

Wnt5a Is a Transcriptional Target of *Dlx* Homeogenes and Promotes Differentiation of Interneuron Progenitors *In Vitro* and *In Vivo*

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During brain development, neurogenesis, migration, and differentiation of neural progenitor cells are regulated by an interplay between intrinsic genetic programs and extrinsic cues. The *Dlx* homeogene transcription factors have been proposed to directly control the genesis and maturation of GABAergic interneurons of the olfactory bulb (OB), subpallium, and cortex. Here we provide evidence that *Dlx* genes promote differentiation of olfactory interneurons via the signaling molecule *Wnt5a*. *Dlx2* and *Dlx5* interact with homeodomain binding sequences within the *Wnt5a* locus and activate its transcription. Exogenously provided *Wnt5a* promotes GABAergic differentiation in dissociated OB neurons and in organ-type brain cultures. Finally, we show that the *Dlx*-mutant environment is unfavorable for GABA differentiation, *in vivo* and *in vitro*. We conclude that *Dlx* genes favor interneuron differentiation also in a non-cell-autonomous fashion, via expression of *Wnt5a*.

Introduction

In the developing brain, neural progenitor cells (NPCs) exit the cell cycle and leave the germinal layers, while progressively initiating region- and time-specific programs of neuronal differentiation (Nguyen et al., 2006; Doe, 2008). The balance between proliferation and differentiation of NPCs is the result of a complex interplay between genetically determined cellular programs and local environmental cues, largely to be deciphered (Zaki et al., 2003; Fukuda and Taga, 2005; Hämmerle and Tejedor, 2007). The fate of differentiating neurons is established by the patterned expression of transcription factors, often of the homeobox class (Flames et al., 2007; Fogarty et al., 2007; Guillemot, 2007; Hébert and Fishell, 2008; Wigle and Eisenstat, 2008).

GABA⁺ interneurons derive from subpallial NPCs residing in the medial (MGE) and lateral (LGE) ganglionic eminences, which migrate toward the cortex/hippocampus and olfactory bulb (OB), respectively (Wichterle et al., 2001; Marin and Rubenstein, 2003; Métin et al., 2006; Wonders and Anderson, 2006).

The *Dlx* homeogenes play major roles in the control of migration and differentiation of subpallial NPCs, as revealed by the brain phenotypes of mice null for individual genes (*Dlx1*^{−/−}, *Dlx2*^{−/−}, and *Dlx5*^{−/−}) or combinations of them (Anderson et al., 1997; Levi et al., 2003; Long et al., 2003, 2007). In the absence of *Dlx5*, the number of TH⁺, calretinin-positive (CR⁺), and GABA⁺ olfactory interneurons is reduced, although the *Dlx5*⁺ cell lineage is not depleted. Thus, a role of *Dlx* proteins to drive GABA differentiation cell-autonomously (Stühmer et al., 2002) appears well established.

Wnt molecules regulate NPC proliferation, commitment, and differentiation during development (Ciani and Salinas, 2005; Ille and Sommer, 2005; Malaterre et al., 2007). Binding of Wnts to the Frizzled/LRP complex activates the “canonical” β -catenin-dependent pathway. Alternatively, “noncanonical” pathways are known, which include the Ca²⁺/PKC and JnK ones (Eisenmann, 2005; Gordon and Nusse, 2006; Barker, 2008; Huang and He, 2008), and novel receptor/signal routes have recently been uncovered (Lyu et al., 2008).

Several published results indicate that Wnt/ β -catenin signaling is essential to maintain the proliferation potential of hippocampal, cortical, and midbrain NPCs *in vivo* (Zechner et al., 2003; Lie et al., 2005; Adachi et al., 2007; Nusse, 2008); for instance, expression of stabilized β -catenin in cortical NPCs leads to the expansion of NPCs in the forebrain (FB) (Chenn and Walsh, 2003; Woodhead et al., 2006). Likewise, Wnt/ β -catenin signaling in the subpallium is required for proliferation of NPCs, while it does not influence their migration or differentiation (Gulacsi and Anderson, 2008).

Wnt signaling also promotes NPC differentiation (Klüber and Sommer, 2004; Muromiyama et al., 2004). Wnt3 and Wnt7a specif-

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This paper is dedicated to the memory of Giorgio Corte, who passed away before this publication.

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ically favor neuronal differentiation of late, but not of early, cortical NPCs (Hirabayashi et al., 2004; Hirabayashi and Gotoh, 2005; Yu et al., 2006), and Wnt5a stimulates the differentiation of dopaminergic neurons (Castelo-Branco and Arenas, 2006; Andersson et al., 2008). As Wnt ligands elicit such a variety of responses, their functions need to be determined in a cell type- and context-dependent manner.

Here we provide evidence that *Dlx* genes promote differentiation of olfactory interneurons by regulating the expression of *Wnt5a*, hence non-cell-autonomously. We also show that *Wnt5a* favors GABA⁺ differentiation both in dissociated OB neurons and in organ-type cultures.

Materials and Methods

Mouse lines. Mice with targeted disruption of *Dlx5* and *Dlx5;Dlx6* have been previously reported (Acampora et al., 1999; Merlo et al., 2002b). The *Dlx5* null allele harbors a knock-in insertion of the *lacZ* reporter, which allows for detection of the *Dlx5*-expressing cells by β -galactosidase (β -gal) immunostaining. The *Dlx5;Dlx6* double KO mice carry an allele with a large deletion encompassing both genes. β -actin-eGFP mice (Okabe et al., 1997) were used as donors in the grafting experiments. Animal procedures were approved by the Institutional Animal Care Committee and the Italian Ministry of Health.

Culture and transduction of neurospheres. Brains of E18.5 wild-type, *Dlx5*^{+/−}, and *Dlx5*^{−/−} mice were dissected to obtain the rostral periventricular region. The tissue was dissociated in PBS with 10 mM glucose and 0.1% trypsin, cells were collected and resuspended in serum-free NSA medium (Euroclone) supplemented with N2 (Invitrogen), 2 mM glutamine, 20 ng/ml EGF, and 10 ng/ml bFGF (Pepro-tech). Neurospheres formed after 3–5 d and were mechanically dissociated twice a week and replated. Neurosphere from *Dlx5*^{−/−} brains were transduced with a retroviral vectors expressing wild-type murine *Dlx5*, as previously reported (Perera et al., 2004), to obtain the cells denominated *Dlx5*^{−/−}^{*Dlx5exp*}.

Microarray profiling and validation. Neurospheres cells from wild-type and *Dlx5*^{−/−} brains, as well as *Dlx5*^{−/−}^{*Dlx5exp*} cells, were used to purify total RNA. cDNA and biotinylated cRNA samples were generated as previously described (Gangemi et al., 2006), and used to hybridize on Affymetrix murine genome U74Av2 GeneChips. Quality controls and normalization were done using standard statistical methods. Only genes whose expression was altered at least twofold ($\log_2 = 1$) were considered. Next, genes were filtered for coherent expression changes in the *Dlx5*^{−/−}^{*Dlx5exp*} cells, i.e., a downregulation in *Dlx5*^{−/−} cells corresponding to an upregulation in *Dlx5*^{−/−}^{*Dlx5exp*} cells, and vice versa. The concordance between the two comparisons was highly significant (for downregulated, $p = 8.8 \times 10^{-111}$; for upregulated, $p = 8.1 \times 10^{-135}$). Differential expression of 20 selected mRNAs was confirmed by reverse transcription (RT)-PCR on total RNA from wild-type and *Dlx5*^{−/−} neurospheres. The following genes survived this technical validation analysis: *Wnt5a*, *Fzd9*, *R-cadherin*, *Neurexophilin*, *Sema6a*, *PlexinB2* (downregulated), *Fibulin2*, *Thy1.2*, *Ulip4*, and *Dhh* (upregulated). *In situ* hybridization was done to detect *Wnt5a*, *Fzd9*, *R-cadherin*, *Neurexophilin*, *Sema6a*, *PlexinB2*, *Fibulin2*, *Thy1.2*, and *Ulip4* mRNA on sections of normal and *Dlx5*^{−/−} brains. Only *Wnt5a* was validated *in situ*.

Derivation, characterization, and differentiation of brain-derived neural stem cells. Embryos at day 12.5 of gestation were collected by cervical dislocation, genotyped for the presence of the *Dlx5;6*^{−/−} allele, and used to derive adherent cultures of neural stem (NS) cells, as described previously (Conti et al., 2005; Pollard et al., 2006; Spiliotopoulos et al., 2009). Briefly, the entire forebrain was dissected, the tissue was dissociated with Accutase (Sigma), and cells were plated on plastic dishes in Euromed-N Medium (Euroclone) supplemented with 1% N2 (Invitrogen) and 20 ng/ml of both FGF2 and EGF and expanded adherently for 10 passages. Three clones were selected, expanded, and shown to have identical properties. Absence of *Dlx5* and *Dlx6* expression in mutant cells was verified by qPCR. Cells at the passage 12 were examined for the expression of Nestin (Millipore Bioscience Research Reagents, 1:10), Sox2 (Millipore Bioscience Research Reagents, 1:1000), BLBP (Millipore Bioscience Research

Reagents, 1:500), Olig2 (Millipore Bioscience Research Reagents, 1:2000), GFAP (DAKO, 1:2000), β III-tubulin (TuJ1, Promega, 1:2000), glutamic acid decarboxylase 67 (GAD67) (Millipore Bioscience Research Reagents, 1:2000), GABA (Sigma, 1:1000), CR (Millipore Bioscience Research Reagents, 1:2000), and anti-dll (pan-Dlx, gift from Dr. Grace Boekhoff-Falk, University of Wisconsin Medical School, Madison, WI, 1:1000). Immunofluorescence was performed using mouse AlexaFluor-488 (Invitrogen) and rabbit AlexaFluor-568 (Invitrogen) secondary antibodies, followed by nuclear counterstaining with DAPI. Cells were also examined for expression of *Dlx2*, *Pax6*, *Emx1*, *Emx2*, and *Oct4* by RT-PCR. NS cells were induced to differentiate following the described protocols (Spiliotopoulos et al., 2009), essentially consisting in adhesion on laminin-coated slides, removal of the growth factors, and addition of BDNF.

Coculture experiments were done by mixing 20% of LC1-GFP cells (Conti et al., 2005) with 80% of either wild-type (WT) or *Dlx5;Dlx6* mutant (GFP[−]) brain-derived NS cells, followed by standard differentiation protocol, immunostaining, and counting the number of GFP⁺/GAD67⁺ cells over the number of GFP⁺ cells. Results are expressed as percentages.

Organotypic cultures of embryonic FB. Cultures of brain slices were prepared essentially as described previously (Zaghetto et al., 2007). Briefly, embryonic heads at E16.5 were dissected in cold PBS+0.6% glucose, embedded in warm 3% LMP agarose, and let harden at room temperature. The gel blocks were vibratome-sectioned (thickness 200 μ m), kept in cold PBS-glucose for 1 h, and placed on Millicell CM culture plate membranes (Millipore) in 35 mm plastic dishes containing Neurobasal medium (Invitrogen), supplemented with 1% N2, 25 μ g/ml gentamycin (Sigma), and 0.5 mM glutamine, and maintained for 48–72 h at 37°C in standard conditions. For the coculture experiment, COS7 cells were transfected with 4 μ g of Wnt4-, Wnt5a-, and Wnt7b-mycTAG expression vectors (provided by Dr. Yimin Zou, University of California, San Diego, San Diego, CA) and used as feeder layer. Slices were placed on top and incubated for 48 h. Acrylic beads were saturated for 12 h at 4°C with 500 ng/ml purified recombinant Wnt5a protein (R&D Systems) or PBS, then applied onto the OB slices using a thin needle. Slices were incubated for 72 h, and the OB was then excised and used to extract total RNA for real-time analyses. For the graft experiments, a small portion of the LGE near the ventricle was excised from E14.5 β -actin-eGFP embryos and transferred on the ascending section of the RMS of neonatal brain slices prepared on the same day. Following 7 d *in vitro*, the slices were fixed and used for immunofluorescent staining to detect GAD67.

Culture and differentiation of dissociated OB cells. WT and *Dlx5*^{−/−} OBs were excised from E16.5 brains, mechanically dissociated, and plated on poly-lysine-coated slides in Neurobasal medium supplemented with 1% B27, gentamycin, and glutamine, for 48 h. Cells were then fixed with PFA 4% and stained for β III-tubulin, GAD67, and CR as above. Positive cells were counted, and the number of positive cells was expressed relative to the number of DAPI⁺ nuclei. For coculture experiments, 24 h before OB dissociation HeLa cells were transfected with 4 μ g of Wnt5a-mycTAG expression vectors (above), plated on six-well cluster dishes, and allowed to adhere for 12 h. Dissociated OB cells were then placed on top, maintained for 48 h, and processed.

