Wnt5a is a transcriptional target of Dlx genes and promotes differentiation of olfactory interneuron progenitors.

Development/Plasticity/Repair

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; Marín 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 Ca^{2+}/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 (Kléber and Sommer, 2004; Muroyama et al., 2004). Wnt3 and Wnt7a specif-

Received June 17, 2010; revised Oct. 8, 2010; accepted Dec. 7, 2010.

G.R.M. is recipient of a Career Award from Telethon-Italy (TCP99003), and is supported by Fondazione Cariplo (S00083FCRC) and Fondazione Ricerca Biomedica (University of Torino). We thank Dr. Yingzi Yang (National Institutes of Health) for the *Wnt5a* and *Wnt5b* probes, Dr. V. Broccoli (Università Vita-Salute San Raffaele) for the *Arx*-luc reporter, and Dr. Yimin Zou (University of California, San Diego) for the Wnt-mycTAG expression vectors. We thank Dr. F. DiCunto, P. Provero, and G. Garaffo (University of Torino) for bioinformatic analyses, and Dr. A. P. Puche (University of Maryland School of Medicine) for comments and criticism on the manuscript.

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

†Deceased, October 23, 2010.

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DOI:10.1523/JNEUROSCI.3110-10.2011

 0270-6474/11/312675-13$15.00/0

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 (Peprotech). 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), βIII-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 μm), 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 μg/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 μg 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 βIII-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 μg 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 μm. 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 μm cryostatic sections, processed as described. Briefly, sections were permeabilized with 3 μg/ml proteinase K, washed in PBS, and deacetylated with 1.3% triethanolamine and 0.25% acetic anhydrate at room temperature. Sections were prehybridized in 50% formamide at 60°C, hybridized with the DIG-labeled probes

