporation (IUE), neocortical progenitors started to generate immature OB interneurons instead of cortical pyramidal projection neurons. These OB interneurons express *Gad1* (*Gad67*), *Sp8*, *Sp9*, *Arx*, *Pbx3*, *Etv1* (*Er81*), *Tshz1*, and *Prokr2*. We propose that *DLX1/2* promote OB interneuron development mainly through activating the expression of *Sp8/9*, which further promote *Tshz1* and *Prokr2* expression. This study, in combination with earlier studies, presents evidence for a transcriptional regulatory network that controls the multi-step process of OB interneuron development (Fig. 10N).

## Materials and Methods

### Mice

*Dlx1/2*<sup>+/-</sup> (Qiu et al. 1997), *Pax6*<sup>Sey/+</sup> (small eye mice carrying the *Sey*<sup>neu</sup> mutant allele of *Pax6*) (Hill et al. 1991), *Ascl1-GFP* knockin knockout (Kim et al. 2007; Leung et al. 2007), *Sp9*<sup>LacZ/+</sup> (Zhang et al. 2016), *Sp9* floxed (Zhang et al. 2016), *Sp8* floxed (Bell et al. 2003), *Dlx1/2* floxed (Silbereis et al. 2014), *hGFAP-Cre* (Zhuo et al. 2001), and *Dlx5/6-Cre-Ires-EGFP* (Stenman et al. 2003) mice were previously described. These mice were maintained in a mixed genetic background of C57BL/6J and CD1. The day of vaginal plug detection was regarded as embryonic day 0.5, and the day of birth was considered as postnatal day 0. All animal experiments described in this study were approved in accordance with institutional guidelines at Fudan University Shanghai Medical College.

### Tissue Preparation

E17–E18 embryos and postnatal mice were perfused intracardially with 4% PFA in 1× phosphate buffered saline (PBS, pH 7.4); embryonic brains (earlier than E17) were immersion fixed in 4% PFA. Postnatal brains were fixed overnight in 4% PFA, cryoprotected in 30% sucrose for at least 24 h, frozen in the embedding medium and cryosectioned.

### In Utero Electroporation

In utero electroporation (IUE) of wild type CD1 embryos was performed at E14.5 as described (Saito and Nakatsuji 2001). In brief, plasmids *pCAGIG-Ires-GFP* (control) or (*pCAGIG-Dlx1-Ires-GFP* and *pCAGIG-Dlx2-Ires-GFP*, 1.0 μg/μL, 1:1) or (*pCAGIG-Dlx1-Ires-GFP*, *pCAGIG-Dlx2-Ires-GFP* and *pCAGIG-Cre*, 1.0 μg/μL, 1:1:2) were mixed with 0.05% Fast Green (Sigma), and injected through the uterine wall into the lateral ventricle using a beveled pulled glass micropipette. Five electrical pulses were applied at 35 V (50 ms duration) across the uterine wall at 950 ms intervals using 7-mm platinum electrodes (Tweezertrode 45-0488, BTX,

Harvard Apparatus) connected to an electroporator (ECM830, BTX). Embryos were then analyzed at different time points.

### Immunohistochemistry

Immunohistochemistry was performed on 6, 12, and 20  $\mu\text{m}$  cryostat sections on slides. For SP9, DLX2, and BCL11b immunohistochemistry, sections were boiled in 10 mM sodium citrate briefly for antigen retrieval. The following primary antibodies were used: goat anti-SP8 (1:3 000, Santa Cruz, sc-104 661), rabbit anti-SP9 (1:500) (Zhang et al. 2016), chicken anti-GFP (1:3 000, Aves Labs, GFP-1020), goat anti-DCX (1:1 000, Santa Cruz, sc-8066), rabbit anti-DCX (1:1 000, Abcam, ab18723), mouse anti-NeuN (1:500, Millipore, MAB-377), rabbit anti-ASCL1 (1:2 000, Cosmo Bio, SK-T01-003), rabbit anti-GSX2 (1:2 000, Millipore, ABN162), rabbit anti-OLIG2 (1:500, Millipore, AB9610), rabbit anti-Ki67 (1:500, Vector Labs, VP-K451), rabbit anti-PAX6 (1:2 000, MBL, PD022), goat anti-EBF (1:200, Santa Cruz, sc-15 888), rabbit anti-EBF1 (1:5 000, Millipore, AB10523), goat anti-SOX2 (1:500, Santa Cruz, sc-17 320), rabbit anti-ISL1 (1:500, Abcam, ab20670), rabbit anti-GFAP (1:500, Dako, Z0334), rabbit anti-EOMES (TBR2, 1:500, Abcam, ab23345), rat anti-BCL11b (1:1 000, Abcam, ab18465), and guinea pig anti DLX2 (1:2000) (Kuwajima et al. 2006; Hansen et al. 2013).

Secondary antibodies against the appropriate species were incubated for 60 min at room temperature (all from Jackson, 1:400). Fluorescently stained sections were then washed, counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma, 200 ng/mL) for 2–5 min and coverslipped with Gel/Mount (Biomedex, Foster City, CA). Omission of primary antibodies eliminated the staining signals.

### In Situ RNA Hybridization

All in situ RNA hybridization experiments were performed using digoxigenin riboprobes on 20  $\mu\text{m}$  cryostat sections. Riboprobes were described in previous studies (Zhang et al. 2016; Li et al. 2018; Liu et al. 2018; Xu et al. 2018) or made from cDNAs amplified by PCR using the following primers:

1. *ErbB4* Fwd: GCACCGATATTTGCCCAA  
*ErbB4* Rev: CAGTCATGACTAGTGGGACCGTTAC
2. *Etv1* Fwd: TTCATGGCCTCCACTGAAAATC  
*Etv1* Rev: CCTTCGTTGTAGGGGTGAGGGTT
3. *Dlx5* Fwd: CAGCTTTCAGCTGGCCGCTT  
*Dlx5* Rev: CAAGGACCATTGATAGTGTCCACA
4. *Bcl11b* Fwd: GTAAAGATGAGCCTTCCAGCTACAT  
*Bcl11b* Rev: TTAGTCTCTCAGCCTGCTC
5. *Sox1* Fwd: CCCTGTGAAATCGAAACGTGCT  
*Sox1* Rev: TCCAATGTAGGTTGAGCTCTGGTCT
6. *Pou3f4* Fwd: ATGGCCACAGCTGCCTCGAA  
*Pou3f4* Rev: GCTCACCATGGTCTGGAGGCTC
7. *Pax6* Fwd: TGCAGACCCATG  
*Pax6* Rev: CGGTCTGCCGTTCAACATCCTTAG

### Microscopy

Bright field images (in situ hybridization results) and some fluorescent images were imaged with Olympus BX 51 microscope using a  $\times 4$  or  $\times 10$  objective. Other fluorescent images were taken with Olympus FV1000 confocal microscope system using  $\times 10$ ,  $\times 20$ ,  $\times 40$ , or  $\times 60$  objectives. Z-stack confocal images were reconstructed using the FV10-ASW software. All images were

merged, cropped and optimized in Photoshop CS5 without distorting the original information.

### RNA Sequencing Analysis

RNA sequencing (RNA-Seq) analysis was performed as previously described (Xu et al. 2018). The LGE (including VZ, SVZ, and MZ-mantle zone/striatum) from E16.5 *Dlx1/2*<sup>-/-</sup> null mutant mice and littermate wild type controls were dissected ( $n = 3$  mice, each group). The neocortex (including VZ, SVZ, CP-cortical plate, and MZ-marginal zone) from E16.5 *Pax6*<sup>Sey/Sey</sup> mice and littermate wild type controls were dissected ( $n = 2$  mice, each group). In brief, total RNA was extracted using RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol, quantified using NanoDrop ND-2000, and checked for RNA integrity by an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). RNA-Seq libraries were prepared according to the Illumina TruSeq protocol. Levels of gene expression were reported in FPKM (fragments per kilobase of transcript per million mapped reads) (Trapnell et al. 2012; Xu et al. 2018). A gene was considered to be expressed if it had an FPKM  $> 1$ . For a gene to be called as differentially expressed, it required a  $P$ -value  $< 0.05$ . Data from this experiment have been deposited in the GEO database (GSE121215).

### Quantification

DLX2 expression and its colocalization with SP8/9 in the E16.5 dLGE SVZ1 and SVZ2, and adult SVZ, RMS, and OB core (34 292  $\mu\text{m}^2$  area from the lateral SVZ and RMS; 29 000  $\mu\text{m}^2$  from the OB core) were quantified in 2–3 randomly chosen 6- $\mu\text{m}$  sections for E16.5 mice and 12- $\mu\text{m}$  sections for P21 mice, and 3 mice were used for each group.

GSX2<sup>+</sup>, ASCL1<sup>+</sup>, ISL1<sup>+</sup>, SP8<sup>+</sup>, EBF1<sup>+</sup>, EBF3<sup>+</sup>, SP9<sup>+</sup>, BCL11b<sup>+</sup>, SP9<sup>+</sup>/BCL11b<sup>+</sup> cells in the dLGE at E16.5 were quantified in 3 randomly chosen 12- $\mu\text{m}$  sections for each group of mice ( $n = 3$ ).

DLX2, GSX2, KI67, ASCL1, SP8, SP9, and OLIG2 expressions in the SVZ were quantified in 3 randomly chosen 12- $\mu\text{m}$  sections for each group of mice (P50 wild type mice and hGFAP-Cre, *Dlx1/2*<sup>F/-</sup> mice were used,  $n = 3$ ). We counted the positive cells and cell numbers colabeled with DCX within a 200 000  $\mu\text{m}^2$  area in the SVZ per section.