In situ hybridization. For *in situ* hybridization (ISH), embryos were collected between ages E14.5 and P0, and heads were either immersion-fixed in 4% PFA/0.1 M phosphate buffer (PB, pH 7.4) or perfused. The brains were dissected, washed in PBS, cryoprotected in 30% sucrose for 24 h, frozen in OCT, and sectioned at 12 μ m. Hybridization was performed with DIG-labeled riboprobes corresponding to the antisense sequence of the following murine cDNAs: *Dlx2* (1600 nt of the ORF), *Dlx5* (780 nt of the ORF), *Wnt5a* (360 nt of the ORF, provided by Dr. Yingzi Yang, National Institutes of Health, Bethesda, MD), and *Wnt5b* (1600 nt of the ORF, from Dr. Yang). The hybridization signal obtained with these probes was consistent with published and online data (www.genepaint.org). Hybridization was done on 14 μ m cryostatic sections, processed as described. Briefly, sections were permeabilized with 3 μ g/ml proteinase K, washed in PBS, and deacetylated with 1.3% triethanolamine and 0.25% acetic anhydride at room temperature. Sections were prehybridized in 50% formamide at 60°C, hybridized with the DIG-labeled probes

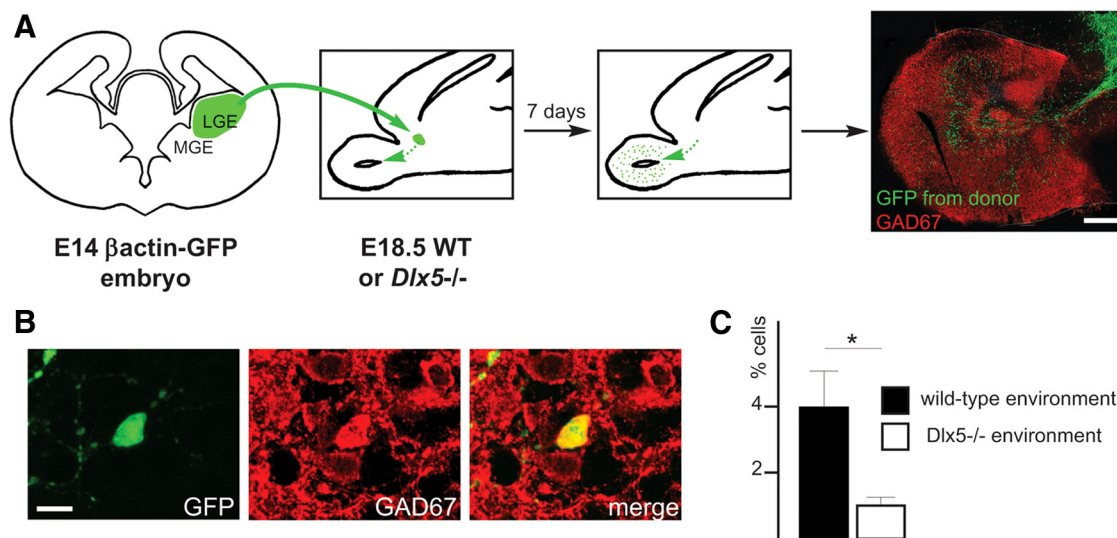


Figure 1. Grafting of normal subpallial progenitors in *Dlx5*^{−/−} forebrain, to reveal non-cell-autonomous regulations. **A**, Scheme illustrating the experimental strategy. Fragments of the LGE from GFP⁺ (normal) E14.5 embryos were used as donors to graft onto cultures of brain slices set up from neonatal WT or *Dlx5*^{−/−} mice. After 7 d in culture, GFP⁺/GAD67⁺ cells present in the host OB were counted. A comparable number of GFP⁺ cells were found in the WT and in the *Dlx5*^{−/−} OB; thus, migration seems to be unaffected. Scale bar, 100 μ m. **B**, Micrograph of a GFP/GAD67 double-positive cell, within the host OB, detected by confocal microscopy. Merged signal is in yellow. Scale bar, 5 μ m. **C**, Number of GFP⁺/GAD67⁺ donor cells in WT (solid black bars) or *Dlx5*^{−/−} (open bars) OB. Error bars represent the mean $\pm$ SD of five independent experiments. Differences indicated with * were statistically significant ($p < 0.05$).

for 16 h, washed, incubated with an anti-DIG-AP antibody (Roche), and developed with NBT-BCIP (Sigma). Digital micrographic images were contrast balanced, color matched, and rotated using Photoshop CS3 (Adobe).

RT and real-time qPCR. OB and GE were excised from the E14.5 or neonatal brains of WT and *Dlx5*^{−/−} animals, transferred in Trizol (Invitrogen), and used to extract total RNA according to standard procedures. Cultured cells were harvested in PBS, centrifuged, resuspended in Trizol, and processed. For real-time qPCR, 1 μ g of total RNA was reverse-transcribed at 42°C for 50 min in the presence of 500 ng/ μ l random hexamers and 10 mM each of dNTPs, RNasin, and SuperScript II Reverse Transcriptase (Invitrogen). Relative cDNA abundance was determined using the LightCycler System and the TaqMan Master kit (Roche). Specific cDNAs were amplified using primers and probes designed according to the Universal ProbeLibrary system (Roche). Dilution curves were set up for every amplicon. Experiments were repeated at least twice on independent samples, every point was done in triplicate, and results were normalized to the level of *TATA-binding protein* (TBP) mRNA. Data analysis was performed with ABI software, version 2.1 (Applied Biosystems). All primer sequences are provided as supplemental Tables I–III (available at www.jneurosci.org as supplemental material).

Expression vectors, transfection, and luciferase assay. The *DLX5*-myc and *DLX2*-myc expression vectors (OriGene) containing, respectively, the full-length human *DLX5* and *DLX2* cDNA with an in-frame insertion of the myc-TAG at the C terminus, were used. From these, N terminus-tagged versions of the same vectors were generated. To verify expression and activity of the exogenous *DLX2* and *DLX5* proteins, we performed immunostaining and Western analyses of transfected cells with anti-myc antibody (Santa Cruz Biotechnology) and luciferase reporter assays using the *Dlx2* cis-response conserved enhancer from *Arx* (provided by Dr. Broccoli, Università Vita-Salute San Raffaele, Milano, Italy). *Wnt4*-, *Wnt5a*-, and *Wnt7b*-mycTAG expression vectors were previously reported and tested for biological activity (Lyuksyutova et al., 2003; Zaghetto et al., 2007), and kindly provided by Dr. Yimin Zou.

The following *Wnt5a*-promoter-*luc* reporter vectors were constructed (further details are provided as supplemental Fig. 4, available at www.jneurosci.org as supplemental material): a 2000 bp fragment of the murine *Wnt5a* promoter and 5' UTR was inserted upstream of the *luc* expression cassette of pGL3-basic (Promega) to generate the reporter “A.” Upstream of this, three *Wnt5a* genomic fragments, comprising respectively intron II, intron III, and intron IV, and containing putative

homeodomain binding sites (Iler and Abate-Shen, 1996), were inserted to generate reporters named A1, A2, and A3. Initial transfection assays were done on HeLa (human) cells, by Lipofectamine (Invitrogen). For the NS cells, nucleofection (mouse NSC Nucleofector Kit, Amaxa) was used to transduce 500 ng of expression plasmid, 1 μ g of reporter vector, and 50 ng of pRL-TK vector (Renilla luciferase control reporter vector) as a control for transfection efficiency. Results are expressed as fold activation compared to the same reporter vectors cotransfected with empty expression plasmids.

ChIP for *Dlx* binding. ST14A rat striatal cells (Cattaneo and Conti, 1998), which express *Dlx5* and *Wnt5a* mRNA endogenously, were used to transfect 8 μ g of the *DLX2*- and *DLX5*-myc TAG expression vectors (above). Transfection consistently yielded an efficiency of 35% and 20%, respectively (number of myc-positive cells over total number of nuclei). Chromatin was crosslinked, sonicated, immunoprecipitated with anti-myc TAG, and de-crosslinked, according to instructions (EZ Magna ChipG, Millipore). Fragments of the murine *Wnt5a* locus were PCR-amplified and analyzed by electrophoresis. Total chromatin was used as positive control (input). Irrelevant IgG was used as negative control. Primer sequences are provided in supplemental Tables I–III (available at www.jneurosci.org as supplemental material).

Immunoblotting analyses of JnK and β -catenin. The OB and hippocampus were quickly dissected from neonatal mouse brains and either frozen in liquid N₂ or directly extracted in RIPA lysis buffer, supplemented with phosphatase and protease inhibitors. As positive control for phosphorylated JnK, HeLa cells were UV irradiated (40 mJ/cm²) and lysed 20 min later. As a control for β -catenin activation, NS cells were treated with 200 ng/ml purified Wnt3 (R&D Systems) for 12 h in growth medium. Total proteins were separated and blotted by standard Western methods, and developed with the ECL system (GE Healthcare). The antibodies used were as follows: anti-active β -catenin (Millipore Biotechnology), anti-pan JnK1–3, anti-phospho-JnK1–3 (9252 and 9251, respectively, Cell Signaling Technology), and anti-actin (Santa Cruz Biotechnology).

Microscope and image capture. Fluorescence images were acquired using an inverted microscope (Axio Observed Z1, Zeiss). Images were digitally captured using a cooled color 16-bit camera (Axio MRM, Zeiss) with Axio Vision Release 4.7.1 software. Images were optimized for color balance, brightness, and contrast using Photoshop CS3 (Adobe) and assembled into figures using QuarkXPress 7.2 (Quark). No additional digital manipulation was done.

Results

The environment of a *Dlx5* null OB is unfavorable for GABA+ differentiation

To get insight on how *Dlx* genes may control interneuron differentiation, we grafted fragments of the embryonic LGE taken from the forebrain of β -actin-GFP (normal) E14.5 embryos onto thick longitudinal brain slices, maintained in culture. The donor tissue was excised as near to the ventricle as possible, was placed at the base of the RMS of the host slices, and contained little or no CR+, CB+, or GABA+ neurons [confirmed by immunostaining (supplemental Fig. 1, available at www.jneurosci.org as supplemental material)]. The host slices were set up either from WT or from *Dlx5*^{−/−} neonatal brains, maintained for 7 d after the graft, and examined for the number GFP+/GAD67+ cells (an example is shown in Fig. 1*B*). The cumulative result of five independent experiments indicate that, while the total number of GFP+ cells in the OB did not significantly differ, the number of GFP+/GAD67+ over the total number of GFP+ cells was significantly reduced in the *Dlx5*^{−/−} OB, compared to the WT OB (Fig. 1*C*). This result suggests that the *Dlx5*^{−/−} OB constitutes an environment unfavorable for GABA+ differentiation.

Wnt5a is downregulated in neurospheres from *Dlx5* mutant brain

Comparing expression profiles of neurospheres derived from WT and *Dlx5*^{−/−} embryonic brains [G.R.M., M.P., G.C., and P. Provero (University of Torino), unpublished work, supplemental Tables IV, V, available at www.jneurosci.org as supplemental material, and Gene Ontology files], *Wnt5a* was found to be downregulated (twofold reduction) in the *Dlx5* null cells, and upregulated (threefold increase) in *Dlx5* null cells re-expressing *Dlx5*. Next, using an algorithm that surveys large sets of expression profiles (Pellegri et al., 2004), *Wnt5a* ranked in the first 1% of genes coregulated with *Dlx5* (data not shown). These data represented the starting point of our search to evaluate whether *Wnt5a* could be a putative transcriptional target of *Dlx* proteins in NPCs.