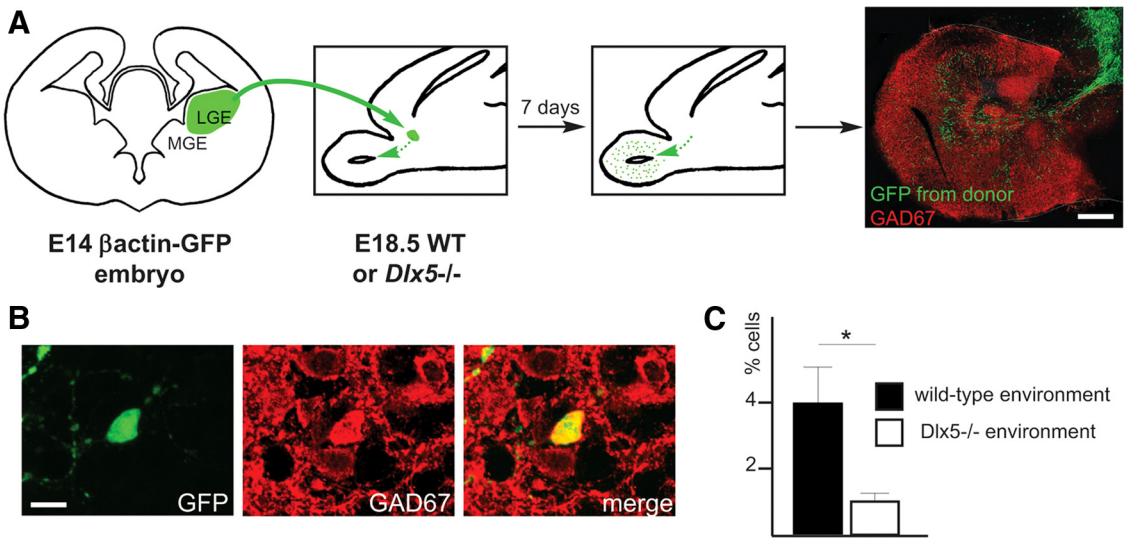

Figure 1. Grafting of normal subpallial progenitors in *Dlx5*−/− forebrain, to reveal non-cell-autonomous regulations. ***A***, Scheme illustrating the experimental strategy. Fragments of the LGE from GFP+ (normal) E14.5 embryos were used as donors to graft onto cultures of brain slices set up from neonatal 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 μm. ***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 μm. ***C***, Number of GFP+/GAD67+ donor cells in WT (solid black bars) or *Dlx5*−/− (open bars) OB. Error bars represent the mean ± 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 μg of total RNA was reverse-transcribed at 42°C for 50 min in the presence of 500 ng/μl 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 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 μg 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 μg 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 lyses buffer, supplemented with phosphatase and protease inhibitors. As positive control for phosphorylated JnK, HeLa cells were UV irradiated (40 mJ/cm^2) 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 (Pellegrino 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*−/−