### Statistics

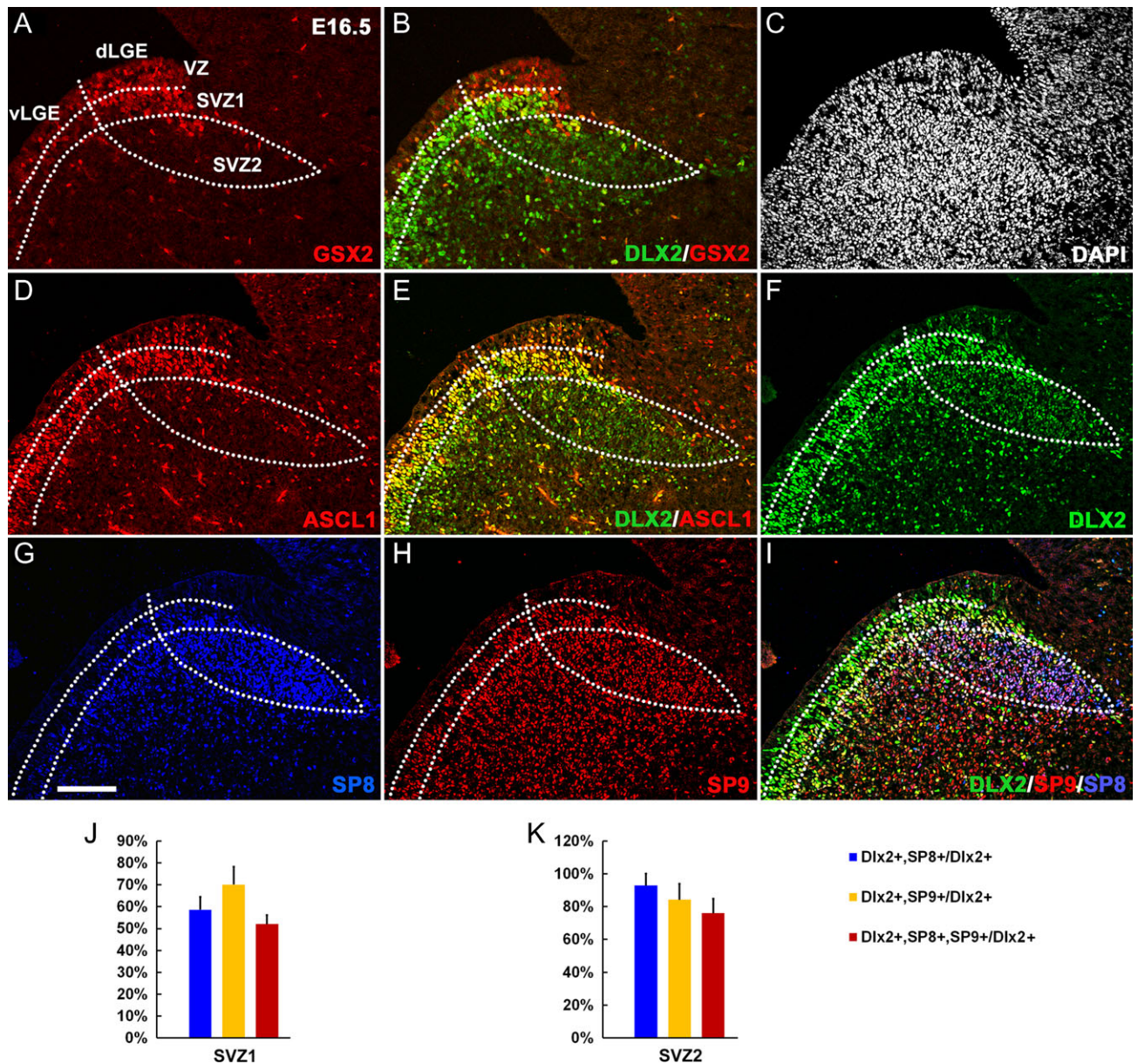
Statistical significance was assessed using unpaired Student's  $t$  test. All quantification results were presented as the mean  $\pm$  SEM.  $P < 0.05$  were considered significant.

## Results

### Coexpression of DLX2, SP8, and SP9 in Immature OB Interneurons

GSX2, DLX2, ASCL1, SP8, and SP9 are essential for OB interneuron development. We compared GSX2, DLX2, ASCL1, SP8, and SP9 protein expression in the dLGE and postnatal SVZ, RMS, and OB using triple immunofluorescence analysis and confocal microscopy. The germinal zone of dLGE consists the VZ, SVZ1 (adjacent to the VZ) and SVZ2 (Fig. 1A) (Petryniak et al. 2007; Wang et al. 2013; Xu et al. 2018). Strong GSX2 expression in the dLGE and weak GSX2 expression in the vLGE was detected at E12.5 (Toresson et al. 2000; Wang et al. 2009), but became more evident at E14.5 (Supplementary Fig. S1A–C). In the dLGE at E16.5, most progenitors in the VZ and SVZ1 expressed strong GSX2, whereas





**Figure 1.** SP8, SP9, and DLX2 are coexpressed in the E16.5 dLGE SVZ. (A, B) GSX2 was strongly expressed in the dLGE VZ and SVZ1; only a few GSX2<sup>+</sup> cells were in the SVZ2, whereas DLX2 was mainly expressed in the SVZ1&2. Note weak expression of GSX2 in the vLGE. (C) The section was stained with DAPI. (D, E) Most ASCL1<sup>+</sup> cells expressed DLX2. (F–I) DLX2 was strongly expressed in the SVZ1 and relatively weakly expressed in the SVZ2 and DLX2 expression began before SP8/9 expression. Most cells in the SVZ2 expressed DLX2/SP8/SP9. (J, K) Quantification of DLX2<sup>+</sup> cells that expressed SP8, SP9, or SP8/9 in the dLGE SVZ1 and SVZ2 at E16.5. Scale bar: 100  $\mu$ m in G for (A–I).

the vLGE expressed relatively weak GSX2 (Fig. 1A). A few ASCL1<sup>+</sup> and DLX2<sup>+</sup> cells were scattered in the VZ (Fig. 1D–F), but most dLGE SVZ1 progenitors strongly coexpressed ASCL1, DLX2, and GSX2 (Fig. 1B,E) (Eisenstat et al. 1999). In the dLGE SVZ2, only a few GSX2<sup>+</sup> and ASCL1<sup>+</sup> cells were detected (Fig. 1A,D). However, DLX2 expression was maintained in nearly all SVZ2 cells. DLX2 expression was even higher in SVZ1 (Fig. 1E,F). SP8 and SP9 were robustly expressed in the dLGE SVZ1&2 (Fig. 1G–I); in SVZ2 ~80% of DLX2<sup>+</sup> cells coexpressed SP8 and SP9 (Fig. 1J,K). Thus DLX2 expression begins before SP8 and SP9 in the dLGE VZ/SVZ1 and most DLX2<sup>+</sup> cells in the SVZ2 coexpressed SP8/9, and vice versa.

Expression of DLX2, SP8, and SP9 in the postnatal SVZ, RMS, and OB core showed similar results (Supplementary

Fig. S2A–K). In the SVZ and RMS at P21, intermediate progenitors (type C cells) expressed strong DLX2<sup>+</sup> but not SP8 or SP9 (Supplementary Fig. S2A–H) (Doetsch et al. 2002; Waclaw et al. 2006; Wei et al. 2011; Li et al. 2018). However, most neuroblasts (type A cells) in the SVZ, RMS, and OB core coexpressed DLX2 and SP8/9 (Supplementary Fig. S2D, H, K, L). Because primary neural stem cells first generate intermediate progenitor cells, which in turn generate neuroblasts that tangentially migrate from SVZ to the OB, based on the DLX2/SP8/SP9 expression patterns, we hypothesized that *Dlx2* (and *Dlx1*) promotes *Sp8/9* expression during the process of OB interneuron development, from embryonic to postnatal stages.



### Ascl1 Does not Regulate the Expression of *Gad1*, *Sp8/9*, *Tshz1*, and *Prokr2* in the dLGE

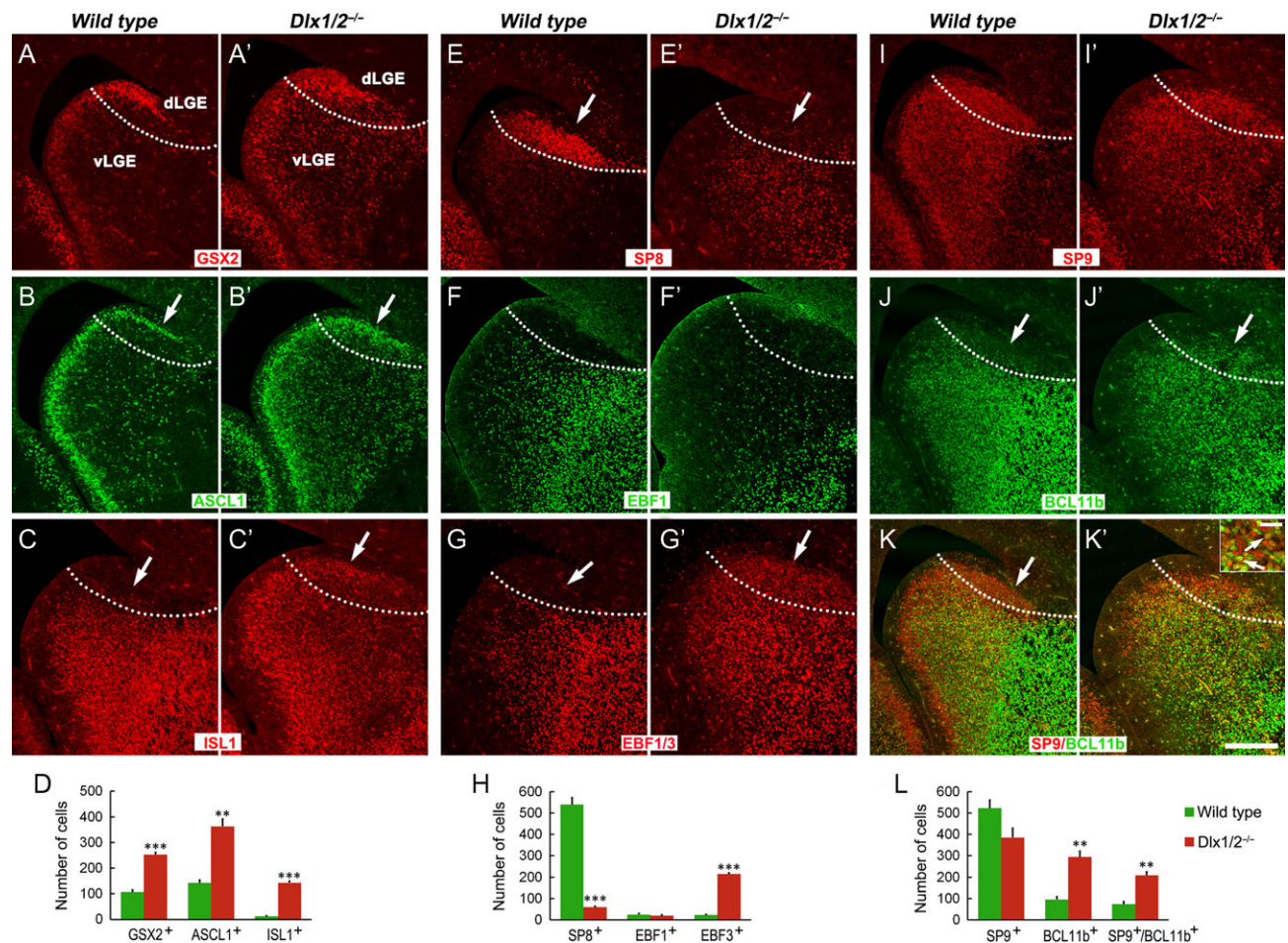
Proneural transcription factor ASCL1 promotes the maintenance of neural progenitors through activating Notch signaling and lateral inhibition (Casarosa et al. 1999; Long et al. 2009; Castro et al. 2011; Wang et al. 2013). Here, we re-examined *Ascl1* function in the dLGE and confirmed that *Gad1* expression were increased in the LGE VZ/SVZ in *Ascl1*<sup>GFP/GFP</sup> knockin knockouts (null mutants) (Supplementary Fig. S3A–C'). However, expression of *Sp8*, *Sp9*, *Tshz1*, and *Prokr2* in the mutant dLGE was comparable with controls (Supplementary Fig. S3D–O'), suggesting that *Ascl1* is not required for expression of these key regulators of OB interneuron development. However, consistent with our previous study (Zhang et al. 2016), *Sp9* expression was reduced in the vLGE of *Ascl1* mutants (Supplementary Fig. S3H–I'), where it regulates striatal MSN development.