Wnt5a expression in normal and *Dlx5* mutant brains

We examined the expression and localization of *Wnt5a*, *Wnt5b*, *Dlx2*, and *Dlx5*, by ISH on sections of murine embryonic (E14.5) and neonatal OB, and compared the expression in normal versus *Dlx5*^{−/−}

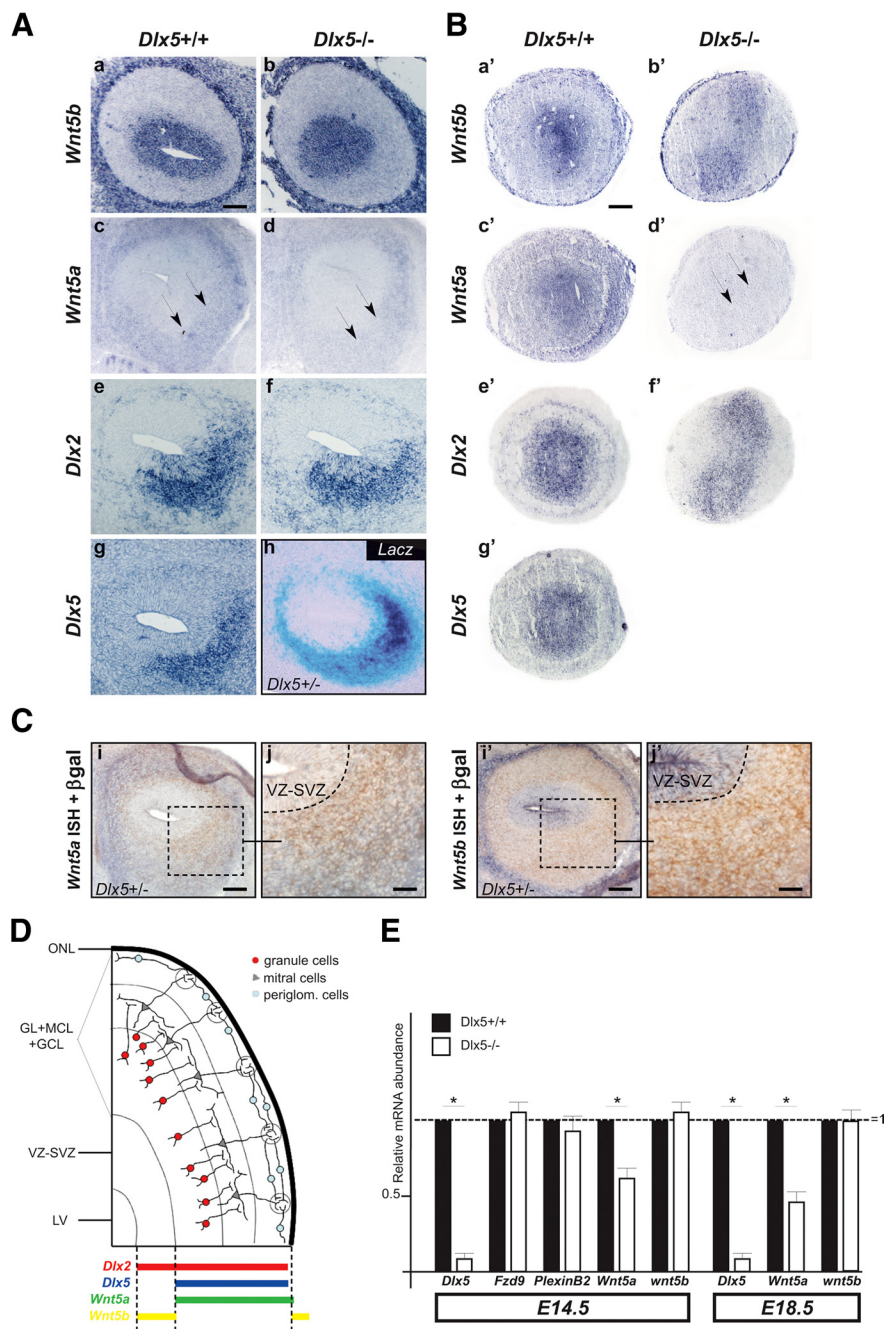


Figure 2. Expression of *Wnt5a* and *Wnt5b* in normal and *Dlx5*^{−/−} OB. ***A, B***, ISH on coronal sections of E14.5 (***A***) and neonatal (***B***) OB of WT and *Dlx5*^{−/−} specimens (genotypes are reported on top). Expression of *Wnt5b* (***a, b, a', b'***), *Wnt5a* (***c, d, c', d'***), *Dlx2* (***e, f, e', f'***), *Dlx5* (***g, g'***), and *Dlx5-lacZ* (X-gal staining) (***h***) is shown. In the WT embryonic OB, *Wnt5b* is expressed in VZ–SVZ region; conversely, *Wnt5a* is not expressed in this region and instead is expressed in a region overlapping with both *Dlx2* and *Dlx5* in the ventral–medial OB. In the neonatal brain (left), coexpression of *Wnt5a*, *Dlx2*, and *Dlx5* is observed in the differentiated layers of the OB. The expression of *Wnt5a* is reduced in the *Dlx5*^{−/−} specimens at both ages (black arrows). ***C***, ISH for *Wnt5a* (left) or *Wnt5b* (right) on coronal sections of E14.5 OB from *Dlx5-lacZ*^{+/−} embryos (blue signal), followed by immunostaining for β -gal (brown signal). Lower (***i, i'***) and higher (***j, j'***) magnification micrographs are shown. ***D***, Diagram illustrating the overlapping expression of *Wnt5a* (green bar), *Wnt5b* (yellow bar), *Dlx2* (red bar), and *Dlx5* (blue bar) in the embryonic OB, and their position relative to the VZ–SVZ. The layers and neurons indicated in the scheme refer to the situation at P0. GCL, Granule cell layer; GL, glomerular layer; LV, lateral ventricle; MCL, mitral/tufted cell layer; ONL, olfactory nerve layer. ***E***, Real-time qPCR analyses on RNA extracted from WT and *Dlx5*^{−/−} OBs at E14.5 (left) and at birth (right). Reduced expression of *Wnt5a*, but not *Wnt5b*, is observed in both cases. Samples are from pools of tissues (at least 3). Error bars represent the mean $\pm$ SD of two independent experiments. Differences were statistically significant as measured by a two-tailed Student's *t* test (**p* < 0.001). Scale bars: 400 μ m (***a–h, i, i'***), 200 μ m (***a'–g'***), 100 μ m (***j, j'***).

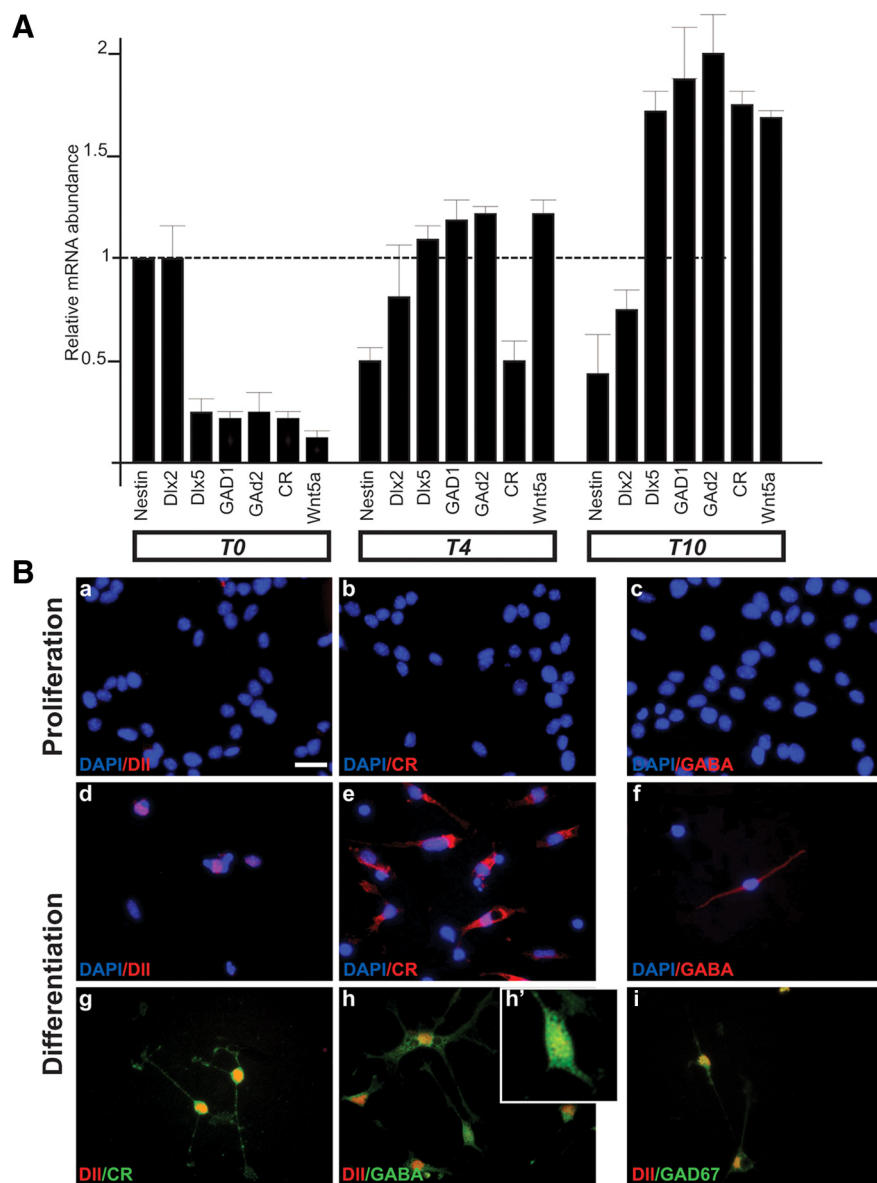


Figure 3. Expression of *Dlx*, *Wnt5a*, and differentiation markers in NS cells. **A**, Real-time qPCR analysis for the expression of *Nestin*, *Dlx2*, *Dlx5*, *GAD1*, *GAD2*, *CR*, and *Wnt5a* mRNA in NS cells, at two time points (T4 and T10, indicating 4 and 10 d *in vitro*) following the application of a differentiation stimulus (see Materials and Methods). T0 corresponds to proliferating cells. *Dlx2* is expressed in proliferating cells, while *Dlx5* is upregulated during differentiation, followed by *GAD-1*, *GAD-2*, *CR*, and *Wnt5a*. One “referee” sample was introduced in every experiment, and used for normalization. Error bars represent the mean $\pm$ SD of three independent experiments. Differences were statistically significant as measured by a two-tailed Student’s *t* test ($p < 0.05$). **B**, Top row, Immunostaining on proliferating NS cells (maintained in EGF + FGF2) to show absence of pan-Dlx staining (**a**), CR (**b**), and GABA (**c**). Middle row, Immunostaining on differentiating cells (10 DIV) showing expression of pan-Dlx proteins (**d**), CR (**e**), and GABA (**f**). Bottom row, Double immunostaining to confirm the coexpression of Dlx proteins (nuclear staining) with CR (**g**), GABA (**h**), and GAD67 (**i**). Few GAD67+ /Dlx− cells were observed in these cultures (**h'**). Scale bar (in **a**), 50 μ m.

specimens. In the embryonic OB, *Wnt5b* is expressed in the periventricular [ventricular zone (VZ)–subventricular zone (SVZ)] region, where neurogenesis occurs; conversely, *Wnt5a* is not present in this region and instead is expressed in the differentiating anlage (future granule, mitral, and glomerular layers). In the ventral–medial OB *Dlx2*, but not *Dlx5*, is expressed in the VZ–SVZ region, and overlaps with *Wnt5b*. *Dlx2* and *Dlx5* are both expressed in the differentiating anlage of the OB, overlapping with *Wnt5a* (Fig. 2A). More posteriorly, *Wnt5b* expression is detected in the VZ–SVZ of the GE of the embryonic subpallium, while *Wnt5a* is nearly undetectable in this region (supple-

mental Fig. 3, available at www.jneurosci.org as supplemental material). In the neonatal OB, we observed a similar colocalization of *Wnt5a*, *Dlx2*, and *Dlx5* expression in the differentiated layers (Fig. 2B), while expression of *Wnt5b* was mainly restricted to the VZ–SVZ.

We next examined coexpression of *Dlx2*, *Dlx5*, *Wnt5a*, and *Wnt5b* with greater details, by carrying out ISH on sections of *Dlx5-lacZ*+/− (heterozygous, phenotypically normal) embryonic brains, followed by immunohistochemical detection of the *Dlx5*-expressing cells with anti- β -gal antibody. *Dlx5* and *Wnt5a* were found to be coexpressed in the differentiating region of embryonic OB, but neither one was found to be expressed in the VZ–SVZ (neurogenic) region. On the contrary, expression of *Wnt5b* was detected in the VZ–SVZ region but not in the differentiating region (Fig. 2C). All these results, summarized in the scheme in Figure 2D, are fully compatible with the possibility that *Dlx2* and *Dlx5* may regulate *Wnt5a* expression in differentiating cells of the OB.