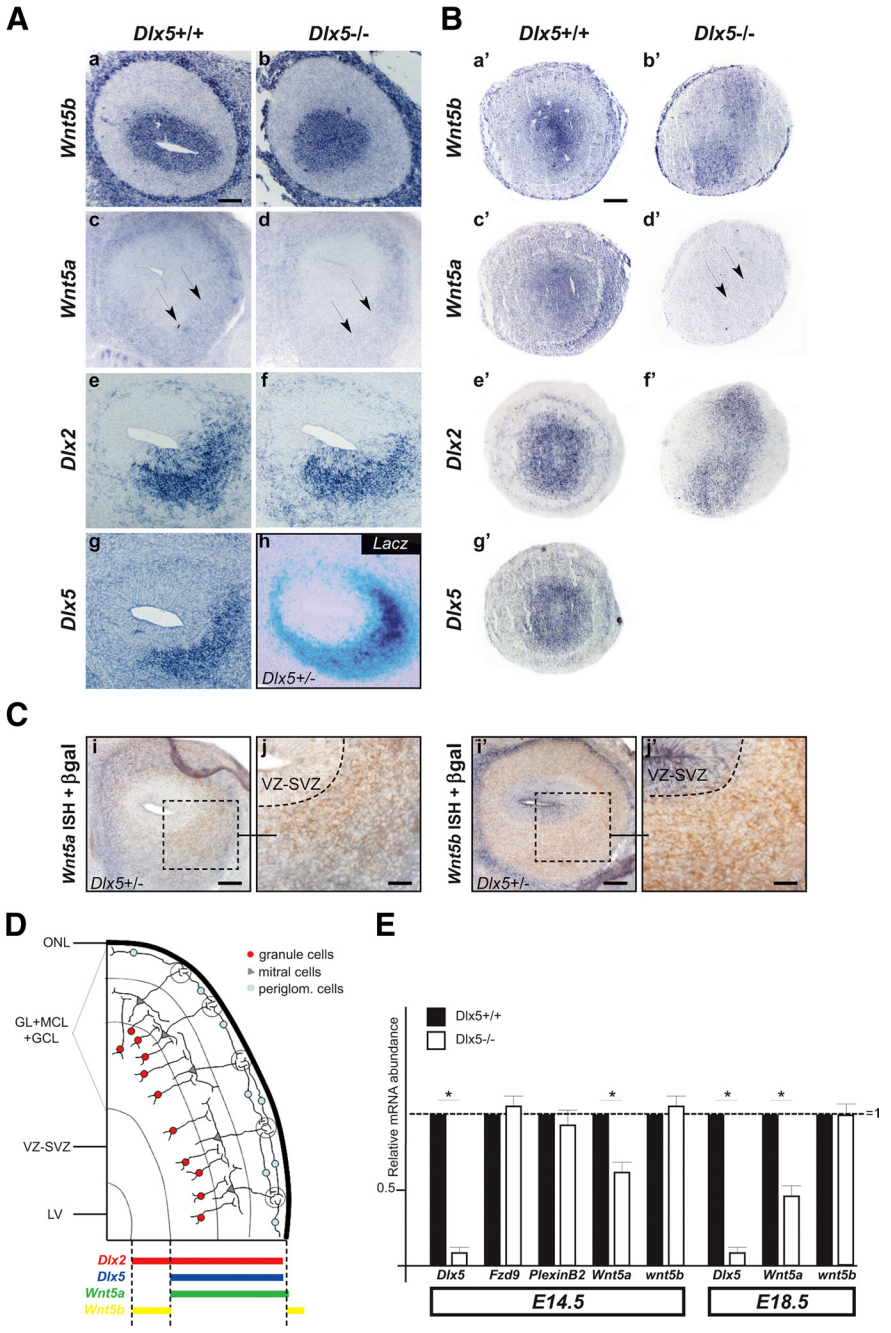

Figure 2. Expression of *Wnt5a* and *Wnt5b* in normal and *Dlx5*−/− OB. ***A***, ***B***, ISH on coronal sections of E14.5 (***A***) and neonatal (***B***) OB of WT and *Dlx5*−/− specimens (genotypes are reported on top). Expression of *Wnt5b* (***a***, ***b***, ***a′***, ***b′***), *Wnt5a* (***c***, ***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 ± 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 μm (***a–h***, ***i***, ***i′***), 200 μm (***a′–g′***), 100 μm (***j***, ***j′***).