### Neural Progenitors Fail to Differentiate Into Immature OB Interneurons in the dLGE of *Dlx1/2*<sup>-/-</sup> Mice

We previously demonstrated that *Dlx1/2*<sup>-/-</sup> mice lack virtually all OB interneurons (Anderson et al. 1997; Bulfone et al. 1998;

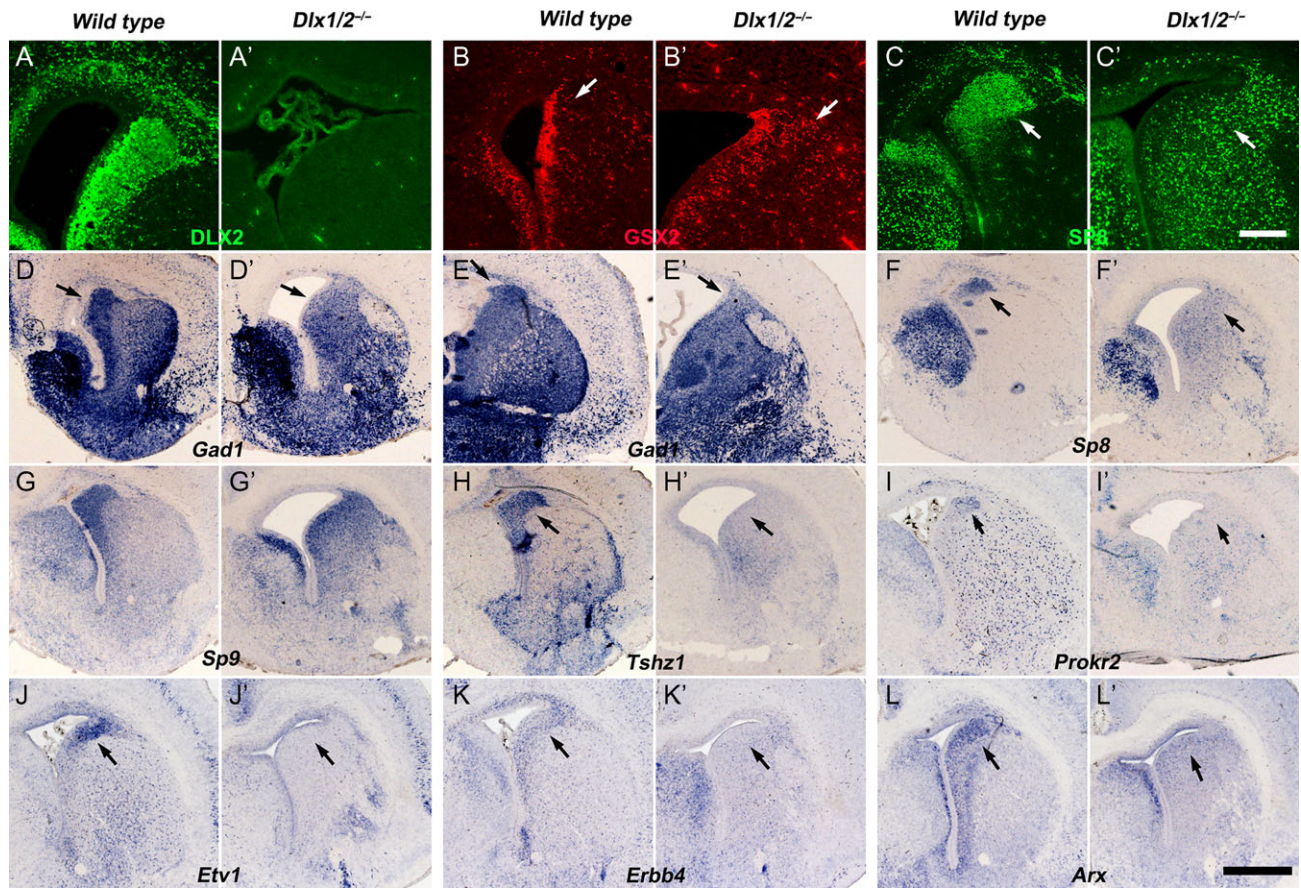
Long et al. 2007). Currently, there are many more known markers for the OB interneuron lineage; thus we used this more definitive gene set to re-examine the generation of OB interneurons in the dLGE of *Dlx1/2*<sup>-/-</sup> mutants. We first examined the LGE at E16.5. *Dlx1/2*<sup>-/-</sup> mutants accumulated *GSX2*<sup>+</sup> and *ASCL1*<sup>+</sup> cells in the SVZ of both dLGE and vLGE (Fig. 2A–B',D), consistent with the known block in neural differentiation (Long et al. 2007, 2009). *SP8* expression was largely lost in the dLGE, whereas *SP8*<sup>+</sup> immature (or undifferentiated) MSNs remained in the vLGE SVZ (Fig. 2E,E',H) (Xu et al. 2018). This suggests that the expression of *SP8* in the dLGE, but not in the vLGE, is critically dependent on *DLX1/2*. On the other hand, *SP9* expression was only partially reduced (Fig. 2I–I',L).

Since *SP9* is expressed in both MSNs and in OB interneurons, the *SP9*<sup>+</sup> cells in the dLGE suggested that the dLGE in the *Dlx1/2*<sup>-/-</sup> mice might generate MSNs. Thus, we examined expression of the genes that are associated with MSN development. Most of these *SP9*<sup>+</sup> cells in the mutant dLGE expressed strong *BCL11b* (Fig. 2I–K',L), a marker of immature and mature MSNs (Arlotta et al. 2008), whereas *BCL11b* was not expressed in the dLGE of control mice (Fig. 2J). Furthermore, the expression of *ISL1*, a *Drd1* MSN marker (Ehrman et al. 2013; Lu et al. 2014), was also observed in the mutant dLGE (Fig. 2C,C',D). Notably, the dLGE in neither the



**Figure 2.** Neural progenitors accumulate in the dLGE of *Dlx1/2*<sup>-/-</sup> mice at E16.5. (A–B) Compared with controls, there were more *GSX2*<sup>+</sup> and *ASCL1*<sup>+</sup> progenitors in the d/vLGE of *Dlx1/2*<sup>-/-</sup> mice (arrows). (C, C') *ISL1* expression was increased in the mutant dLGE (arrows). (E–G') *EBF1* was not expressed in the control and mutant dLGE, but the pan EBF antibody immunostaining showed that *EBF3* was expressed in the mutant dLGE (arrows). (I–K') *SP9* expression was reduced in the dLGE; most *SP9*<sup>+</sup> cells coexpressed the immature MSN marker *BCL11b* (arrows in the inset of K'). (D, H, L) Quantification of cell numbers in the dLGE. Scale bars: 200  $\mu$ m in K' for A–K'; 20  $\mu$ m in the inset of K'.





**Figure 3.** Immature OB interneurons are not generated in the dLGE of *Dlx1/2*<sup>-/-</sup> Mice at E18.5. (A–A') No DLX2 protein was detected in the DLX1/2 mutant telencephalon. (B–B') More GSX2<sup>+</sup> cells were in the mutant dLGE SVZ (arrows). (C–C', F–F') The expression of Sp8 mRNA and SP8 protein was greatly reduced in the mutant dLGE (arrows). (D–E') In situ hybridization showed *Gad1* expression was reduced in the dLGE SVZ of mutants compared with controls (arrows). (G–G') Sp9 expression was maintained in the mutant dLGE. (H–L') Expression of OB interneuron lineage markers, *Tshz1*, *Prokr2*, *Etv1*, *Erbb4*, and *Arx*, were either lost or severely reduced in the mutant dLGE (arrows). Scale bars: 200  $\mu$ m in C' for A–C'; 600  $\mu$ m in L' for D–L'.

control, nor the mutant, expressed EBF1, another marker of immature *Drd1*<sup>+</sup> MSNs (Fig. 2F,F') (Lobo et al. 2006). However, the mutant dLGE did ectopically express EBF3 (Fig. 2 G,G',H), a transcription factor that was not expressed in the normal dLGE.

*Dlx1/2*<sup>-/-</sup> mice die within a few hours after birth (Qiu et al. 1997), therefore we analyzed molecular features in the VZ/SVZ of dLGE at E18.5, especially for OB interneuron lineage markers. The *Dlx1/2*<sup>-/-</sup> mutants had more GSX2<sup>+</sup> cells in the LGE SVZ (Fig. 3A–B'). Once again, SP8 expression was greatly reduced in the mutant dLGE SVZ, whereas many SP8<sup>+</sup> immature MSN-like cells were observed throughout the LGE SVZ (Fig. 3C,C',F,F'). *Sp9* expression was still observed in the mutant dLGE SVZ (Fig. 3G,G'). OB immature interneuron markers *Gad1*, *Tshz1*, *Prokr2*, *Etv1*, *Erbb4*, and *Arx* were either lost or severely reduced in the dLGE (Fig. 3D–E',H–L'). Thus, the *Sp9*<sup>+</sup> cells in the dLGE SVZ are not immature OB interneurons.

Previously we have reported that SP8 and SP9 coordinately promote the differentiation of OB interneurons by activating *Tshz1* and *Prokr2* expression (Li et al. 2018). The loss or reduced expressions of *Sp8*, *Sp9*, *Tshz1*, and *Prokr2* in the dLGE of *Dlx1/2*<sup>-/-</sup> mice (Fig. 3C–I'), and the persistent expression of *Dlx1/2/5* in the dLGE of *Sp8/9* single or double conditional mutants (Supplementary Fig. 4A–F''') suggested that DLX1/2 act upstream of *Sp8/Sp9* to promote the differentiation of OB interneurons. Thus, in the absence of *Dlx1/2*, the dLGE fail to produce OB immature interneurons. In contrast, some of dLGE progenitors began to generate immature

striatal MSN-like cells that express ISL1, SP9, and BCL11b (Fig. 2C', I–K').

### RNA-Seq Analysis Reveals Key Molecular Defects in the LGE of *Dlx1/2*<sup>-/-</sup> Mice

To further characterize the molecular changes in the LGE of *Dlx1/2*<sup>-/-</sup> mice, we performed RNA-Seq analysis. Gene expression profiles from the E16.5 LGE (including VZ, SVZ, MZ-striatum) of *Dlx1/2*<sup>-/-</sup> mice and littermate wild type controls were analyzed ( $n = 3$  biological replicas each group, GEO accession number: GSE121215). The levels of gene expression were reported in FPKM (Trapnell et al. 2012). For example, the expression levels (FPKM) of *Dlx1* and *Dlx2* genes in E16.5 wild type LGE were 80.2 and 139.4, respectively, whereas they were 11.6 and 5.5 in the null mutants, respectively ( $P$ -value = 4.29 E-100 and 2.02E-232, respectively) (Supplementary Table S1). The expression levels of *Dlx5/6* genes, direct downstream targets of *Dlx1/2*, were almost undetectable (*Dlx5*: 49.0 vs. 1.4; *Dlx6*: 22.3 vs. 0.6; wild types vs. *Dlx1/2* mutants) (Supplementary Table S1).

Our RNA-Seq analysis revealed about 3300 dysregulated genes in the mutants (1890 with reduced RNA expression and 1410 with increased RNA expression), suggesting that *Dlx1/2* have profound effects on the LGE development. To further analyze the impact of *Dlx1/2* deletion on different cell types of OB interneuron lineage in the dLGE, such as neural stem/progenitors and immature OB

interneurons, we explored expression levels for genes that are enriched in these populations (Supplementary Table S1). Compared with controls, we found that mRNA levels of LGE progenitor-enriched genes in *Dlx1/2<sup>-/-</sup>* mice were significantly increased, such as *Gsx1/2*, *Ascl1*, *Egfr*, *Lhx2*, *Dll1/3*, *Hes5/6*, *Nr2e1*, *Nr2f1*, *Notch1*, *Gli2/3*, *Sox9*, and *Nes* (Supplementary Table S1). However, mRNA levels of immature OB interneuron-enriched genes were all significantly reduced or undetectable, such as *Dlx1/2/5/6*, *Arx*, *Etv1*, *Erbp4*, *Sp8*, *Neto1*, *Meis1/2*, *Gad1/2*, *Vax1*, *Tshz1*, and *Errbb4* (Supplementary Table S1). In general, this is consistent with our RNA in situ hybridization results and previous transcriptomic analyses using microarrays (Long et al. 2009). Thus, our results confirm that lack of OB interneuron genesis in the dLGE of *Dlx1/2<sup>-/-</sup>* mice.