Next, we compared expression of *Dlx2*, *Wnt5a*, and *Wnt5b* on sections of WT and *Dlx5*−/− OB from E14.5 and neonatal brains. In the *Dlx5* null OB, the nerve layer is absent and cellular layering is disorganized (evident in Fig. 2Bb',Bf') (Levi et al., 2003). The expression of *Wnt5b* and *Dlx2* did not change in the absence of *Dlx5*, while the expression of *Wnt5a* was visibly reduced in the neonatal OB (Fig. 2B). Reduced *Wnt5a* expression was also observed in the embryonic OB, although less evident (Fig. 2A). Considering that ISH is not quantitative, we performed real-time qPCR to quantify expression of *Wnt5a* on RNA extracted from WT or *Dlx5*−/− OB, at E14.5 and at birth. *Wnt5a* expression was significantly reduced in the *Dlx5*−/− OB at both ages (−30% and −55%, respectively) (Fig. 2E), while expression of *Wnt5b* did not significantly change. Expression of *PlexinB2* and *Fzd9*, two genes found to be differentially expressed in the expression profiles from neurospheres, did not change in the *Dlx5*−/− samples.

The role of *Dlx5* and *Wnt5a* in neural stem cells

Neurospheres are not an adequate model to address the role of *Dlx5* and *Wnt5a* during NPC differentiation because they are heterogeneous and differentiate more efficiently in astrocytes. Therefore we switched to adherent NS cells: a stable, homogeneous, symmetrically dividing multipotent NPC population, prone to GABA+ differentiation (Conti et al., 2005; Pollard et al., 2006; Spiliotopoulos et al., 2009). Originally obtained from embryonic stem (ES) cells, these cells can be directly derived from

embryonic and neonatal brains, with indistinguishable properties (Pollard et al., 2006; Fig. 3; supplemental Fig. 2, available at www.jneurosci.org as supplemental material). First we used murine ES-derived NS cells to determine whether this cell model is adequate to examine the *Dlx5*-*Wnt5a* regulation and the function of *Wnt5a* for interneuron differentiation. We measured by real-time qPCR the mRNA abundance of *Nestin*, *Dlx2*, *Dlx5*, *GAD-1*, *GAD-2*, and *CR* at different time points following a differentiation stimulus. *Dlx2* is expressed in proliferating cells, while *Dlx5* is upregulated during differentiation, followed by *GAD-1*, *GAD-2*, and *CR*. In the same assay, we examined the expression of *Wnt5a*, observing that also *Wnt5a* is upregulated during differentiation, with kinetics similar to that of *GAD1*, *GAD2*, and *CR* (Fig. 3A). Next, we performed immunocytochemical detection of *Dlx* (anti *Dll*, pan-*Dlx* antibody), GABA, GAD67 (the protein coded by *GAD-1*), and *CR* on differentiating cells. We observed that *Dlx* is coexpressed with GABA, GAD67, and *CR* (Fig. 3B). Note that few GAD67+/dll- cells were observed in these cultures (<5% of all GAD+ cells) (Fig. 3B). These results indicate that the NS cells are an appropriate model in which to examine the role of *Dlx*-*Wnt* regulation for the initial steps of GABAergic differentiation.

We next derived NS lines from the subpallium (LGE+MGE) of E12.5 WT, *Dlx5* null, or *Dlx5*/*Dlx6* double-null embryos (Merlo et al., 2002a), following described procedures (Conti et al., 2005; Pollard et al., 2006). This was done to confirm that the observed properties are not due to ES-NS conversion, and to have an *in vitro* model in which to study the role of *Dlx5*. All the obtained NS cell lines appeared very similar to the ES-derived NS in terms of immunochemical properties: in proliferating conditions, they express Sox2, BLBP, Nestin, and Olig2, and lack expression of β III-tubulin, GFAP, GAD67, and GABA. Under differentiation conditions, the WT cells readily express β III-tubulin, GABA, and GAD67 (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) and *CR* (data not shown), and display dynamic changes of *GAD1*, *GAD2*, and *CR* mRNA abundance similar to that of ES-derived NS cells (data not shown). *Dlx5* null cells after few passages showed only minor differences in their GABA+ differentiation efficiency, compared to WT. Instead, the *Dlx5*/*Dlx6* mutant NS cells, after 15 and 20 d of culture in differentiation conditions showed, respectively, a 55% and a 70% reduction of GAD67+ neurons (Fig. 4A). Under the same conditions, expression of *Wnt5a*, but not *Wnt5b*, mRNA was reduced by 45% in *Dlx* mutant NS cells compared to WT ones.

To show that *Dlx*-dependent non-cell-autonomous regulations take place *in vitro* and influence cell differentiation, we set up a coculture experiment in which a minority (20%) of normal

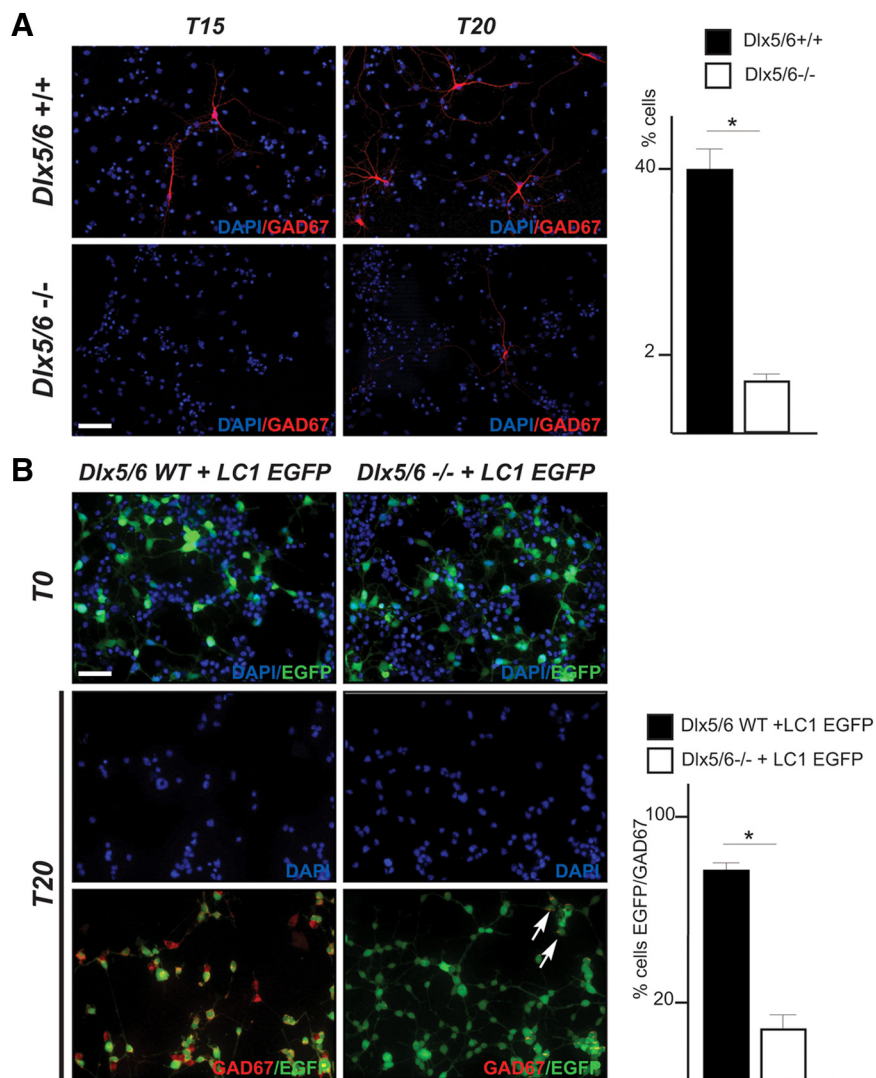


Figure 4. GABA+ differentiation of normal and *Dlx* mutant NS cells reveals non-cell-autonomous regulations. **A**, GAD67 expression of NS cells derived from WT (top row) or *Dlx5*/*Dlx6* null (bottom row) embryonic brains, following 15 or 20 d in differentiating medium. The histogram on the left reports the number of GAD67+ cells over the total number of nuclei, as a percentage. **B**, Coculture experiments in which 20% of EGFP-labeled LC1 (normal) NS cells were mixed with 80% of either WT or *Dlx5*/*Dlx6* mutant NS cells (top row); after 20 d in differentiating medium, cells are immunostained for GAD67 (bottom row). Only double GFP+/GAD67+ cells were counted and expressed as a percentage of the total number of GFP+ cells (histogram on the right). Scale bar, 5 μ m.

EGFP-expressing NS cells (denominated LC1-EGFP) were used as “sensors” to monitor the environment provided by the presence of either WT or *Dlx5*/*Dlx6* mutant NS cells, representing the remaining 80% (majority) of the initial population (as shown in Fig. 4B, top). After 20 d in differentiation conditions, many fewer EGFP/GAD67 double-positive cells were observed in the *Dlx* mutant cell environment, compared to the WT cell environment (~70%) (Fig. 4B). In the same experiment, the *Dlx5*/*Dlx6* mutant (EGFP-) cells yielded fewer GAD67+ cells, as expected.

DLX2 and DLX5 transcriptionally regulate *Wnt5a* expression

The *Wnt5a* locus contains several predicted homeobox binding sites, conserved in the mammalian and in the chicken genomes, some of which are also predicted with a *Dlx*-specific Position Weight Matrix [G.R.M. and P. Provero (University of Torino), unpublished work; supplemental Fig. 4, available at www.jneurosci.org as supplemental material]. Furthermore, *Wnt5a* has been shown experimentally to be a downstream target for the