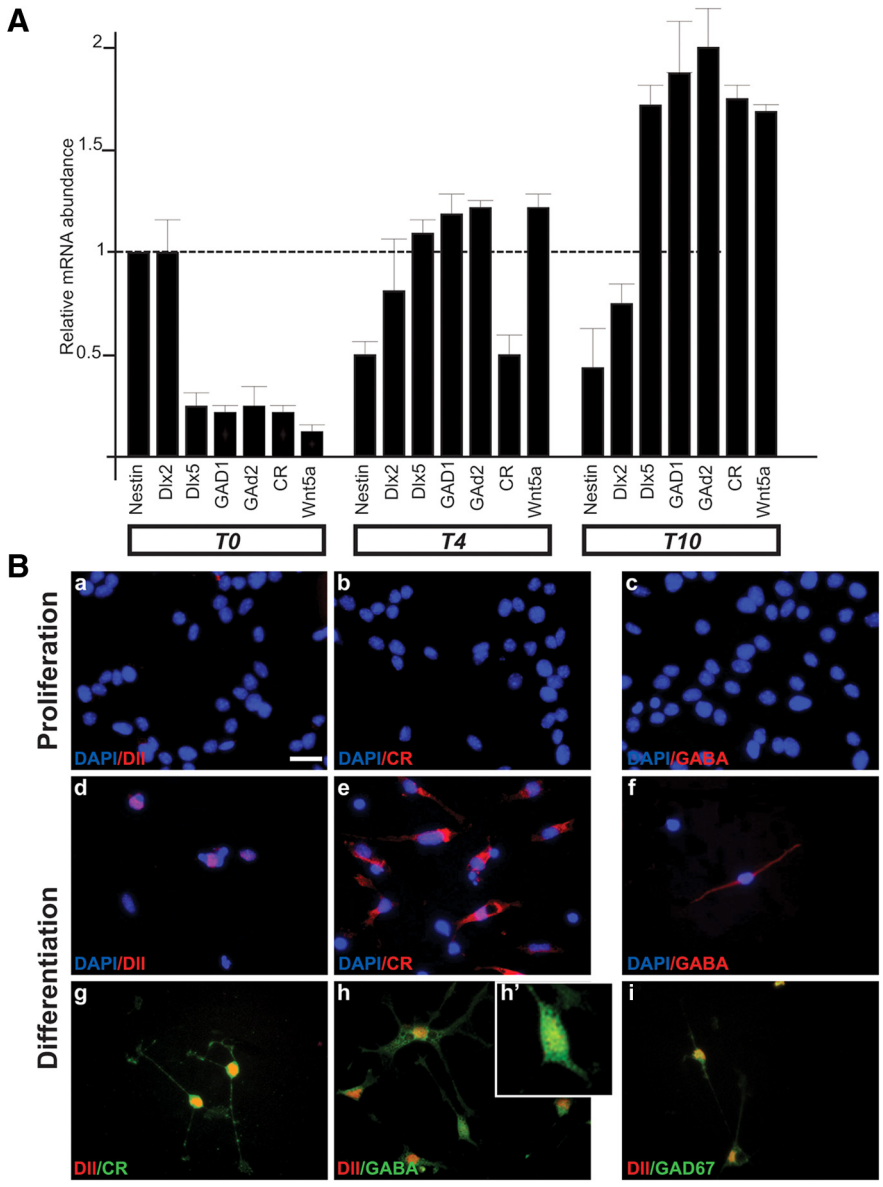

Figure 3. Expression of *Dlx*, *Wnt5a*, and differentiation markers in NS cells. ***A***, Real-time qPCR analysis for the expression of *Nestin*, *Dlx2*, *Dlx5*, *GAD1*, *GAD2*, *CR*, and *Wnt5a* mRNA in NS cells, at two time points (T4 and T10, indicating 4 and 10 d *in vitro*) following the application 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 ± 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 μm.

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. 2*A*). 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 (supplemental 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. 2*B*), 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. 2*C*). All these results, summarized in the scheme in Figure 2*D*, 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. 2*Bb'*,*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. 2*B*). Reduced *Wnt5a* expression was also observed in the embryonic OB, although less evident (Fig. 2*A*). 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. 2*E*), 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. 3*A*). 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. 3*B*). Note that few GAD67+/dll− cells were observed in these cultures (<5% of all GAD+ cells) (Fig. 3*B*). 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 βIII-tubulin, GFAP, GAD67, and GABA. Under differentiation conditions, the WT cells readily express βIII-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. 4*A*). 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 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. 4*B*, 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. 4*B*). In the same experiment, the *Dlx5;Dlx6* mutant (EGFP−) cells yielded fewer GAD67+ cells, as expected.