### ***Pax6<sup>Sey/Sey</sup>* Mice Have a Large OB Interneuron Ectopia in the Neocortex**

*Pax6<sup>Sey/Sey</sup>* mice lack functional PAX6 protein, lack an OB and die at birth (Hill et al. 1991). However, the generation of OB interneurons in the embryos of *Pax6<sup>Sey/Sey</sup>* mice has not been fully investigated. At E16.5, in situ RNA hybridization and immunohistochemistry staining revealed that both dLGE and neocortex generated a large numbers of immature OB interneurons as they expressed the following markers of OB interneurons: *Gad1*, SP9, SP8, *Tshz1*, *Prokr2*, *Etv1* (Fig. 4A–J’). Perhaps because *Pax6<sup>Sey/Sey</sup>* mice do not form OBs, these interneurons lost their target destination, and thus formed ectopic collections in the dLGE and neocortex (Fig. 4A–J’) (Kroll and Leary 2005). To assess whether vLGE fate was also generated in the dLGE and cortex, we assessed expression of *Bcl11b<sup>+</sup>*, a marker of striatal MSNs. Strong *Bcl11b<sup>+</sup>* expression was not present in the ectopias (Fig. 4K–L’), suggesting that neocortical progenitors in *Pax6<sup>Sey/Sey</sup>* mice acquired the dLGE fate, but not the vLGE fate.

### **RNA-Seq Analysis Provided Further Molecular Evidence for Ectopic Generation of OB Interneurons in the Cortex of *Pax6<sup>Sey/Sey</sup>* Mice**

Gene expression profiles from the E16.5 cortex (including VZ, SVZ, CP, and MZ) of *Pax6<sup>Sey/Sey</sup>* mice and littermate wild type controls were analyzed using RNA-Seq ( $n = 2$  biological replicas each group, GEO accession number: GSE121215). The expression levels (FPKM) of *Pax6* gene in E16.5 wild type cortex was 27.9, whereas in E16.5 *Pax6<sup>Sey/Sey</sup>* cortex was 110.5 ( $P = 3.41E-13$ ) (Supplementary Table S2), indicating that loss of functional PAX6 protein greatly increased *Pax6* transcripts. This is consistent with previous observations (Stoykova et al. 1996). On the other hand, the expression levels of *Neurog2*, the direct downstream target of *Pax6* (Scardigli et al. 2003; Sansom et al. 2009), in wild type cortex was 32.6, whereas in *Pax6<sup>Sey/Sey</sup>* cortex was reduced to 1.8 ( $P = 3.75E-8$ ). Accordingly, we observed reduced expression of *Eomes* (*Tbr2*), a gene expressed highly in intermediate progenitors of cortical pyramidal projection neurons (Bulfone et al. 1995; Englund et al. 2005; Elsen et al. 2018), and *Tbr1*, a gene expressed in postmitotic cortical pyramidal neurons (Hevner et al. 2001; McKenna et al. 2011; Fazel Darbandi et al. 2018) (Wild types vs. Mutants; *Eomes*: 53.7 vs. 11.2;  $P = 1.76E-07$ ; *Tbr1*: 114.3 vs. 62.4;  $P = 1.62E-04$ ) (Supplementary Table S2).

Compared with controls, RNA levels of OB interneuron lineage-enriched genes (from genes that highly expressed in progenitors to genes that highly expressed in immature OB interneurons) in the *Pax6<sup>Sey/Sey</sup>* cortex were significantly increased,

levels ranging from a 1.5- to 18-fold change (Supplementary Table S2). These include *Gsx2*, *Ascl1*, *Dlx1/2/5/6*, *Cend2*, *Cdca7*, *Mki67*, *Nr2e1*, *Nr2f1*, *Pcna*, *Pax6*, *Sox1/2*, and *Etv1*, *Gad1/2*, *Arx*, *Sp8/9*, *Tshz1*, *Pbx3*, *Pou3f4*, *Prokr2*, *Errbb4*, and others. In contrast, the expression levels of maturing/mature OB interneuron-enriched genes *Th* (tyrosine hydroxylase), *Pvalb*, *Calb1* (calbindin 1) and *Calb2* (calbindin 2, calretinin, CR) were very low in the *Pax6<sup>Sey/Sey</sup>* cortex (Supplementary Table S2). Likewise, the *Pax6<sup>Sey/Sey</sup>* cortex had low cortical expression of MSNs-enriched genes, such as *Drd1*, *Chrm4*, *Ebf1*, *Isl1*, *Pdyn*, *Tac1*, and *Drd2*, *Adora2a*, *Penk*, *Ptpm*, *Gpr6* (Supplementary Table S2). Thus, we conclude that cortical progenitors in *Pax6<sup>Sey/Sey</sup>* mice acquired the dLGE fate and produced cells with properties of immature OB interneurons.

### **Dlx1/2 are Required for the Differentiation of Ectopic Immature OB Interneurons in *Pax6<sup>Sey/Sey</sup>* Mice**

Our results demonstrated that the *Dlx1/2<sup>-/-</sup>* dLGE fails to produce OB immature interneurons. However, it is quite difficult to further analyze the functions of *Dlx1/2* in OB interneuron development, as the dLGE domain is much smaller than the vLGE domain. In addition, the dLGE and vLGE share many common molecular features and both exhibit blocking neuronal differentiation phenotypes in *Dlx1/2<sup>-/-</sup>* mice (Anderson et al. 1997; Long et al. 2007, 2009). However, the enlarged dLGE and dLGE-like neocortex of *Pax6<sup>Sey/Sey</sup>* mice provide an excellent model to analyze *Dlx1/2* functions in OB interneuron development.

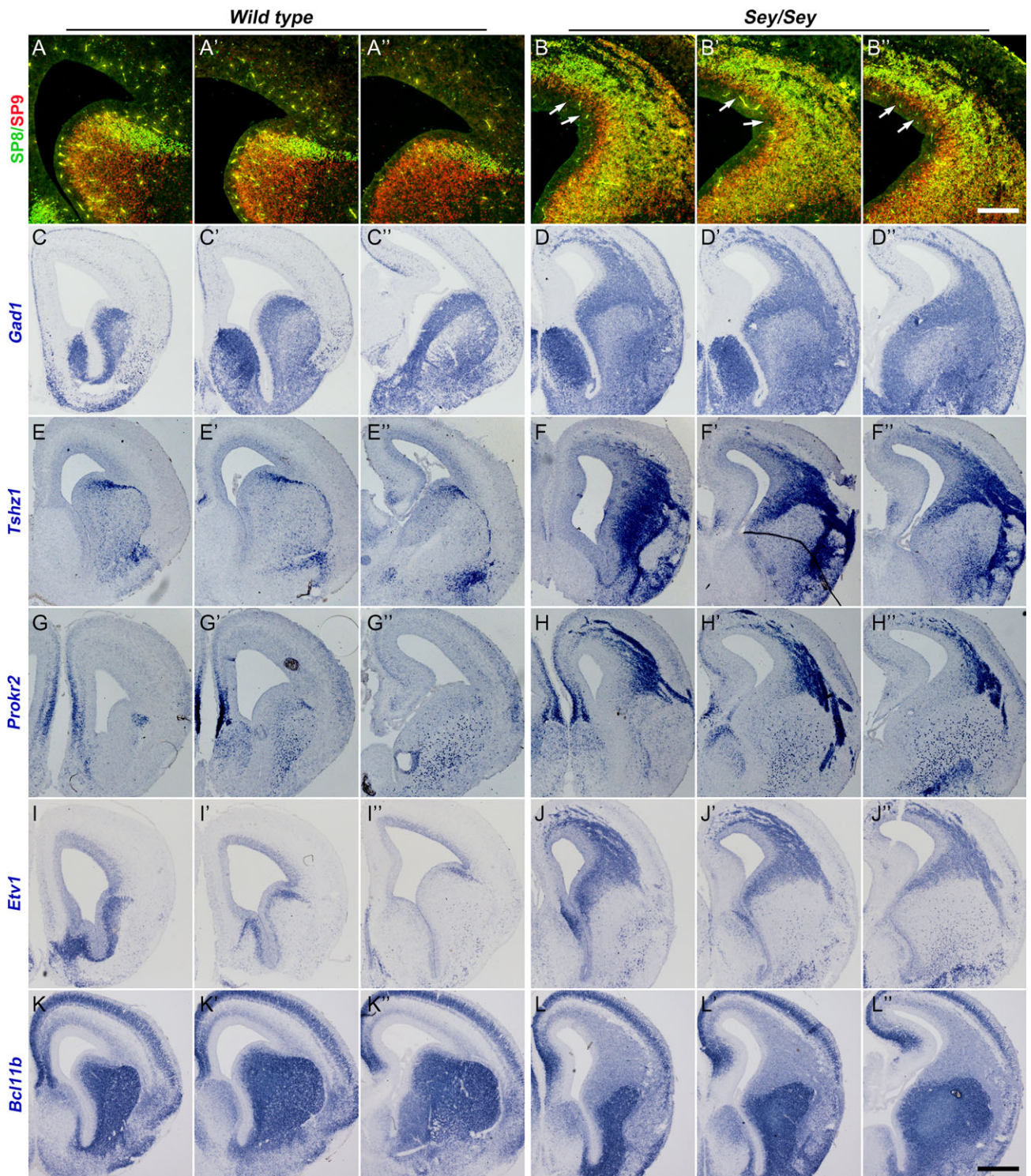
The mouse *Pax6* and *Dlx1/2* genes are both located in chromosome 2 and separated by ~12 cm. We obtained *Pax6<sup>Sey/+</sup>*; *Dlx1/2<sup>+/-</sup>* mice through meiotic recombination. We then generated *Pax6<sup>Sey/Sey</sup>*; *Dlx1/2<sup>-/-</sup>* and studied their cortex and dLGE. We found that the *Pax6<sup>Sey/Sey</sup>*; *Dlx1/2<sup>-/-</sup>* mutants lacked PAX6, EOMES, and DLX2 proteins, and had increased GSX2, ASCL1 and OLIG2 expression (Fig. 5A–F’). *Sox1<sup>+</sup>* progenitors were also increased (Fig. 6L–L’). Some SP8<sup>+</sup>/SP9<sup>+</sup> cells were observed in the *Pax6<sup>Sey/Sey</sup>*; *Dlx1/2<sup>-/-</sup>* cortex, but these cells coexpressed *BCL11b*, a striatal MSN marker, providing evidence that they were not OB interneurons (Fig. 5G–L’). However, we did not observe the expression of *ISL1* and *EBF1* in the cortex of *Pax6<sup>Sey/Sey</sup>*; *Dlx1/2<sup>-/-</sup>* triple mutant mice (Fig. 5M–N’), suggesting that MSNs were not generated either.