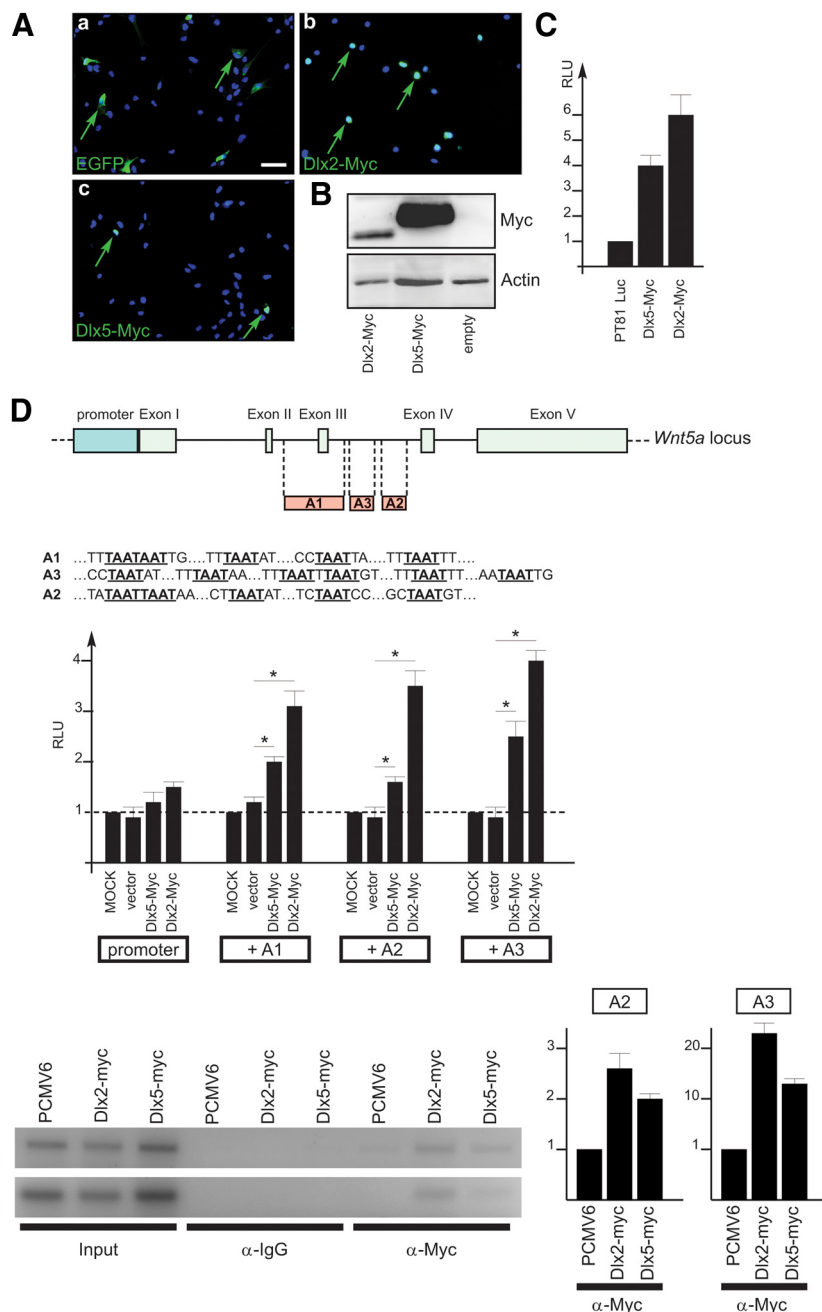


Figure 5. Dlx proteins regulate *Wnt5a* transcription and bind to the *Wnt5a* locus in native chromatin. **A**, NS cells nucleofected with EGFP (**a**), *Dlx2-mycTAG* (**b**), or *Dlx5-mycTAG* (**c**) expression vector, stained with anti-mycTAG antibody (green arrows). Scale bar (in **a**), 100 μ m. **B**, Western blot analysis on total proteins from NS cells nucleofected with *Dlx2-mycTAG*, *Dlx5-mycTAG*, or the empty vector (mock), revealed with anti-mycTAG antibody. **C**, Luc-reporter transcription activity of Dlx2- and Dlx5-mycTAG proteins on the Dlx-responsive element of *Arx*. Experiments were done by nucleofecting NS cells. Values are expressed as relative Luc activity (RLU), normalized against mock nucleofection and *Renilla* expression. Mean values of three experiments are shown. **D**, Top, Diagram of the *Wnt5a* locus to report the position and sequence of the putative homeobox binding sites in the A1, A2, and A3 fragments. Bottom, Luc transactivation activity of Dlx2- and Dlx5-mycTAG proteins on the A1, A2, and A3 fragments of the *Wnt5a* locus, inserted in combination with the *Wnt5a* promoter. Error bars represent the mean $\pm$ SD of three independent experiments. Differences were statistically significant as measured by a two-tailed Student's *t* test (**p* < 0.05). **E**, ChIP analysis on chromatin from the ST14A cells transfected with the Dlx2- and Dlx5-mycTAG vectors, precipitated with anti-mycTAG antibody, followed by PCR analysis with primers for fragments in the A2 and A3 regions of the *Wnt5a* locus. As negative control, cells were transfected with the empty vector, and the ChIP was performed with anti-IgG alone. As input, unprecipitated chromatin was used for PCR amplification. The signals were quantified by digital densitometry using the Quantity One software (version 4.5.2). Enrichment is observed upon transfection with both Dlx2-myc and Dlx5-myc with both primers used. Quantification is reported on the histograms on the right; values were normalized against empty vector. Error bars represent the mean $\pm$ SD of two independent experiments.

Msx homeoproteins (Iler and Abate-Shen, 1996), although *Msx* genes are not expressed in the subpallium. *Dlx* proteins are closely related to the *Msx* proteins; therefore, we raised the hypothesis that in the OB *Wnt5a* may be regulated by Dlx proteins, via these conserved homeobox sites. To address this, we constructed a series of luciferase-reporter vectors containing a 1100 kb fragment of the murine *Wnt5a* promoter, alone or in combination with one of three evolutionarily conserved fragments within a 3.4 kb intron of *Wnt5a*, named A1, A2, and A3, harboring several homeobox binding sequences (Fig. 5D). We transfected brain-derived NS cells with the reporter vectors indicated above and either *DLX5*- or *DLX2*-mycTAG expression vectors (Fig. 5A–C). These vectors were previously tested for protein expression and for transcriptional activity on the UAS3 *Arx* enhancer-luc vector (Cobos et al., 2005; Colasante et al., 2008), as a control. The results indicate that in brain-derived NS cells, both *DLX2* and *DLX5* act on *Wnt5a* expression through these three fragments. Exogenous expression of *DLX5* resulted in a 2-, 1.5-, and 2.5-fold increase in reporter transcription in the presence of the A1, A2, and A3 fragments, respectively, while expression of *DLX2* induced a 3-, 3.5-, and 4-fold increase on the same fragments (Fig. 5D).

We next performed ChIP analysis, to determine whether Dlx proteins directly associate with the Dlx-response elements in the *Wnt5a* locus. We used the ST14A cell line, consisting in immortalized embryonic subpallial neural precursors capable of migration and neuronal differentiation, and easily transfectable (Cattaneo and Conti, 1998). ST14A cells were transfected with *DLX2-mycTAG* or *DLX5-mycTAG*, followed by ChIP with anti-myc. Fragments A2 and A3 were precipitated by the anti-myc antibody after transfection with either expression vectors (Fig. 5E). In the presence of *DLX2* the fragments A2 and A3 were detected, respectively, with a 2.5- and a 2-fold increase, while in the presence of *DLX5* the same fragments were detected with a 22- and a 12-fold increase, compared to empty vector. These results indicate that *DLX5* and *DLX2* are physically associated to homeobox binding sites present in the *Wnt5a* locus, in the chromatin of subpallial precursors.

Reduced JnK activation in the *Dlx5*^{−/−} OB

We then determined whether the loss of *Dlx5* and reduced *Wnt5a* expression in the

OB results in altered intracellular Wnt signaling. It is widely assumed that noncanonical ligands act independently of the β -catenin pathway and signal either via JnK phosphorylation or via Ca^{2+} release and activation of calmodulin kinase II (Zou, 2004; Lyu et al., 2008; Farias et al., 2009). We therefore compared normal and *Dlx5*^{−/−} OB specimens for possible reductions in JnK and β -catenin activity, as previously done (Shihabuddin et al., 2000; Miquelajauregui et al., 2007). At P0, the OB of the *Dlx5*^{−/−} mice showed a 50% decrease of the phosphorylated form of JnK1–3 when compared with normal samples, while the total level of JnK1–3 protein was unchanged (Fig. 6A). As further controls, we detected an increase of phospho-JnK1–3 in HeLa cells irradiated with UV (known to induce JnK phosphorylation), and we easily detected phospho-JnK1–3 in the hippocampus, a location where it is abundant. On the contrary, the level of dephosphorylated (active) β -catenin protein was unchanged between the WT and the *Dlx5*^{−/−} OB samples. We also examined the level of active β -catenin in normal NS cells treated with purified Wnt3a or Wnt5a. We observed an increase in active β -catenin upon treatment with Wnt3a, but not upon treatment with Wnt5a (Fig. 6B). These results indicate that the β -catenin pathway is unaffected by the absence of *Dlx5* and reduced *Wnt5a* expression.

To further support a link between *Wnt5a* expression and JnK phosphorylation, we examined the level of phospho-JnK in WT brain-derived NS cells treated with a conditioned medium prepared from cells transfected with either an empty vector or a *Wnt5a*-mycTAG expression vector. The level of phospho-JnK increased by 50% in cells transfected with the *Wnt5a*-mycTAG vector, relative to the control sample (Fig. 6C).

Wnt5a promotes interneuron differentiation in cultured OB slices

To test whether *Wnt5a* stimulates interneuron differentiation *in vivo*, we set up organotypic slice cultures of the FB of E16.5 embryos, obtained from either WT or *Dlx5*^{−/−} mice. The cultures were exposed to exogenous *Wnt5a* and examined for expression of the interneuron markers *GAD-1*, *GAD-2*, and *CR*, by real-time qPCR. *Wnt5a* was provided in two ways: in the first, we layered brain slices on top of a feeder layer of COS7 cells previously transfected with vectors expressing *Wnt5a*-, *Wnt4*-, and *Wnt7a*-mycTAG fusion proteins (Lyuksytova et al., 2003; Zaghetto et al., 2007). *Wnt5a*-, but not *Wnt4*- or *Wnt7a*-myc, resulted in significant increased expression of *GAD-1*, *GAD-2*, and *CR*, both in the normal and in the *Dlx5*^{−/−} OB (respectively, +30%, +70%, and +30% for the WT and +60%, +80%, and +40% for the mutant) (Fig. 7A). In the second strategy, we applied purified recombinant *Wnt5a* directly onto cultured *Dlx5*^{−/−} OBs; in the presence of (500 ng/ml) *Wnt5a*, *GAD-1*, *GAD-2*, and *CR* expression was increased (+50%, +60%, and +70%, respectively) as compared to control (PBS only) (Fig.

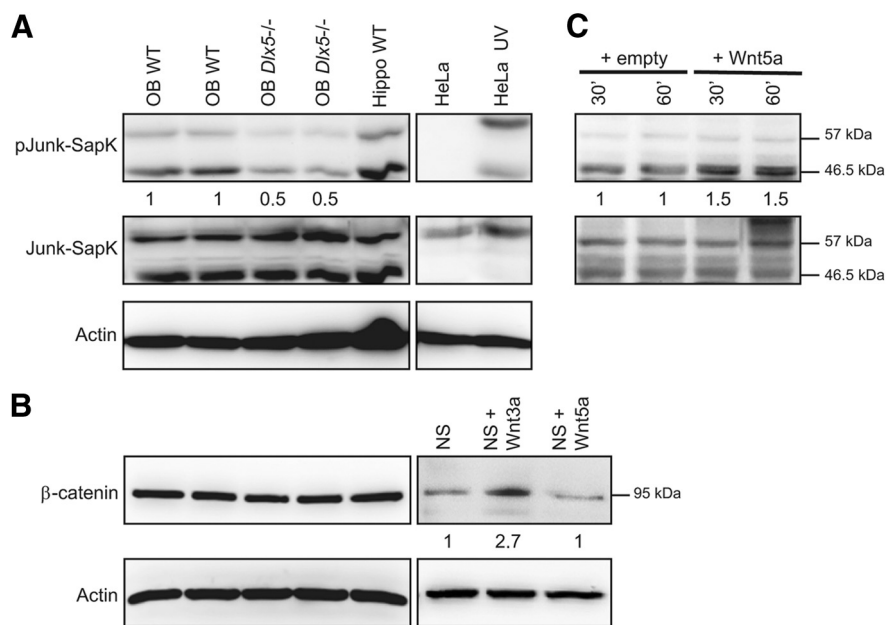


Figure 6. Reduced phospho-JnK in *Dlx5*^{−/−} OB. **A**, Western blot analysis of total proteins extracted from the OBs of two individual WT or *Dlx5*^{−/−} animals, and a normal hippocampus, to detect phospho-JnK1–3 (pJunk-SapK, top panel), or to detect all forms of JnK1–3 (Junk-SapK, middle panel) with a pan-JnK antibody. Actin was used as loading control (bottom panel). While the total level of JnK1–3 is unchanged, the level of pJunk-SapK is reduced ~50% in the *Dlx5*^{−/−} extracts. (Numbers below each lane indicate the densitometric ratios; the signals were quantified by the Quantity One 4.5.2 software.) As a further control, we verified the expected increase of pJnK1–3 in UV-irradiated HeLa cells. **B**, Western blot analysis to detect the level of active (dephosphorylated) β -catenin protein. No change is observed in the *Dlx5* mutant OBs compared to WT. Right, As a control, we verified the increase of active β -catenin in brain-derived NS cells exposed to recombinant Wnt3a, but not Wnt5a. **C**, Western blot analysis to detect the level phospho-JnK (top blot) following treatment (for 30 or 60 min) of brain-derived NS cells with a conditioned medium prepared from HeLa cells transfected with an empty vector or with a *Wnt5a*-mycTAG expression vector, normalized with the level of total JnK (middle blot).

7B). In a similar experimental setting, we examined whether *Wnt5a* may induce endogenous expression of *Dlx2* and *Dlx5*. Using cultures of brain slices from WT animals, we found that treatment with *Wnt5a* had no effect on *Dlx2* expression but resulted in a reduced (−35%) *Dlx5* expression (supplemental Fig. 5, available at www.jneurosci.org as supplemental material), suggesting the possibility that these molecules participate in a negative feedback loop.

Wnt5a promotes interneuron differentiation in primary cultures of dissociated OB cells

We compared the efficiency of β III-tubulin⁺, GABA⁺, and CR⁺ differentiation of primary cultures of OB cells obtained from WT or *Dlx5*^{−/−} E16.5 embryos, maintained in differentiating medium. Compared to WT cells under the same conditions, *Dlx5*^{−/−} cells consistently displayed a reduced number of GABAergic neurons (−40%) (Fig. 8A), while the number of β III-tubulin⁺ and CR⁺ neurons did not significantly change. Next, we cocultured dissociated cells from WT or *Dlx5*^{−/−} OB with HeLa cells previously transfected with *Wnt5a*-mycTAG expression vector. In the presence of *Wnt5a*-expressing cells, the number of GABA⁺ cells increased both in the WT (+50%) and in the *Dlx5*^{−/−} (+50%) cultures, compared to the same cultures exposed to mock-transfected cells (Fig. 8B). The number of CR⁺ cells both in the WT and in the *Dlx5*^{−/−} cultures did not significantly change. Thus, *Wnt5a* promotes GABA⁺ differentiation and rescues the lower differentiation efficiency observed in the absence of *Dlx5*^{−/−}.