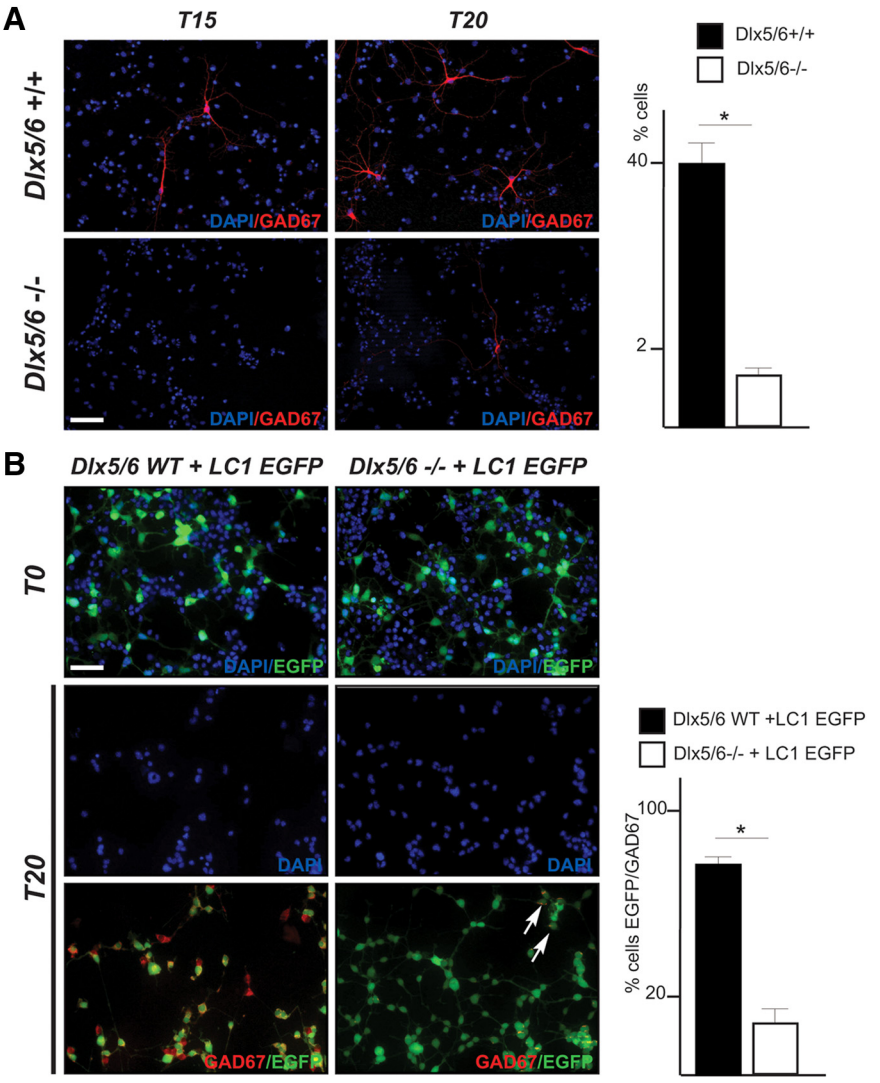

Figure 4. GABA+ differentiation of normal and *Dlx* mutant NS cells reveals non-cell-autonomous regulations. ***A***, GAD67 expression of NS cells derived from WT (top row) or *Dlx5;Dlx6* null (bottom row) embryonic brains, following 15 or 20 d in differentiating medium. The histogram on the left reports 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 μm.