*Pax6<sup>Sey/Sey</sup>* *Dlx1/2<sup>-/-</sup>* mutants lacked the ectopic cortical expression of *Gad1*, *Arx*, *Etv1*, *Pou3f4*, *Pbx3*, *Vax1*, *Tshz1*, *Prokr2*, and *Erbp4* that were observed in the cortex of *Pax6<sup>Sey/Sey</sup>* mice (Fig. 6A–K’). This provides strong evidence for a block of interneuron generation. Thus, the analysis of *Pax6<sup>Sey/Sey</sup>*; *Dlx1/2<sup>-/-</sup>* triple mutant mice further demonstrated that *Dlx1/2* are absolutely required for making nearly all OB interneurons.

### **Postnatal SVZ Progenitors Fail to Generate OB Interneurons in *hGFAP-Cre*; *Dlx1/2<sup>F/F</sup>* Conditional Knockout Mice**

Next, we assessed *Dlx1/2* function in the postnatal generation of OB interneurons. To this end we use a *Dlx1/2* conditional allele (*Dlx1/2<sup>F/F</sup>* mice) (Silbereis et al. 2014; Pla et al. 2018). *Dlx1/2<sup>+/-</sup>* mice were crossed with *hGFAP-Cre* mice (Zhuo et al. 2001), and male *hGFAP-Cre*; *Dlx1/2<sup>+/-</sup>* mice were crossed female *Dlx1/2<sup>F/F</sup>* mice to generate *hGFAP-Cre*; *Dlx1/2<sup>F/F</sup>* conditional knockouts (*Dlx1/2-CKO*). *hGFAP-Cre* starts to be expressed in the E13.5 cortical VZ and E18.5 LGE VZ (Zhuo et al. 2001; Malatesta et al. 2003), and can delete floxed alleles from ~95% postnatal SVZ stem cells (Lim et al. 2009). Indeed, DLX2 expression was almost undetectable in the SVZ of *Dlx1/2-CKO* mice at P50 (Fig. 7A–B’,M). Furthermore, SP8/9 SVZ



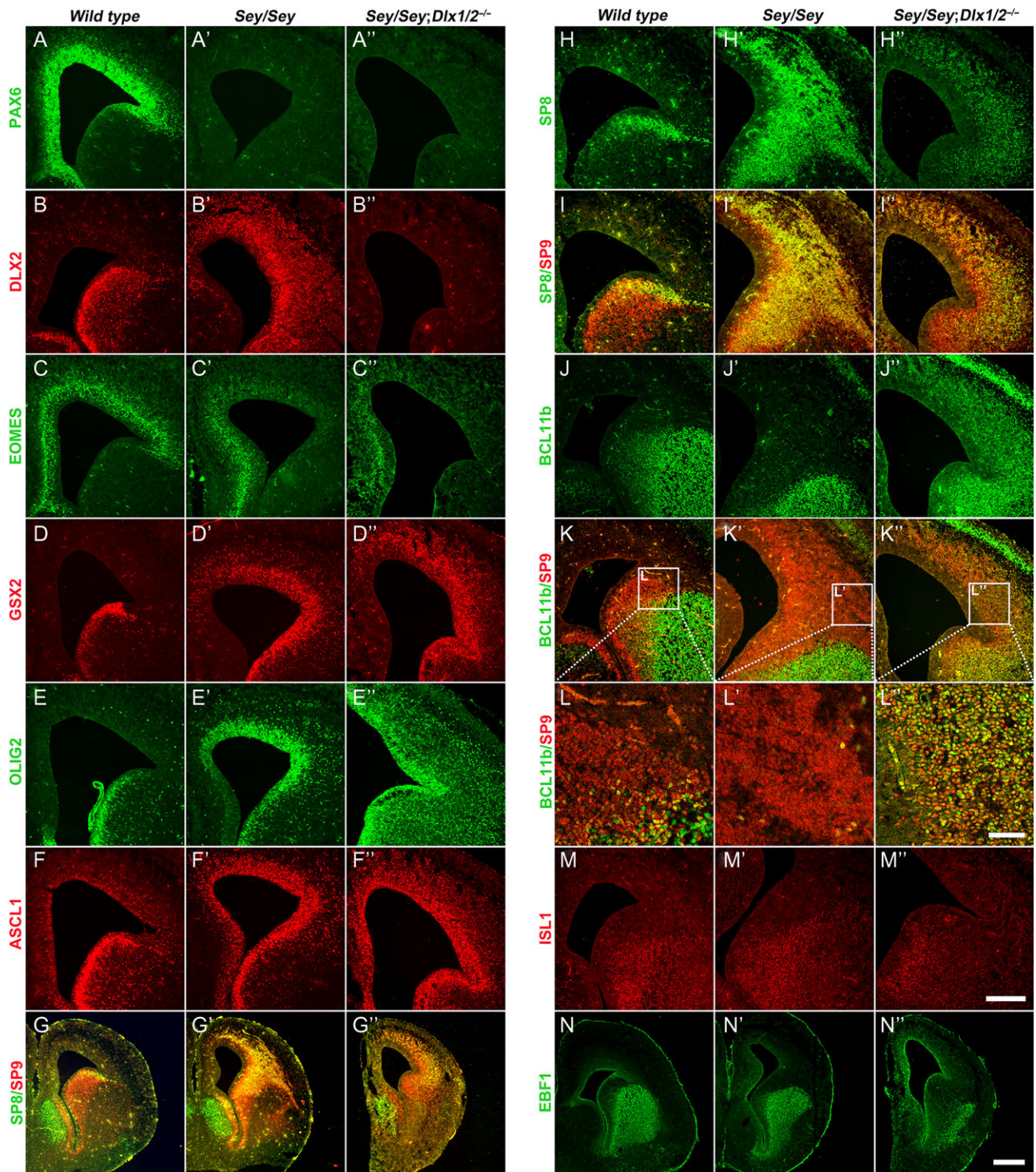


**Figure 4.** *Pax6<sup>Sey/Sey</sup>* mice have a large ectopia containing OB interneurons in the cortex and dLGE at E16.5. (A–B') SP8 and SP9 were ectopically expressed in the cortex of *Pax6<sup>Sey/Sey</sup>* mice. Note SP9 expression appears in more immature cells (i.e., are closer to the ventricle) than SP8 in the cortical SVZ (arrows). (C–J') Increased *Gad1*, *Tshz1*, *Prokr2*, and *Etv1* expression was seen in the mutant dLGE and cortex. (K–L') Strong *Bcl11b* expression was not observed in the region of the ectopia mutant cortex. Scale bars: 200  $\mu$ m in (B') for (A–B'); 400  $\mu$ m in (L') for (C–L').

expression was greatly reduced (Fig. 7I–L',Q,R). In contrast, there were increased numbers of SVZ cells expressing progenitor markers: *GSX2*<sup>+</sup>, *Ki67*<sup>+</sup>, *ASCL1*<sup>+</sup>, *GSX2*<sup>+</sup>/*DCX*<sup>+</sup>, *Ki67*<sup>+</sup>/*DCX*<sup>+</sup>, and *ASCL1*<sup>+</sup>/*DCX*<sup>+</sup> (Fig. 7C–H',N–P). Similar expression patterns of these genes were also observed in the RMS and the core of the OB

at P50 (Supplementary Fig. S5A–L'). Notably, while wild type controls had very few *GSX2*<sup>+</sup>, *Ki67*<sup>+</sup>, *ASCL1*<sup>+</sup> cells in the OB core, these cells were abundant in the OB core of *Dlx1/2*-CKO mice at P50 (Supplementary Fig. S5D',F',H'). Consistent with a block in neuronal differentiation in the SVZ, there was a large increase of *GFAP*<sup>+</sup>