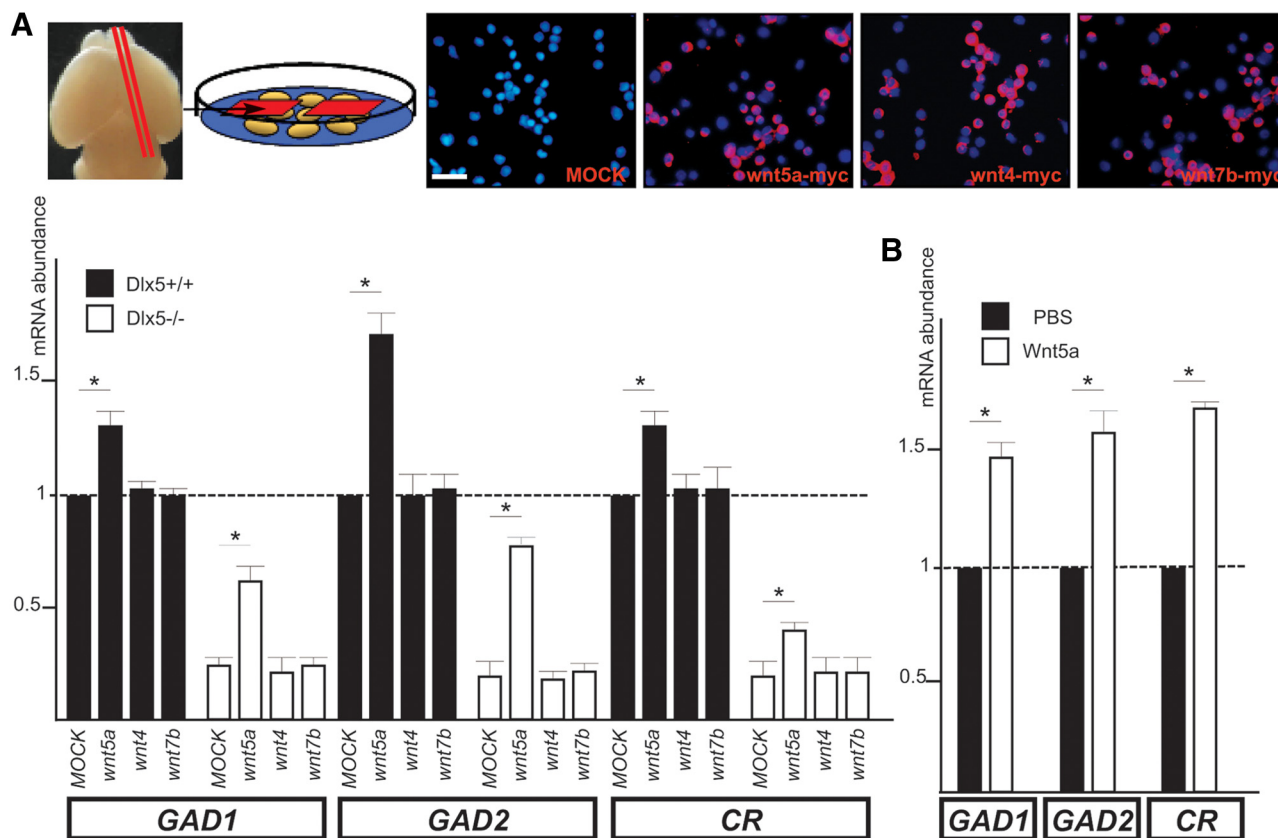


Figure 7. Wnt5a promotes expression of interneuron differentiation markers *in vitro*. **A**, Top panel, COS7 cells transfected with Wnt5a-, Wnt4-, and Wnt7b-mycTAG expression vectors, immunostained with anti-mycTAG antibody, and used as a feeder layer for organotypic cultures. Thick longitudinal sections from the FB of WT and *Dlx5*^{-/-} embryos, at the age of E16.5, were cultured on top of transfected COS7 cells. Bottom panel, Real-time analysis for the expression of the IN differentiation markers *GAD1*, *GAD2*, and *CR* in the cultured OBs comparing WT (black bars) and *Dlx5*^{-/-} (open bars). In the presence of Wnt5a, but not Wnt4 or Wnt7b, expression of *GAD1*, *GAD2*, and *CR* increased both in the WT and in the *Dlx5*^{-/-} samples. Values are normalized against mock/WT. **B**, Sagittal slices from *Dlx5*^{-/-} E16.5 FB, maintained in organotypic culture and exposed to purified Wnt5a protein (PBS is used as negative control), followed by real-time analysis for the expression of the IN differentiation markers *GAD1*, *GAD2*, and *CR* in the OB. Wnt5a resulted in increased expression of *GAD1*, *GAD2*, and *CR*. Values are normalized against PBS. Error bars represent the mean $\pm$ SD of four independent experiments. Asterisks indicate $p < 0.05$. Scale bar, 100 μ m.

Discussion

In the developing forebrain, environmental cues and transcription factor-based cell-autonomous regulations cooperate to enable interneuron precursors to undertake region- and time-specific programs of migration and differentiation (Morrison, 2001; Allen et al., 2007; Balordi and Fishell, 2007; Batista-Brito et al., 2008). How these modes of regulation result in the generation of a fixed number and variety of neurochemically defined subtypes is far from being understood. Published works have so far emphasized the role of cell-autonomous regulation, and in particular the *Dlx* homeoproteins have been shown to play a key role in the control of migration and differentiation of subpallial interneuron precursors (Panganiban and Rubenstein, 2002; Stenman et al., 2003; Kohwi et al., 2005; Waclaw et al., 2006; Flames et al., 2007; Kohwi et al., 2007). On the other side, the identity and activity of signaling molecules and environmental cues participating in these processes are seldom examined and remain underestimated, as these cues cannot be easily reproduced or manipulated *in vitro*. Here we uncover a transcriptional link between the *Dlx* activity and the differentiation-promoting function of Wnt5a, *in vitro* and *in vivo*, establishing that the *Dlx*-dependent control over interneuron differentiation entails non-cell-autonomous functions. In addition we show that the noncanonical ligand Wnt5a contributes to GABAergic differentiation. A model of the interplay between *Dlx* genes and Wnt signaling is proposed in Figure 9.

Dlx genes are essential to orchestrate interneuron migration and differentiation in the subpallium, acting at distinct time points and cell stages. While *Dlx1*^{-/-} and *Dlx2*^{-/-} mice show only minor deficiencies in GABA⁺ neuron formation, *Dlx5*^{-/-} mice show a more severe interneuron deficit in the OB, and *Dlx1*; *Dlx2* double-null mice show extensive defects in their migration and differentiation (Anderson et al., 1997; Levi et al., 2003; Long et al., 2003; Perera et al., 2004; Cobos et al., 2005). The observation that forced expression of *Dlx2* or *Dlx5* in embryonic cortical NPCs induces the expression of *GAD* genes (Stühmer et al., 2002) has justified the proposition that *Dlx* genes cell-autonomously confer to NPCs the ability to differentiate toward the GABAergic phenotype. However, other observations challenge this simplistic view. First, the role of *Dlx5* is not strictly specific for GABA⁺ phenotype, as in the *Dlx5*^{-/-} OB both CR⁺ and GABA⁺ interneurons are affected (Levi et al., 2003; Long et al., 2003), and these markers are known to identify two widely distinct populations (5% double labeling in late embryos, 10% postnatally) (Kosaka et al., 1995, 1997; Kosaka and Kosaka, 2005; Parrish-Aungst et al., 2007). Second, published results (Long et al., 2007) and our (unpublished) data indicate that *Dlx* genes are dispensable for GABA⁺ and CR⁺ differentiation in the OB, as few differentiated interneurons can be detected that lack expression of *Dlx* gene (*dll*⁻). Third, GABA⁺ differentiation takes place in *Dlx*-negative territories of the CNS (Leto et al., 2009). Fourth, we show here that the mutant environment of the *Dlx5* null OB is

unfavorable to GABAergic differentiation. Therefore, our key finding that *Dlx* genes work (at least in part) via regulation of *Wnt5a*, hence non-cell-autonomously, is very compatible with previous observations.

Wnt ligands participate in non-cell-autonomous regulations and influence nearly all aspects of neurogenesis, from NPC proliferation to neuronal differentiation and maturation (Ille and Sommer, 2005). Non-cell-autonomous signals are typically region- and time/age-specific, and are best examined in experimental systems that closely resemble the brain tissue in which endogenous NPCs are found. For this reason, we have adopted three distinct assays to show the GABA-promoting function of the *Dlx*-*Wnt5a* regulation and obtained equivalent results: (1) organotypic slice cultures of late embryonic FB; (2) primary cultures of dissociated OB cells at late embryonic ages, and (3) adherent NS cells derived from WT or *Dlx5*^{−/−} embryonic brains, capable of GABA⁺ differentiation *in vitro*.

It is widely accepted that “canonical” β -catenin-dependent Wnt signaling (typically initiated by Wnt1, Wnt3, and Wnt7) promotes the expansion of neural progenitors, and in particular contributes to maintain subpallial NPC proliferation without grossly altering the fate of differentiating interneurons (Gulacsi and Anderson, 2008). Conversely, a role of “noncanonical” β -catenin-independent Wnt signaling (typically initiated by Wnt5a and Wnt11) to promote neuronal differentiation is increasingly being recognized. Treatment with Wnt5a induces neuronal differentiation of ES cells and of telencephalic NPCs (Muroyama et al., 2004; Israsena et al., 2004; Otero et al., 2004; Yu et al., 2006; Lyu et al., 2008), promotes differentiation of mesencephalic DA neurons in a β -catenin-independent fashion (Schulte et al., 2005; Castelo-Brando and Arenas, 2006; Andersson et al., 2008), and promotes olfactory GABAergic differentiation (our data). Thus, noncanonical Wnt signaling participates in neuronal differentiation, an issue to be further investigated on other neuronal cell types.

However, a general rule cannot be drawn as yet. In fact, and surprisingly, the activation of the β -catenin pathway can induce neuronal differentiation of cortical NPCs derived from late developmental ages (Hirabayashi et al., 2004; Hirabayashi and Gotoh, 2005). To explain this apparent paradox, it has been proposed that the cellular response to a Wnt signal relies to a limited extent on the identity of the ligand and to a larger extent on the cell type, developmental stage, receptor context/availability, and the presence of concomitant signals (Viti et al., 2003; Israsena et al., 2004; Kléber and Sommer, 2004; Mikels and Nusse, 2006; Li et al., 2009). A logical consequence of this notion is that Wnt function/signaling should be worked out in each specific cell type. We show here that during GABA⁺ differentiation of subpallial NPC, Wnt5a activates a phospho-JnK-dependent pathway, while the β -catenin pathway is not (directly) involved. Further experimental work is needed to finely dissect the Wnt5a-JnK pathway in