DLX2 and *DLX5* transcriptionally regulate *Wnt5a* expression

The *Wnt5a* locus contain 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

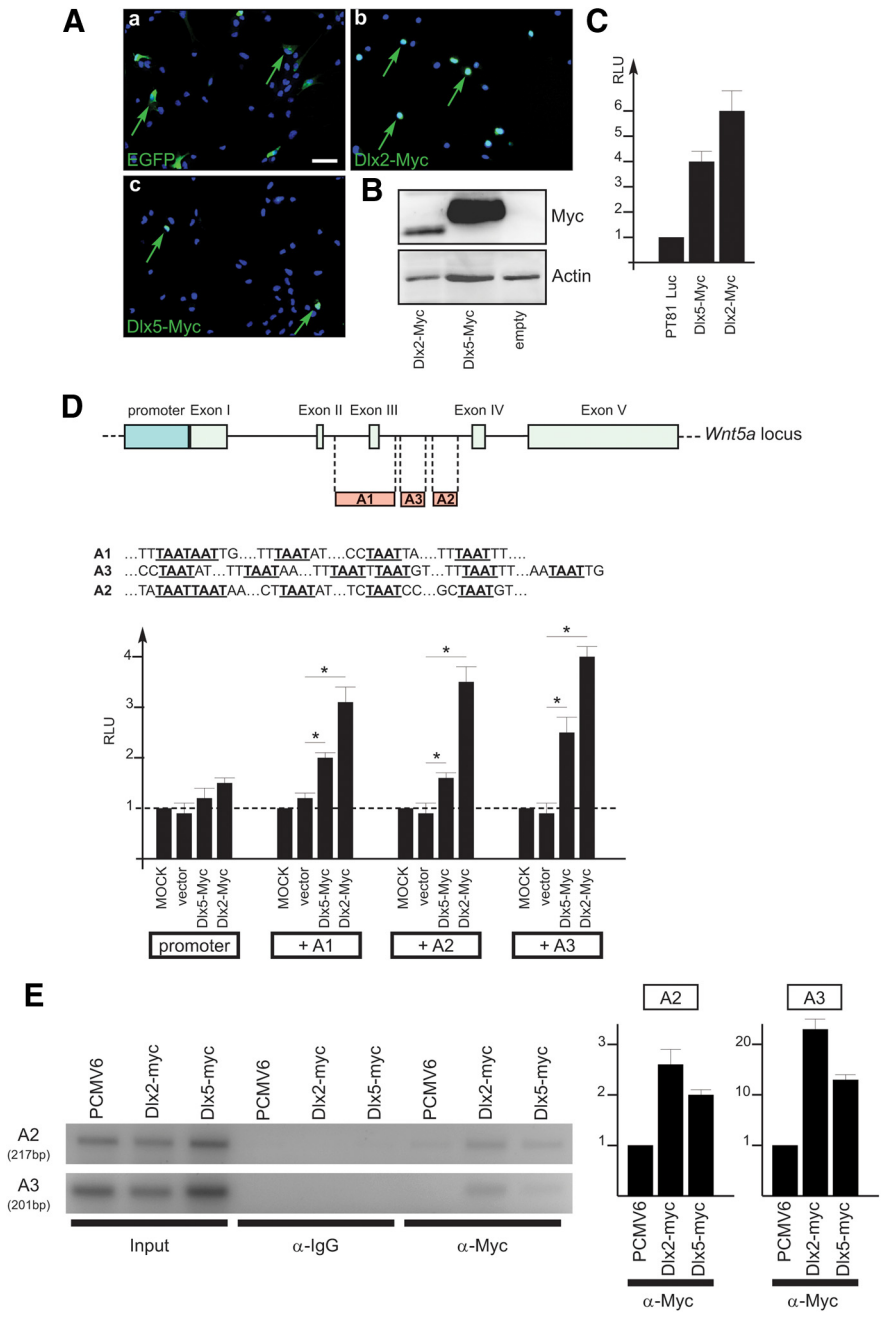

Figure 5. Dlx proteins regulate *Wnt5a* transcription and bind to the *Wnt5a* locus in native chromatin. ***A***, NS cells nucleofected with *EGFP* (***a***), *Dlx2-mycTAG* (***b***), or *Dlx5-mycTAG* (***c***) expression vector, stained with anti-mycTAG antibody (green arrows). Scale bar (in ***a***), 100 μm. ***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 homeodomain 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 ± 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 the 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. Errors bars represent the mean ± 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. 5*D*). We transfected brain-derived NS cells with the reporter vectors indicated above and either *DLX5*- or *DLX2*-mycTAG expression vectors (Fig. 5*A–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. 5*D*).

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. 5*E*). 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; Farías 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. 6*A*). 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. 6*B*). These results indicate that the β-catenin pathway is unaffected by the absence of *Dlx5* and reduced *Wnt5a* expression.

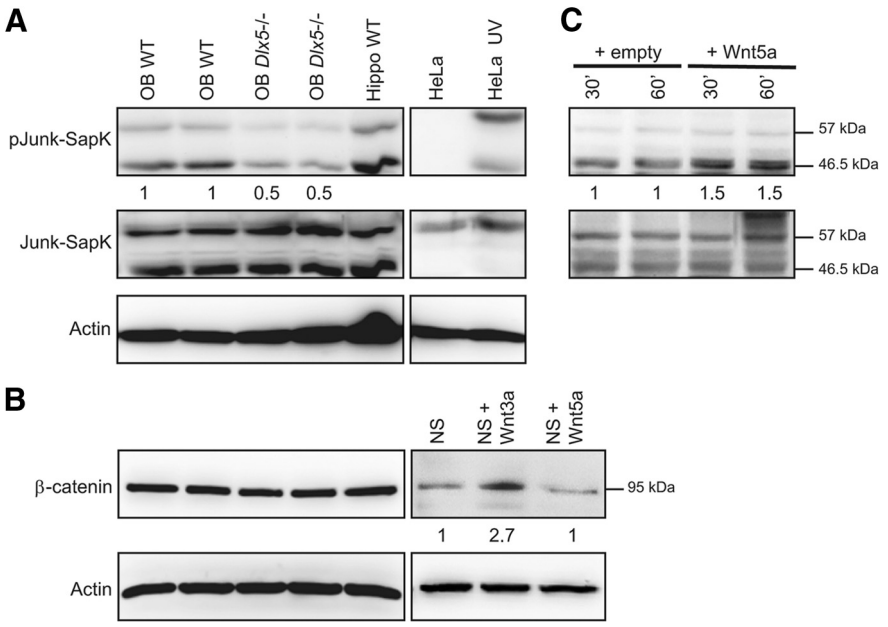

Figure 6. Reduced phospho-JnK in *Dlx5*−/− OB. ***A***, Western blot analysis of total proteins extracted from the OBs of two individual WT or *Dlx5*−/− animals, and a normal hippocampus, to detect phospho-JnK1–3 (pJunk-SapK, top panel), or to detect all forms of JnK1–3 (Junk-SapK, middle panel) with 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).