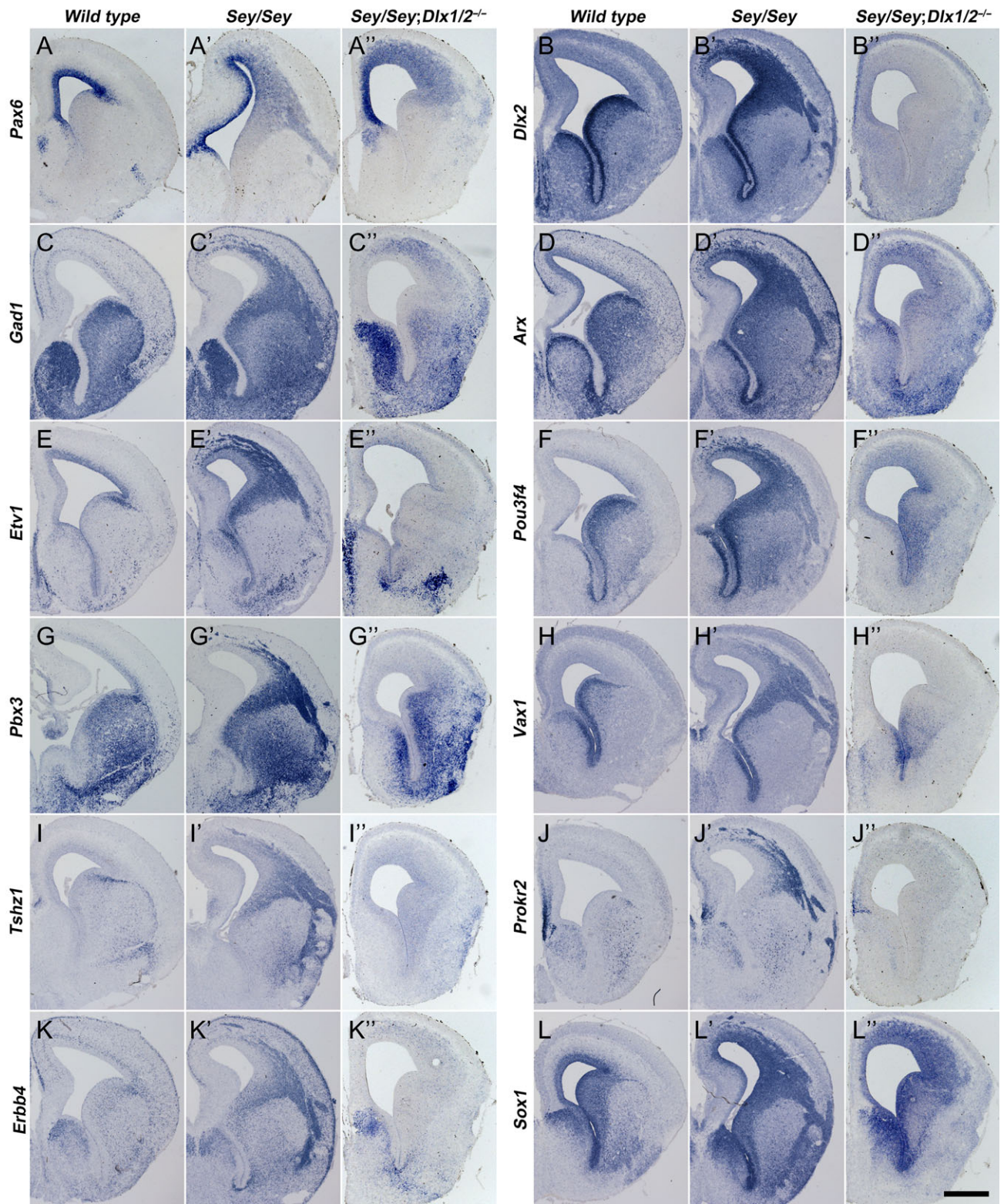


**Figure 5.** More subpallial neural progenitors are in the cortex of *Pax6<sup>Sey/Sey</sup>; Dlx1/2<sup>-/-</sup>* Mice at E16.5. (A–B'') Immunostaining of PAX6 and DLX2 confirmed the genotypes of mice used. (C–C'') EOMES expression was greatly reduced in both *Pax6<sup>Sey/Sey</sup>* and *Pax6<sup>Sey/Sey</sup>; Dlx1/2<sup>-/-</sup>* mice. (D–F'') GSX2<sup>+</sup>, OLIG2<sup>+</sup>, and ASCL1<sup>+</sup> progenitors ectopically accumulated in the neocortex of *Pax6<sup>Sey/Sey</sup>* mice; their expression increased further in *Pax6<sup>Sey/Sey</sup>; Dlx1/2<sup>-/-</sup>* mice. (G–I'') The ectopic SP8/9 cortical expression in the *Pax6<sup>Sey/Sey</sup>* mice was reduced in the *Pax6<sup>Sey/Sey</sup>; Dlx1/2<sup>-/-</sup>* cortex; furthermore, the SP8/9<sup>+</sup> cells showed a scattered pattern. (J–L'') Most SP9/8<sup>+</sup> cells coexpressed BCL11b in the cortex of *Pax6<sup>Sey/Sey</sup>; Dlx1/2<sup>-/-</sup>* mice. (M–N'') ISL1 and EBF1 expression was not observed in the neocortex of these mutants. Scale bars: 70  $\mu$ m in L'' for L–L'', 250  $\mu$ m in M'' for A–F'', H–K'', M–M'', 500  $\mu$ m in N'' for G–G'', N–N''.

(based on strong GFAP expression) and OLIG2<sup>+</sup> cells ( $7.23 \pm 1.18$  vs.  $23.23 \pm 0.27$  per section; wild type vs. *Dlx1/2*-CKO,  $P = 0.0004$ ) (Supplementary Fig. S6A–F'). Finally, the expression of OB interneuron-marker genes, *Gad1*, *Arx*, *Etv1*, *Sp9*, *Prokr2*, *Tshz1*, and

*Pbx3*, in the adult SVZ, RMS, and OB core, were either severely reduced or undetectable (Fig. 8A–G). Thus, *Dlx1/2* function remains essential in the adult SVZ and RMS for the differentiation of OB neural progenitors.



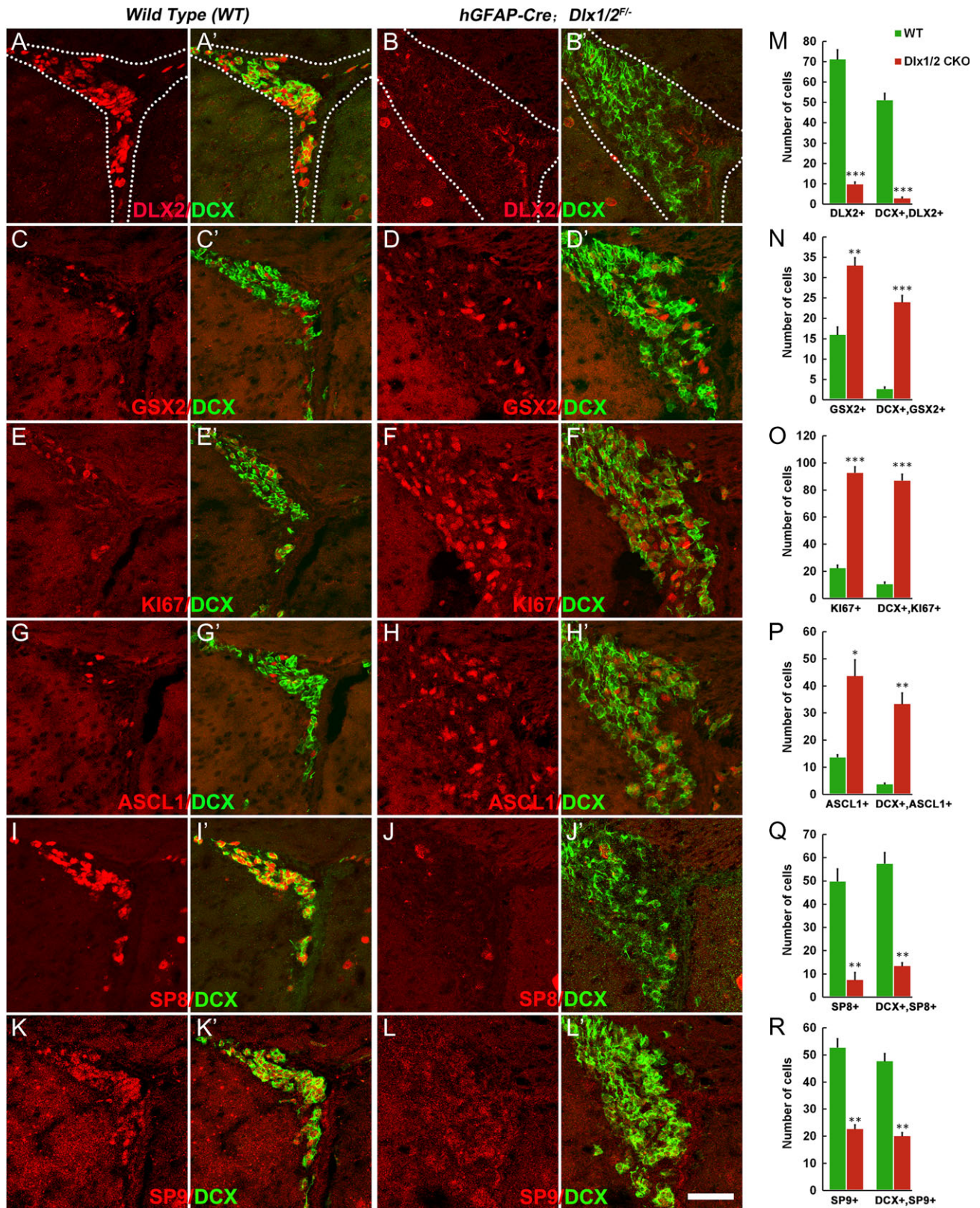


**Figure 6.** Immature OB interneurons are not generated in the cortex of *Pax6<sup>Sey/Sey</sup>; Dlx1/2<sup>-/-</sup>* mice at E16.5. (A–L') In situ RNA hybridization indicated that immature OB interneuron markers (*Gad1*, *Etv1*, *Pbx3*, *Tshz1*, *Erbb4*, *Arx*, *pou3f4*, *Vax1*, *Prokr2*) were not detected in *Pax6<sup>Sey/Sey</sup>; Dlx1/2<sup>-/-</sup>* mice compared with *Pax6<sup>Sey/Sey</sup>* mice. Note that *Pax6* transcripts and *Sox1* expression were increased in the cortex of these mutants. Scale bar: 600  $\mu$ m in L' for A–L'.

We then examined DCX<sup>+</sup> cell migration in the lateral wall of the lateral ventricle in adult mice (P50) using wholemount immunostaining (Doetsch and Alvarez-Buylla 1996; Mirzadeh

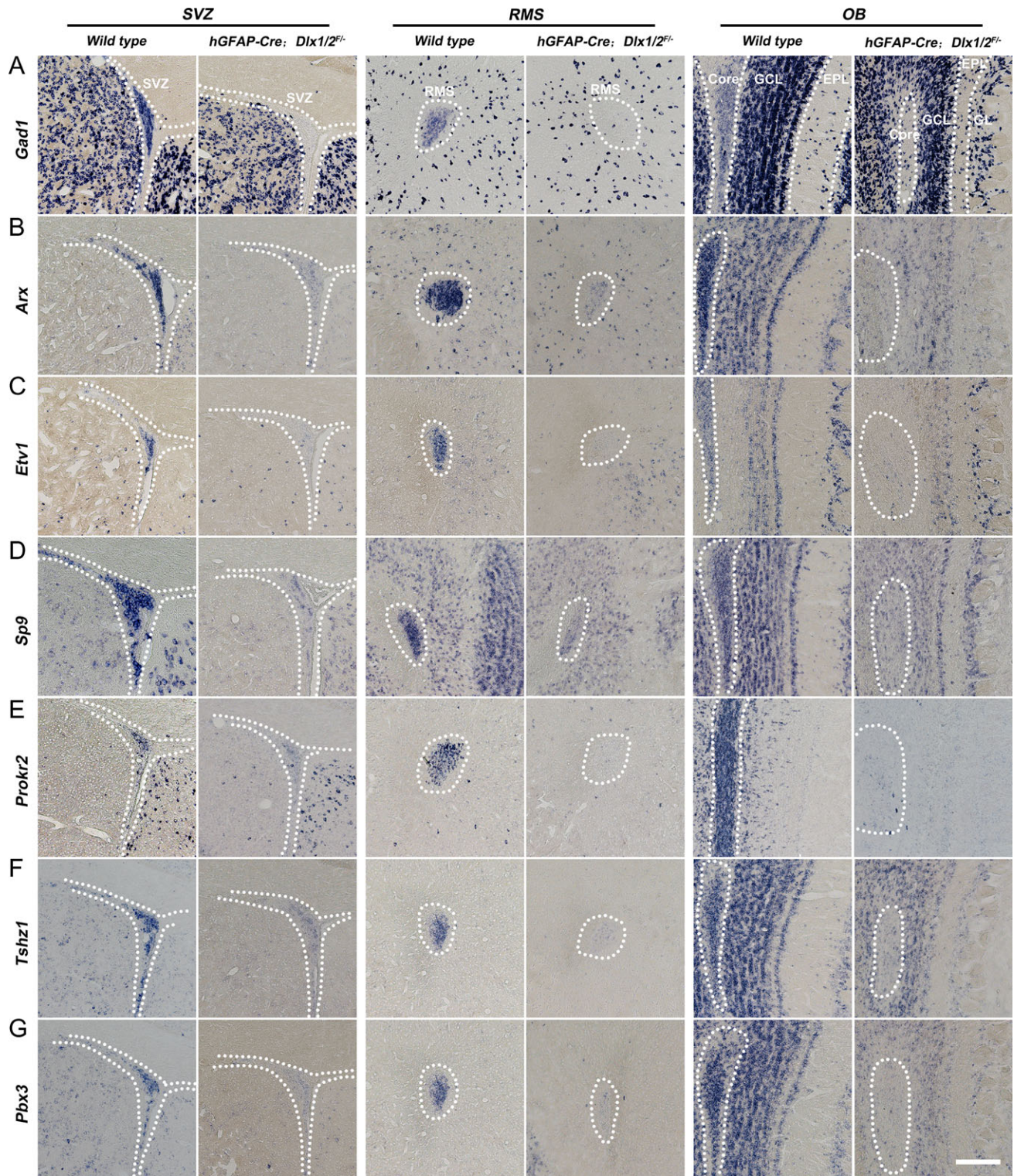
et al. 2010). DCX<sup>+</sup> cells in the normal adult mouse SVZ are dividing and nondividing neuroblasts (Ponti et al. 2013), but DCX<sup>+</sup> cells in the SVZ of adult *Dlx1/2*-CKO mice are mainly





**Figure 7.** Neuronal differentiation of stem/progenitors in the adult SVZ (P50) of *hGFAP-Cre; Dlx1/2<sup>F/-</sup>* conditional knockout (*Dlx1/2*-CKO) mice was blocked. (A–L) *DLX2<sup>+</sup>*, *SP8<sup>+</sup>*, *SP9<sup>+</sup>*, *DLX2<sup>+</sup>/DCX<sup>+</sup>*, *SP8<sup>+</sup>/DCX<sup>+</sup>*, and *SP9<sup>+</sup>/DCX<sup>+</sup>* cells were greatly reduced whereas *GSX2<sup>+</sup>*, *Ki67<sup>+</sup>*, *ASCL1<sup>+</sup>*, *GSX2<sup>+</sup>/DCX<sup>+</sup>*, *Ki67<sup>+</sup>/DCX<sup>+</sup>*, and *ASCL1<sup>+</sup>/DCX<sup>+</sup>* cells were significantly increased in the SVZ of *Dlx1/2*-CKO mice compared with wild type control mice at P50. (M–R) Quantification of these experiments. Scale bar: 50  $\mu$ m in *L'* for A–L'.