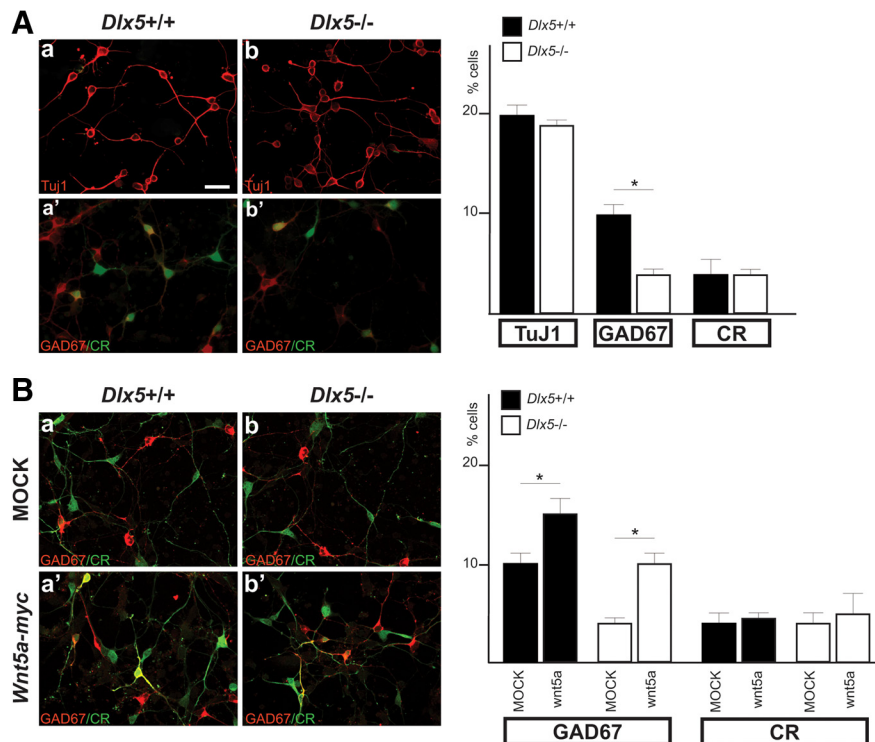


Figure 8. Wnt5a promotes GABA⁺ differentiation of cultured OB cells. **A**, Left, Staining for β III-tubulin (TuJ1) (**a**, **b**) and double staining for GAD67 (red fluorescence) and CR (green) (**a'**, **b'**) of primary cultures of OB cells obtained from WT or *Dlx5*^{−/−} E16.5 embryos, after 48 h in differentiating medium. Right, Counting of the number of GAD67⁺ and CR⁺ neurons, indicated as a percentage of the number of nuclei counted (DAPI staining). Compared to the WT cultures, *Dlx5*^{−/−} ones yielded a reduced number of GAD67⁺ cells (−40%), while the number of β III-tubulin⁺ and CR⁺ cells did not significantly change. **B**, Left, Double staining for GAD67 (red) and CR (green) of primary cultures of OB cells obtained from WT (**a**, **a'**) or *Dlx5*^{−/−} (**b**, **b'**) embryos, cocultured with HeLa cells transfected with Wnt5a-mycTAG expression vector. Right, Counting of the number of GAD67⁺ and CR⁺ neurons after 48 h of differentiating culture, indicated as a percentage of the number of nuclei counted. Compared to mock-transfected cells, Wnt5a-expressing cells yielded an increased number of GAD67⁺ cells both in the WT (+50%) and in the *Dlx5*^{−/−} (+50%) cultures. The number of CR⁺ cells both in the WT and in the *Dlx5*^{−/−} cultures did not change. Error bars represent the mean $\pm$ SD of four independent experiments. Asterisks indicate $p < 0.05$. Scale bar (in **a**), 50 μ m.

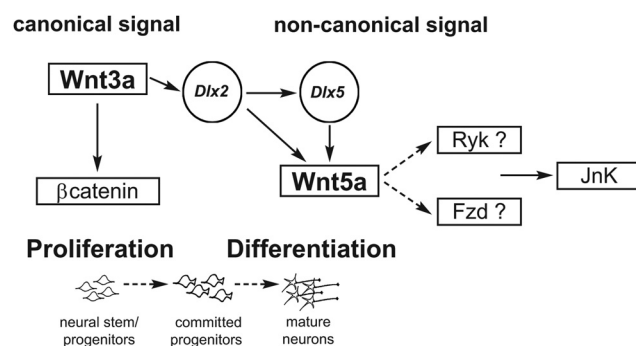


Figure 9. Model of regulation of GABA⁺ neuron differentiation in the olfactory bulb by *Dlx* and *Wnt5a*. The activity of canonical (represented by Wnt3a) and noncanonical (*Wnt5a*) Wnt signaling is regionally and functionally separated (indicated on the top). Transcriptional regulations are represented by solid arrows, and ligand–receptor interactions or other non-cell-autonomous regulations are represented by dashed arrows. *Dlx2* is generally considered transcriptionally upstream of *Dlx5* and *Dlx6*, but our data suggest that it may also regulate transcription of *Wnt5a*.

NPCs. Conversely, we show that Wnt3a activates the β -catenin pathway, a finding that together with the results reported by Gulacsi and Anderson (2008) clearly points to a role of β -catenin transduction to promote proliferation of NPC in the basal forebrain and OB, as depicted in Figure 9.

The identity and activity of Wnt5a receptor(s) has been characterized only in part. Wnt5a binds to ryk and ror2 single-membrane pass receptors as well as to the Frizzled receptors (Bovolenta et al., 2006; Keeble et al., 2006; Mikels and Nusse, 2006; Mikels et al., 2009). Ryk is a *bona fide* Wnt5a receptor both in *Drosophila* (Wouda et al., 2008) and in mammalian immature neurons (Miyashita et al., 2009) and *ryk* mRNA is expressed in the OB and GE (Kamitori et al., 1999), as well as in cultured NS cells (our unpublished data); thus, a role of ryk for interneuron differentiation is plausible but yet to be demonstrated.

Intracellularly, Wnt5a can use a JnK-dependent pathway (Wang et al., 2001; Yamanaka et al., 2002); consistently, we show here that reduced *Wnt5a* expression in the *Dlx5*^{−/−} OB is associated with reduced JnK phosphorylation. We therefore propose that in NS cells Wnt5a operates via JnK phosphorylation. A novel pathway has been recently uncovered, which entails cleavage and nuclear translocation of the ryk C-terminal domain (Lyu et al., 2008). However, in this study Wnt5a was not tested, and therefore the role of ryk to transduce the differentiation-promoting activity of Wnt5a in NPC remains to be defined. We expect that more than one receptor and/or pathway could transduce Wnt5a signaling and that distinct cellular responses can be elicited depending on the cell context and the presence of concomitant signals.

In conclusion, our results strongly support a direct Wnt5a role in GABAergic differentiation and demonstrate that Wnt5a expression is directly regulated by the *Dlx5* transcription factor. However, our data cannot take activity in consideration, while Wnt signaling has been shown to be involved in activity-dependent synapse plasticity and network structure in the hippocampus (Gogolla et al., 2009). Since *Dlx5* null mice have a denervated OB (Levi et al., 2003; Long et al., 2003), we cannot exclude a similar role of Wnt5a *in vivo*.

References

- Acampora D, Merlo GR, Paleari L, Zerega B, Postiglione MP, Mantero S, Bober E, Barbieri O, Simeone A, Levi G (1999) Craniofacial, vestibular and bone defects in mice lacking the Distal-less-related gene *Dlx5*. *Development* 126:3795–3809.
- Adachi K, Mirzadeh Z, Sakaguchi M, Yamashita T, Nikolcheva T, Gotoh Y, Peltz G, Gong L, Kawase T, Alvarez-Buylla A, Okano H, Sawamoto K (2007) Beta-catenin signaling promotes proliferation of progenitor cells in the adult mouse subventricular zone. *Stem Cells* 25:2827–2836.
- Allen ZJ 2nd, Wacław RR, Colbert MC, Campbell K (2007) Molecular identity of olfactory bulb interneurons: transcriptional codes of periglomerular neuron subtypes. *J Mol Histol* 38:517–525.
- Anderson SA, Qiu M, Bulfone A, Eisenstat DD, Meneses J, Pedersen R, Rubenstein JL (1997) Mutations of the homeobox genes *Dlx-1* and *Dlx-2* disrupt the striatal subventricular zone and differentiation of late born striatal neurons. *Neuron* 19:27–37.
- Andersson ER, Prakash N, Cajanek L, Minina E, Bryja V, Bryjova L, Yamaguchi TP, Hall AC, Wurst W, Arenas E (2008) Wnt5a regulates ventral midbrain morphogenesis and the development of A9–A10 dopaminergic cells *in vivo*. *PLoS One* 3:e3517.
- Balordi F, Fishell G (2007) Hedgehog signaling in the subventricular zone is required for both the maintenance of stem cells and the migration of newborn neurons. *J Neurosci* 27:5936–5947.
- Barker N (2008) The canonical Wnt/beta-catenin signalling pathway. *Methods Mol Biol* 468:5–15.
- Batista-Brito R, Close J, Machold R, Fishell G (2008) The distinct temporal origins of olfactory bulb interneuron subtypes. *J Neurosci* 28:3966–3975.
- Bovolenta P, Rodriguez J, Esteve P (2006) Frizzled/Ryk mediated signalling in axon guidance. *Development* 133:4399–4408.
- Castelo-Branco G, Arenas E (2006) Function of Wnts in dopaminergic neuron development. *Neurodegener Dis* 3:5–11.
- Cattaneo E, Conti L (1998) Generation and characterization of embryonic striatal conditionally immortalized ST14A cells. *J Neurosci Res* 53:223–234.
- Chenn A, Walsh CA (2003) Increased neuronal production, enlarged forebrains and cytoarchitectural distortions in beta-catenin overexpressing transgenic mice. *Cereb Cortex* 13:599–606.
- Ciani L, Salinas PC (2005) WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. *Nat Rev Neurosci* 6:351–362.
- Cobos I, Calcagnotto ME, Vilaythong AJ, Thwin MT, Noebels JL, Baraban SC, Rubenstein JL (2005) Mice lacking *Dlx1* show subtype-specific loss of interneurons, reduced inhibition and epilepsy. *Nat Neurosci* 8:1059–1068.
- Colasante G, Collombat P, Raimondi V, Bonanomi D, Ferrai C, Maira M, Yoshikawa K, Mansouri A, Valtorta F, Rubenstein JL, Broccoli V (2008) *Arx* is a direct target of *Dlx2* and thereby contributes to the tangential migration of GABAergic interneurons. *J Neurosci* 28:10674–10686.
- Conti L, Pollard SM, Gorba T, Reitano E, Toselli M, Biella G, Sun Y, Sanzone S, Ying QL, Cattaneo E, Smith A (2005) Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS Biol* 3:e283.
- Doe CQ (2008) Neural stem cells: balancing self-renewal with differentiation. *Development* 135:1575–1587.
- Eisenmann DM (2005) Wnt signaling. In: *WormBook* (The C. elegans Research Community, ed). doi:10.1895/wormbook.1.7.1.
- Fariás GG, Alfaro IE, Cerpa W, Grabowski CP, Godoy JA, Bonansco C, Inestrosa NC (2009) Wnt-5a/JNK signaling promotes the clustering of PSD-95 in hippocampal neurons. *J Biol Chem* 284:15857–15866.
- Flames N, Pla R, Gelman DM, Rubenstein JL, Puelles L, Marín O (2007) Delineation of multiple subpallial progenitor domains by the combinatorial expression of transcriptional codes. *J Neurosci* 27:9682–9695.
- Fogarty M, Grist M, Gelman D, Marín O, Pachnis V, Kessaris N (2007) Spatial genetic patterning of the embryonic neuroepithelium generates GABAergic interneuron diversity in the adult cortex. *J Neurosci* 27:10935–10946.
- Fukuda S, Taga T (2005) Cell fate determination regulated by a transcriptional signal network in the developing mouse brain. *Anat Sci Int* 80:12–18.
- Gangemi RM, Daga A, Muzio L, Marubbi D, Cocozza S, Perera M, Verardo S, Bordo D, Griffero F, Capra MC, Mallamaci A, Corte G (2006) Effects of *Emx2* inactivation on the gene expression profile of neural precursors. *Eur J Neurosci* 23:325–334.
- Gogolla N, Galimberti I, Deguchi Y, Caroni P (2009) Wnt signaling mediates experience-related regulation of synapse numbers and mossy fiber connectivities in the adult hippocampus. *Neuron* 62:510–525.
- Gordon MD, Nusse R (2006) Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. *J Biol Chem* 281:22429–22433.
- Guillemot F (2007) Spatial and temporal specification of neural fates by transcription factor codes. *Development* 134:3771–3780.
- Gulacsi AA, Anderson SA (2008) Beta-catenin-mediated Wnt signaling regulates neurogenesis in the ventral telencephalon. *Nat Neurosci* 11:1383–1391.
- Hämmerle B, Tejedor FJ (2007) A novel function of DELTA-NOTCH signalling mediates the transition from proliferation to neurogenesis in neural progenitor cells. *PLoS One* 2:e1169.
- Hébert JM, Fishell G (2008) The genetics of early telencephalon patterning: some assembly required. *Nat Rev Neurosci* 9:678–685.
- Hirabayashi Y, Gotoh Y (2005) Stage-dependent fate determination of neural precursor cells in mouse forebrain. *Neurosci Res* 51:331–336.
- Hirabayashi Y, Itoh Y, Tabata H, Nakajima K, Akiyama T, Masuyama N, Gotoh Y (2004) The Wnt/beta-catenin pathway directs neuronal differentiation of cortical neural precursor cells. *Development* 131:2791–2801.
- Huang H, He X (2008) Wnt/beta-catenin signaling: new (and old) players and new insights. *Curr Opin Cell Biol* 20:119–125.
- Iler N, Abate-Shen C (1996) Rapid identification of homeodomain binding sites in the Wnt-5a gene using an immunoprecipitation strategy. *Biochem Biophys Res Commun* 227:257–265.
- Ille F, Sommer L (2005) Wnt signaling: multiple functions in neural development. *Cell Mol Life Sci* 62:1100–1108.
- Irasena N, Hu M, Fu W, Kan L, Kessler JA (2004) The presence of FGF2 signaling determines whether beta-catenin exerts effects on proliferation or neuronal differentiation of neural stem cells. *Dev Biol* 268:220–231.
- Kamitori K, Machide M, Osumi N, Kohsaka S (1999) Expression of receptor