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. 6*C*).

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 (Lyuksyutova 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. 7*A*). 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. 7*B*). 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 βIII-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. 8*A*), while the number of βIII-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. 8*B*). 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*−/−.

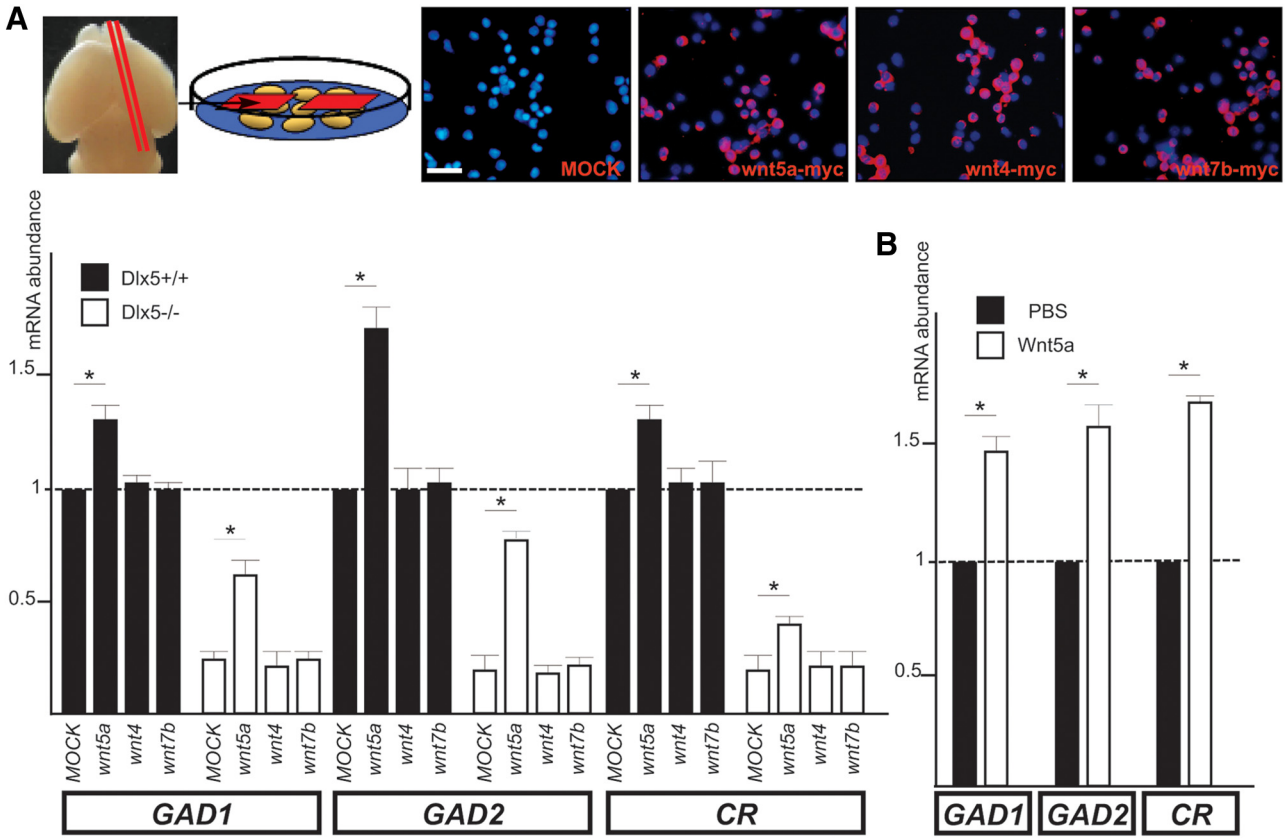

Figure 7. Wnt5a promotes expression of interneuron differentiation markers *in vitro*. ***A***, Top panel, COS7 cells transfected with Wnt5a-, Wnt4-, and Wnt7b-mycTAG expression vectors, immunostained with anti-mycTAG antibody, and used as a feeder layer for organotypic cultures. Thick longitudinal sections 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 ± SD of four independent experiments. Asterisks indicate $p < 0.05$. Scale bar, 100 μm.