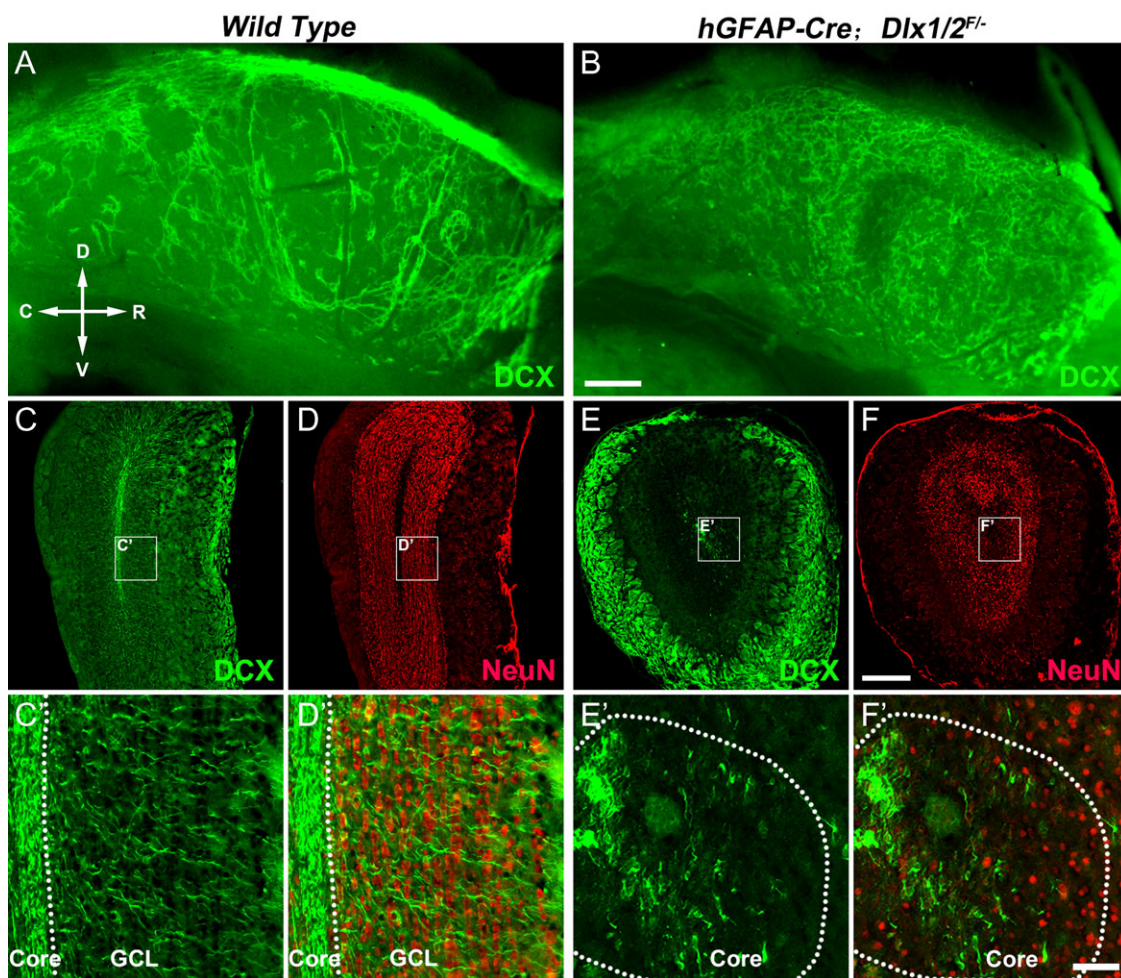


**Figure 8.** Loss of immature OB interneurons in the adult (P50) *Dlx1/2*-CKO SVZ, RMS and the core of the OB. (A–G) In situ RNA hybridization showed that expression of *Gad1*, *Arx*, *Etv1*, *Sp9*, *Prokr2*, *Tshz1*, and *Pbx3* in the adult SVZ, RMS, and OB core of *Dlx1/2*-CKO mice were either severely reduced or undetectable. Scale bar: 100  $\mu$ m in G for A–G.

GSX2<sup>+</sup>, Ki67<sup>+</sup>, ASCL1<sup>+</sup> progenitors (Fig. 7C–H). Control mice displayed DCX<sup>+</sup> cells (neuroblasts) arranged in orderly chains (Fig. 9A), whereas in *Dlx1/2*-CKO mice, DCX<sup>+</sup> cells (progenitors) did not form migration chains, appearing disorganized (Fig. 9B), indicative of a defect in tangential migration ( $n = 7$  mice per

group). The sizes of OBs were reduced in adult *Dlx1/2*-CKO mice and the number of NeuN<sup>+</sup> cells in the OB was significantly decreased compared with wild types (Fig. 9C–F). While laminar structures were present in the *Dlx1/2*-CKO mouse OB, these could correspond the projection neurons and outer nerve layer





**Figure 9.** Tangential and radial migration defects of DCX<sup>+</sup> cells in the adult SVZ and OB of *Dlx1/2*-CKO mice. (A, B) Wholemount DCX immunostaining of the lateral wall of the lateral ventricle showed DCX<sup>+</sup> cells in the SVZ of *Dlx1/2*-CKO mice (P50) did not form migration chains. (C–F) OB coronal sections stained for NeuN and DCX. Note that *Dlx1/2*-CKO mouse OB was smaller than wild type OB. (C'–F') High magnification images of boxed areas in (C–F) showing the defect of the radial migration of DCX<sup>+</sup> cells. R, rostral; C, caudal; D, dorsal; V, ventral. Scale bars: 300 μm in B for A–B'; 350 μm in F for C–F; 50 μm in F' for C'–F'.

(Fig. 9C'–F') (Bulfone et al. 1998). A severe reduction of DCX<sup>+</sup> cells was seen in the mutant OB, and their lack of clear radial orientation suggested that their radial migration may be abnormal (Fig. 9C'–F').

#### Overexpression of *Dlx1*, *Dlx2*, or *Dlx1&2* in the Neocortex Promoted the Generation of Immature OB Interneurons From Cortical Progenitors

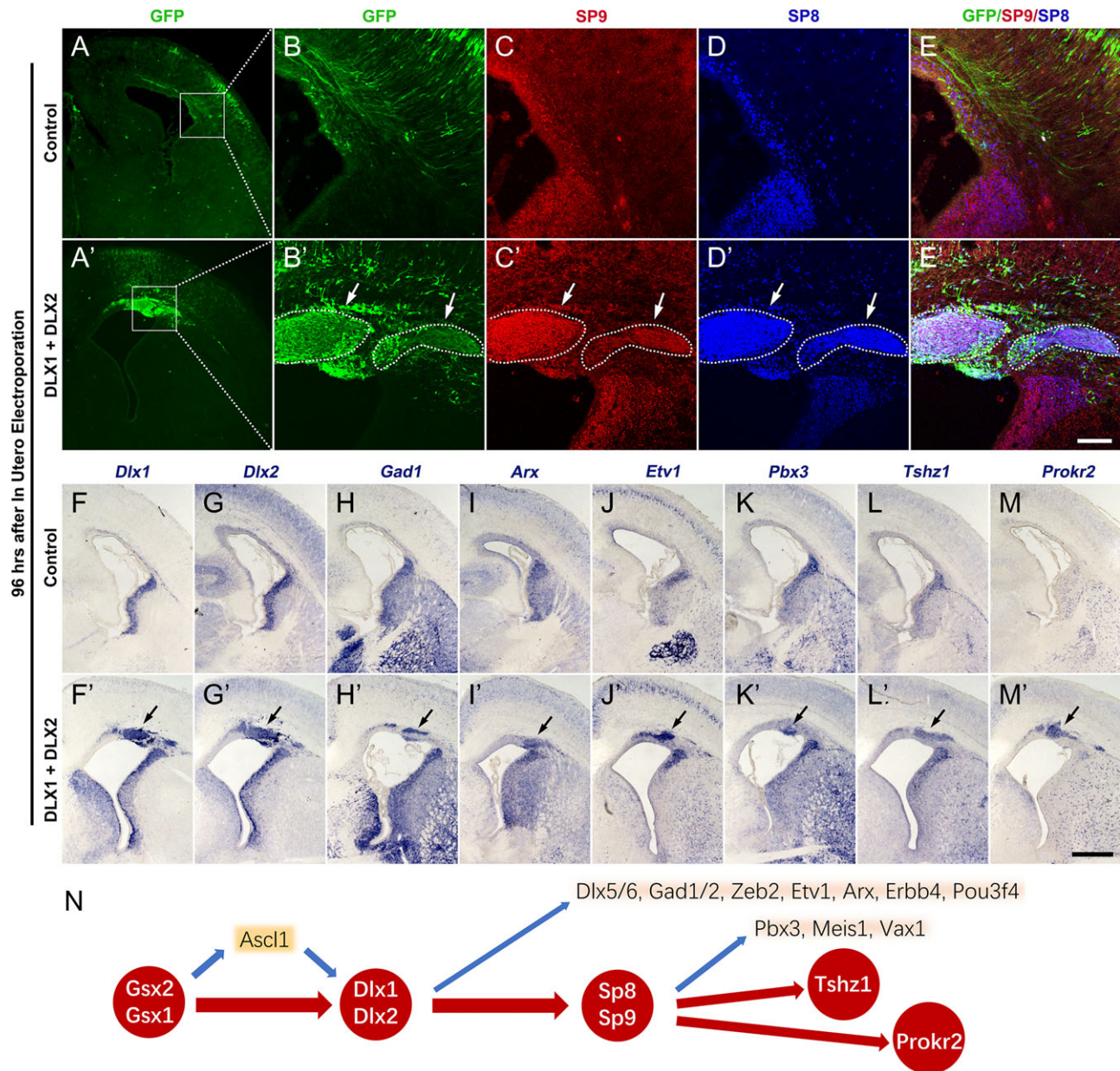
Previous studies have shown that ectopic expression of *Dlx* genes in the developing neocortex is sufficient to induce the ectopic expression of GAD1 (Stuhmer et al. 2002). Thus, we investigated whether overexpression of *Dlx1&2* genes in the neocortex was sufficient for the cortical progenitors to generate OB interneurons. We ectopically expressed *Dlx1&2* by IUE of *pCAGIG-Dlx1-Ires-GFP* plasmids and *pCAGIG-Dlx2-Ires-GFP* plasmids together or control *pCAGIG-GFP* plasmids into the cortical VZ of E14.5 wild type CD1 mice, and examined the brains at different time points after IUE.