- tyrosine kinase RYK in developing rat central nervous system. *Brain Res Dev Brain Res* 114:149–160.
- Keeble TR, Halford MM, Seaman C, Kee N, Macheda M, Anderson RB, Stacker SA, Cooper HM (2006) The Wnt receptor Ryk is required for Wnt5a-mediated axon guidance on the contralateral side of the corpus callosum. *J Neurosci* 26:5840–5848.
- Kléber M, Sommer L (2004) Wnt signaling and the regulation of stem cell function. *Curr Opin Cell Biol* 16:681–687.
- Kohwi M, Osumi N, Rubenstein JL, Alvarez-Buylla A (2005) Pax6 is required for making specific subpopulations of granule and periglomerular neurons in the olfactory bulb. *J Neurosci* 25:6997–7003.
- Kohwi M, Petryniak MA, Long JE, Ekker M, Obata K, Yanagawa Y, Rubenstein JL, Alvarez-Buylla A (2007) A subpopulation of olfactory bulb GABAergic interneurons is derived from Emx1- and Dlx5/6-expressing progenitors. *J Neurosci* 27:6878–6891.
- Kosaka K, Kosaka T (2005) synaptic organization of the glomerulus in the main olfactory bulb: compartments of the glomerulus and heterogeneity of the periglomerular cells. *Anat Sci Int* 80:80–90.
- Kosaka K, Aika Y, Toida K, Heizmann CW, Hunziker W, Jacobowitz DM, Nagatsu I, Streit P, Visser TJ, Kosaka T (1995) Chemically defined neuron groups and their subpopulations in the glomerular layer of the rat main olfactory bulb. *Neurosci Res* 23:73–88.
- Kosaka K, Fujii M, Toida K, Kosaka T (1997) Differentiation of chemically defined neuronal populations in the transplanted olfactory bulb without olfactory receptor innervation. *Neurosci Res* 28:11–19.
- Leto K, Bartolini A, Yanagawa Y, Obata K, Magrassi L, Schilling K, Rossi F (2009) Laminar fate and phenotype specification of cerebellar GABAergic interneurons. *J Neurosci* 29:7079–7091.
- Levi G, Puche AC, Mantero S, Barbieri O, Trombino S, Paleari L, Egeo A, Merlo GR (2003) The Dlx5 homeodomain gene is essential for olfactory development and connectivity in the mouse. *Mol Cell Neurosci* 22:530–543.
- Li XJ, Zhang X, Johnson MA, Wang ZB, Lavaute T, Zhang SC (2009) Coordination of sonic hedgehog and Wnt signaling determines ventral and dorsal telencephalic neuron types from human embryonic stem cells. *Development* 136:4055–4063.
- Lie DC, Colamarino SA, Song HJ, Désiré L, Mira H, Consiglio A, Lein ES, Jessberger S, Lansford H, Dearie AR, Gage FH (2005) Wnt signalling regulates adult hippocampal neurogenesis. *Nature* 437:1370–1375.
- Long JE, Garel S, Depew MJ, Tobet S, Rubenstein JL (2003) DLX5 regulates development of peripheral and central components of the olfactory system. *J Neurosci* 23:568–578.
- Long JE, Garel S, Alvarez-Dolado M, Yoshikawa K, Osumi N, Alvarez-Buylla A, Rubenstein JL (2007) Dlx-dependent and -independent regulation of olfactory bulb interneuron differentiation. *J Neurosci* 27:3230–3243.
- Lyu J, Yamamoto V, Lu W (2008) Cleavage of the Wnt receptor Ryk regulates neuronal differentiation during cortical neurogenesis. *Dev Cell* 15:773–780.
- Lyuksyutova AI, Lu CC, Milanesio N, King LA, Guo N, Wang Y, Nathans J, Tessier-Lavigne M, Zou Y (2003) Anterior-posterior guidance of commissural axons by Wnt-frizzled signaling. *Science* 302:1984–1988.
- Malaterre J, Ramsay RG, Mantamadiotis T (2007) Wnt-Frizzled signalling and the many paths to neural development and adult brain homeostasis. *Front Biosci* 12:492–506.
- Marín O, Rubenstein JL (2003) Cell migration in the forebrain. *Annu Rev Neurosci* 26:441–483.
- Merlo GR, Paleari L, Mantero S, Zerega B, Adamska M, Rinkwitz S, Bober E, Levi G (2002a) The Dlx5 homeobox gene is essential for vestibular morphogenesis in the mouse embryo through a BMP4-mediated pathway. *Dev Biol* 248:157–169.
- Merlo GR, Paleari L, Mantero S, Genova F, Beverdam A, Palmisano GL, Barbieri O, Levi G (2002b) A mouse model of split hand/foot malformation type I. *Genesis* 33:97–101.
- Métin C, Baudoin JP, Rakić S, Parnavelas JG (2006) Cell and molecular mechanisms involved in the migration of cortical interneurons. *Eur J Neurosci* 23:894–900.
- Mikels AJ, Nusse R (2006) Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. *PLoS Biol* 4:e115.
- Mikels A, Minami Y, Nusse R (2009) Ror2 receptor requires tyrosine kinase activity to mediate Wnt5A signaling. *J Biol Chem* 284:30167–30176.
- Miquelajauregui A, Van de Putte T, Polyakov A, Nityanandam A, Boppana S, Seuntjens E, Karabinos A, Higashi Y, Huylebroeck D, Tarabykin V (2007) Smad-interacting protein-1 (Zfhx1b) acts upstream of Wnt signaling in the mouse hippocampus and controls its formation. *Proc Natl Acad Sci U S A* 104:12919–12924.
- Miyashita T, Koda M, Kitajo K, Yamazaki M, Takahashi K, Kikuchi A, Yamashita T (2009) Wnt-ryk signaling mediates axon growth inhibition and limits functional recovery after spinal cord injury. *J Neurotrauma* 26:955–964.
- Morrison SJ (2001) Neuronal potential and lineage determination by neural stem cells. *Curr Opin Cell Biol* 13:666–672.
- Muroyama Y, Kondoh H, Takada S (2004) Wnt proteins promote neuronal differentiation in neural stem cell culture. *Biochem Biophys Res Commun* 313:915–921.
- Nguyen L, Besson A, Roberts JM, Guillemot F (2006) Coupling cell cycle exit, neuronal differentiation and migration in cortical neurogenesis. *Cell Cycle* 5:2314–2318.
- Nusse R (2008) Wnt signaling and stem cell control. *Cell Res* 18:523–527.
- Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y (1997) “Green mice” as a source of ubiquitous green cells. *FEBS Lett* 407:313–319.
- Otero JJ, Fu W, Kan L, Cuadra AE, Kessler JA (2004) Beta-catenin signaling is required for neural differentiation of embryonic stem cells. *Development* 131:3545–3557.
- Panganiban G, Rubenstein JL (2002) Developmental functions of the Distal-less/Dlx homeobox genes. *Development* 129:4371–4386.
- Parrish-Aungst S, Shipley MT, Erdelyi F, Szabo G, Puche AC (2007) Quantitative analysis of neuronal diversity in the mouse olfactory bulb. *J Comp Neurol* 501:825–836.
- Pellegrino M, Provero P, Silengo L, Di Cunto F (2004) CLOE: identification of putative functional relationships among genes by comparison of expression profiles between two species. *BMC Bioinformatics* 5:179.
- Perera M, Merlo GR, Verardo S, Paleari L, Corte G, Levi G (2004) Defective neurogenesis in the absence of Dlx5. *Mol Cell Neurosci* 25:153–161.
- Pollard SM, Conti L, Sun Y, Goffredo D, Smith A (2006) Adherent neural stem (NS) cells from fetal and adult forebrain. *Cereb Cortex* 16 [Suppl 1]:i112–i120.
- Schulte G, Bryja V, Rawal N, Castelo-Branco G, Sousa KM, Arenas E (2005) Purified Wnt-5a increases differentiation of midbrain dopaminergic cells and dishevelled phosphorylation. *J Neurochem* 92:1550–1553.
- Shihabuddin LS, Horner PJ, Ray J, Gage FH (2000) Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus. *J Neurosci* 20:8727–8735.
- Spiliotopoulos D, Goffredo D, Conti L, Di Febo F, Biella G, Toselli M, Cattaneo E (2009) An optimized experimental strategy for efficient conversion of embryonic stem (ES)-derived mouse neural stem (NS) cells into a nearly homogeneous mature neuronal population. *Neurobiol Dis* 34:320–331.
- Stenman J, Toresson H, Campbell K (2003) Identification of two distinct progenitor populations in the lateral ganglionic eminence: implications for striatal and olfactory bulb neurogenesis. *J Neurosci* 23:167–174.
- Stühmer T, Anderson SA, Ekker M, Rubenstein JL (2002) Ectopic expression of the Dlx genes induces glutamic acid decarboxylase and Dlx expression. *Development* 129:245–252.
- Viti J, Gulacsi A, Lillien L (2003) Wnt regulation of progenitor maturation in the cortex depends on Shh or fibroblast growth factor 2. *J Neurosci* 23:5919–5927.
- Waclaw RR, Allen ZJ 2nd, Bell SM, Erdélyi F, Szabó G, Potter SS, Campbell K (2006) The zinc finger transcription factor Sp8 regulates the generation and diversity of olfactory bulb interneurons. *Neuron* 49:503–516.
- Wang H, Ikeda S, Kanno S, Guang LM, Ohnishi M, Sasaki M, Kobayashi T, Tamura S (2001) Activation of c-Jun amino-terminal kinase is required for retinoic acid-induced neural differentiation of P19 embryonal carcinoma cells. *FEBS Lett* 503:91–96.
- Wichterle H, Turnbull DH, Nery S, Fishell G, Alvarez-Buylla A (2001) In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain. *Development* 128:3759–3771.
- Wigle JT, Eisenstat DD (2008) Homeobox genes in vertebrate forebrain development and disease. *Clin Genet* 73:212–226.
- Wonders CP, Anderson SA (2006) The origin and specification of cortical interneurons. *Nat Rev Neurosci* 7:687–696.
- Woodhead GJ, Mutch CA, Olson EC, Chenn A (2006) Cell-autonomous beta-catenin signaling regulates cortical precursor proliferation. *J Neurosci* 26:12620–12630.

- Wouda RR, Bansraj MR, de Jong AW, Noordermeer JN, Fradkin LG (2008) Src family kinases are required for WNT5 signaling through the Derailed/RYK receptor in the *Drosophila* embryonic central nervous system. *Development* 135:2277–2287.
- Yamanaka H, Moriguchi T, Masuyama N, Kusakabe M, Hanafusa H, Takada R, Takada S, Nishida E (2002) JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. *EMBO Rep* 3:69–75.
- Yu JM, Kim JH, Song GS, Jung JS (2006) Increase in proliferation and differentiation of neural progenitor cells isolated from postnatal and adult mice brain by Wnt-3a and Wnt-5a. *Mol Cell Biochem* 288:17–28.
- Zaghetto AA, Paina S, Mantero S, Platonova N, Peretto P, Bovetti S, Puche A, Piccolo S, Merlo GR (2007) Activation of the Wnt-beta catenin pathway in a cell population on the surface of the forebrain is essential for the establishment of olfactory axon connections. *J Neurosci* 27:9757–9768.
- Zaki PA, Quinn JC, Price DJ (2003) Mouse models of telencephalic development. *Curr Opin Genet Dev* 13:423–437.
- Zechner D, Fujita Y, Hülsken J, Müller T, Walther I, Taketo MM, Crenshaw EB 3rd, Birchmeier W, Birchmeier C (2003) β -Catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation in the nervous system. *Dev Biol* 258:406–418.
- Zou Y (2004) Wnt signaling in axon guidance. *Trends Neurosci* 27:528–532.