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;6*−/− 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-Branco 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.

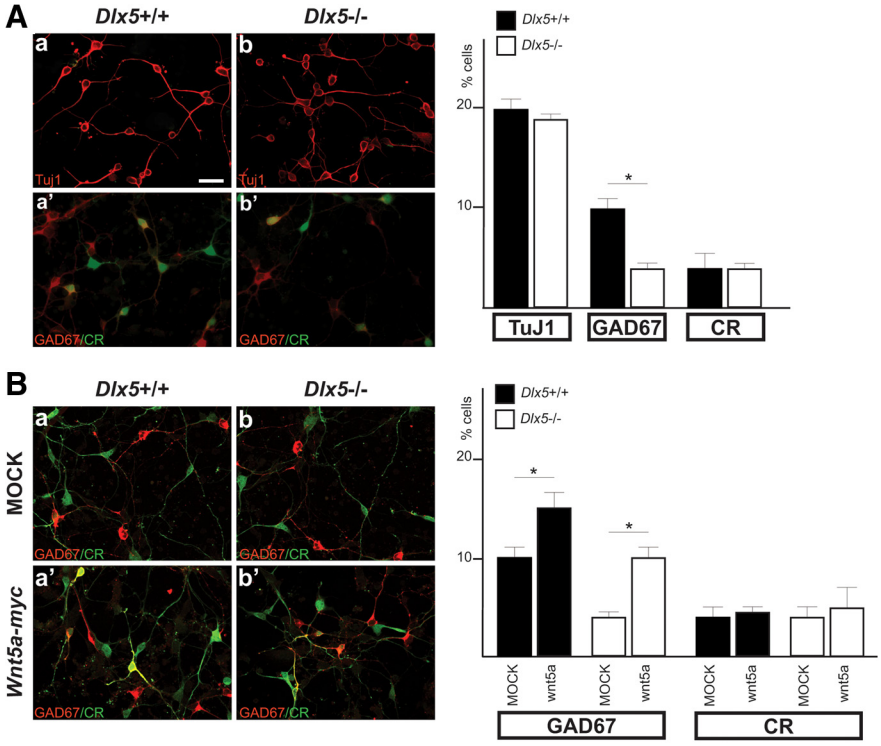

Figure 8. Wnt5a promotes GABA+ differentiation of cultured OB cells. ***A***, Left, Staining for βIII-tubulin (TuJ1) (***a***, ***b***) and double staining for GAD67 (red fluorescence) and CR (green) (***a′***, ***b′***) of primary cultures of OB cells obtained from WT or *Dlx5*−/− E16.5 embryos, after 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 βIII-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 ± SD of four independent experiments. Asterisks indicate $p < 0.05$. Scale bar (in ***a***), 50 μm.

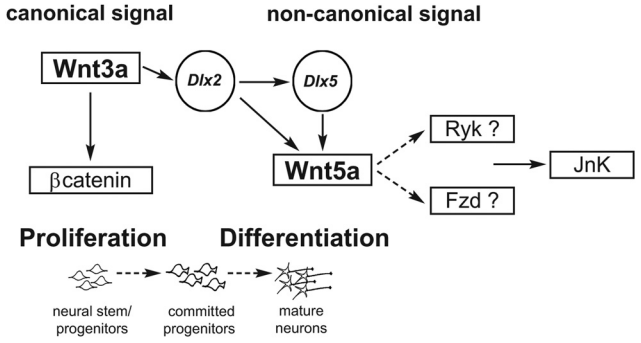

Figure 9. Model of regulation of GABA+ neuron differentiation in the olfactory bulb by Dlx and Wnt5a. The activity of canonical (represented by Wnt3a) and noncanonical (Wnt5a) Wnt signaling is regionally and functionally separated (indicated on the top). Transcriptional regulations are represented by 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*.

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 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*.

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