Twelve hours after *Dlx1&2* IUE, strong DLX1 (data not shown) and DLX2 protein expression was observed in GFP<sup>+</sup> cortical progenitors (Supplementary Fig. S7A–A'); these GFP<sup>+</sup> cells did not express SP8 or SP9 protein (Supplementary Fig. S7B–C'). Twenty-

four hours after *Dlx1&2* IUE, SP8 was strongly expressed in most GFP<sup>+</sup> cells whereas SP9 expression was not turned on yet (Supplementary Fig. S7D–F'). Forty-eight hours later, a subset of GFP<sup>+</sup> cells started to express SP9 (Supplementary Fig. S8A–C'). Sixty hours after IUE, coexpression of SP8 and SP9 was observed in most GFP<sup>+</sup> cells in the cortical SVZ (Supplementary Fig. S8D–F'). Furthermore, *Dlx1&2* IUE induced cortical SVZ expression of *Gad1*, *Arx*, *Etv1*, *Pbx3*, *Tshz1* and *Prokr2*, markers of immature OB interneurons (Supplementary Fig. S8G–L). Ninety-six hours after *Dlx1&2* IUE (at E18.5), DLX1/2<sup>+</sup>/GFP<sup>+</sup> cells appeared to form clusters; these cells expressed *Gad1*, SP8/9, *Arx*, *Etv1*, *Pbx3*, *Tshz1*, and *Prokr2* (Fig. 10A–M', Supplementary Fig. S9A–L). Notably, we observed that SP8 was expressed in nearly all DLX1&2<sup>+</sup> cells, including those cells that already migrated into the different layers of the cortex (Supplementary Fig. 9M–O'), providing further evidence that DLX1&2 can induce SP8 expression. OB interneuron markers were not expressed in the cortical SVZ IUE of the negative control *pCAGIG-GFP* plasmid (Fig. 10A–E,F–M).

Finally, we asked whether IUE of *Dlx1* or *Dlx2* alone in the cortex could induce OB interneuron generation in the cortical SVZ. Consistent with IUE of *Dlx1&2*, 96 h after *Dlx1* or *Dlx2* IUE alone, DLX1<sup>+</sup>/GFP<sup>+</sup> cells or DLX2<sup>+</sup>/GFP<sup>+</sup> cells expressed the OB interneuron markers *Dlx1*, *Dlx2*, *Gad1*, SP8/9, *Etv1*, *Tshz1*, and





**Figure 10.** Overexpression of Dlx1&2 in the dorsal pallium (cortex) induced the generation of immature OB interneurons. (A–E) Overexpression of Dlx1&2 in the neocortex by IUE of plasmids (pCAGIG-Dlx1-Ires-GFP + pCAGIG-Dlx2-Ires-GFP) at E14.5 induced the ectopic expression of SP8&9 (arrows) in the cortical SVZ at E18.5, whereas overexpression of GFP alone (pCAGIG-Ires-GFP) did not. (F–M) In situ RNA hybridization showed that ectopic expression of Dlx1&2 induced *Gad1*, *Arx*, *Etv1*, *Pbx3*, *Tshz1*, and *Prokr2* (arrows). (N) Based on *Dlx1/2* loss of function and gain of function analysis, we propose a transcriptional regulatory network that underlies OB interneuron development. Note that loss of *Gsx2/1*, *Dlx1/2*, *Sp8/9*, or *Prokr2* function results in loss of all or most OB interneurons (see discussion). Scale bars: 500  $\mu$ m in M' for A, A', F–M'; 100  $\mu$ m in E' for B–E'.

*Prokr2* (Supplementary Fig. S10A–T). Thus, overexpression of either *Dlx1*, *Dlx2*, or *Dlx1&2* in cortical neural progenitors induces the genetic program that promotes the generation of immature OB interneurons in the cortical SVZ.

## Discussion

We propose that *Dlx1/2* promote OB interneuron development largely through activating the expression of OB interneuron genes, especially *Sp8/9*, which further activate *Tshz1* and *Prokr2* expression. This study, in combination with earlier studies, presents evidence for a transcriptional regulatory network that controls the multistep process of OB interneuron development (Fig. 10N).

## A Model of the Transcriptional Regulation Network That Regulates OB Interneuron Development

Loss of function and gain of function analysis reveal that *Dlx1/2* are necessary and sufficient for making all OB interneurons. Based on this study and earlier studies, we present a common model of the transcriptional network that regulates OB interneuron development: *Gsx2/1*-*Dlx2/1*-*Sp8/9*-*Tshz1*-*Prokr2* (Fig. 10N).

The fundamental feature of the dLGE is expression of strong *GSX2*, and *GSX2* is at the top of the hierarchy in the dLGE (Wang et al. 2009, 2013). Many OB interneurons were still generated in the *Gsx2* mutant mice (Corbin et al. 2000; Toresson and Campbell 2001; Yun et al. 2001). However, *Gsx1* expression is



upregulated in the dLGE in *Gsx2* mutants; *Gsx1* partially rescues the phenotypes of *Gsx2* mutants (Toresson and Campbell 2001; Yun et al. 2003). Loss of *Gsx1/2* function results in loss of *Ascl1* and *Dlx* genes' expression in the dLGE, and none of interneurons is generated in the OB (Toresson and Campbell 2001; Yun et al. 2003). Ectopic expression of *Gsx2* in the embryonic cortex induces *Dlx* and *Ascl1* expression, which further induces the generation of SP8<sup>+</sup> immature OB interneurons (Waclaw et al. 2009). Therefore, *Gsx2* promotes OB interneuron development through activating *Dlx1/2* and *Ascl1* (Fig. 10N).

*Ascl1* maintains progenitor state (Casarosa et al. 1999; Yun et al. 2002; Long et al. 2009; Castro et al. 2011; Wang et al. 2013), and partially regulates *Dlx1/2* expression through the I12b intergenic enhancer (Poitras et al. 2007). *GSX2*, *ASCL1*, and *DLX2* are coexpressed in the VZ/SVZ1 of the dLGE (Fig. 1A–F). Although *GSX2* and *ASCL1* expression are downregulated in SVZ2 of the dLGE, *DLX1/2* continue to be expressed there prenatally, and in the postnatal SVZ, RMS, and OB. There is evidence that *DLX2* can directly bind 2 of its enhancers (I12b or URE2) and thus may be able to promote its own expression (Poitras et al. 2007; Potter et al. 2009). Consistent with this, in the present study, we also show that overexpression of either *Dlx1* or *Dlx2* alone in the embryonic cortex could induce the expression of *Dlx1/2* in the cortical SVZ, providing another strong evidence that *DLX1/2* can promote their own expression.

Following *Dlx1/2* function, we propose that the next step of OB interneuron development is controlled by *Sp8/9*. *Sp8/9* mutants lack *Tshz1* and *Prokr2* expression and have severely reduced *Pbx3*, *Vax1*, and *Meis1* expression (Li et al. 2018); however, *Dlx1/2/5*, *Etv1*, *Arx*, and *ErbB4* expression are maintained in the dLGE (Supplementary Fig. S4A–F''). There is evidence that *Dlx1/2* directly promote *Sp8/9*, *Gad1/2*, *Etv1*, *Arx*, *ErbB4*, and *Zeb2* expression (Long et al. 2007, 2009; Colasante et al. 2008; McKinsey et al. 2013; Le et al. 2017; Pla et al. 2018) (data not shown) (Fig. 10N).

### The Functions of *Dlx1/2* Genes in the OB Interneuron Development

*Dlx1/2* constitutive null mutants fail to induce *Dlx5/6* expression in most of the forebrain (Anderson et al. 1997; Long et al. 2009) (Supplementary Table S1). Conditional deletion of *Dlx1/2* (probably in SVZ1 or SVZ2), about 24 h after their expression is initiated, does not eliminate *Dlx5/6* expression (Pla et al. 2018). In these mutants the generation and migration of OB and cortical interneurons is largely intact (Pla et al. 2018) (Rubenstein J. L., unpublished data). Thus, the lack of expression of all 4 *Dlx* genes leads to the virtually complete absence of OB interneurons in the *Dlx1/2* mutants. *Dlx1/2* promote OB interneuron genesis through positively regulating a subset of transcription factors (Fig. 10N), but may also through repressing *Gsx2/1*, *Ascl1*, and *Olig2* expression as these transcription factors promote the progenitor and/or glia cell state (Petryniak et al. 2007; Long et al. 2009; Chapman et al. 2013, 2018; Wang et al. 2013).

### *Dlx* Genes and Other Transcription Factors are Required for the Specification of OB Interneuron Subtypes

Regional identity, and the identity of neural stem cells, is determined in the VZ. The LGE may consist of multiple progenitor domains along the dorsoventral axis (Flames et al. 2007; Xu et al. 2018). The dLGE, generates OB interneurons, may consist of pLGE1 and pLGE2, whereas the vLGE, generates largely striatal MSNs and some OB interneurons, may consist of pLGE3 and

pLGE4 (Zhang et al. 2016; Xu et al. 2018). pLGE1 is implicated in the generation of *Dlx2*<sup>+</sup> *Pax6*<sup>+</sup> TH<sup>+</sup> OB interneurons (Long et al., 2007), whereas pLGE2 may generate the majority of OB interneurons. Furthermore, distinct OB interneuron subtypes are produced by the dLGE, vLGE, cortex, and septum (Kohwi et al. 2007; Merkle et al. 2007, 2014; Ventura and Goldman 2007; Young et al. 2007; Kriegstein and Alvarez-Buylla 2009; Lopez-Juarez et al. 2013; Fuentealba et al. 2015; Hoch et al. 2015; Qin et al. 2017). *Gsx2/1-Dlx2/1-Sp8/9-Tshz1-Prokr2* transcriptional network operates in each of these domains, where several of these transcription factors are implicated in specification of OB interneuron subtypes. *Sp8* is required for the generation of Calb2<sup>+</sup> and Pvalb<sup>+</sup> OB interneurons (Waclaw et al. 2006; Li et al. 2011). *Sp9* is required for the generation of a small population of Pvalb<sup>+</sup> OB interneurons (Li et al. 2018). *Pax6*, *Dlx2*, and *Etv1* are required for the generation of the TH<sup>+</sup> subpopulation (Qiu et al. 1995; Dellovade et al. 1998; Hack et al. 2005; Kohwi et al. 2005; Cave et al. 2010). The Calb1<sup>+</sup> subpopulation requires *Tshz1* (Ragancokova et al. 2014). It is likely that the specification of OB interneuron subtypes results from combinatorial transcription factor codes; these remain to be elucidated (Allen et al., 2007; Alvarez-Buylla et al. 2008; Bartolini et al. 2013; Zhou et al. 2015; Fujiwara and Cave 2016).

### Supplementary Material

Supplementary material is available at *Cerebral Cortex* online.

### Authors' Contributions

T.G. and G.L. performed experiments and analysis. H.D., Y.W., S.W., Z.L., G.T., Z.S., X.S., Z.Z., Z.X., and Y.Y. helped conduct experiments and analyze the data. B.C. and J.L.R. helped guide the project and discussed some of results. Z.Y. designed the experiments and analyzed the results. Z.Y. and J.L.R. wrote the article.

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### Notes

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