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ERRATUM

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ORIGINAL PAPER

The role of *Dlx* homeogenes in early development of the olfactory pathway

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Abstract Development of the olfactory pathway requires interaction between cells and signals of different origin. Olfactory receptor neurons (ORN) in the olfactory placodes (OP) extend axons towards the forebrain (FB); with innervation taking place at a later time following degradation of the basement membrane. Cells from the OP migrate along ORN axons and differentiate into various elements, including ensheathing and Gonadotropin Releasing Hormone (GnRH)+ cells. The importance of the olfactory connection and migration is highlighted by the severe endocrine phenotype in Kallmann's patients who lack this migratory pathway. Little is known about the genetic control of intrinsic ORN properties. Inactivation of the *distalless*-related *Dlx5* prevents connections between ORNs and FB. Using a grafting approach we show that misguidance and lack of connectivity is due to intrinsic defects in ORN neurites and migratory cells (MgC), and not to environmental factors. These data point to a cell-autonomous function of *Dlx5* in providing ORN axons with their connectivity properties. *Dlx5* also marks a population of early MgC that partly overlaps with the GnRH+ population. In the absence of *Dlx5* MgCs of the Dlx5+ lineage migrate, associated with PSA-NCAM+ axons, but fail to reach the FB as a consequence of the lack of axonal connection and not an inability to migrate. These data suggests that *Dlx5* is not required to initiate migration and differentiation of MgCs.

Keywords Olfactory · Axon · *Dlx* · Migratory · GnRH · Kallmann

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Introduction

During development of the olfactory pathway a number of processes occur that coordinate axon guidance and connectivity (Scott and Brirley 1999; LaMantia et al. 2000; Bozza et al. 2002; Brunjes and Greer 2003). Beginning at E10 immature olfactory receptor neurons (ORNs) in the olfactory placode (OP), the precursor of the olfactory epithelium (OE) and of the vomero-nasal organ (VNO), extend axons towards the forebrain (FB) to form a mesh of neurites at the anterior-ventral margin of the FB, denominated the migratory mass (MM). A small number of pioneer axons enter the FB at earlier stages (Astic et al. 2002; Key 1998), but their function is still unclear. At later stages (E13.5-E14.5) the basement membrane (BM) separating the MM from the olfactory bulb (OB) is degraded allowing massive entry of ORN axons and the formation of the outer *sublaminae* of the nerve layer (Doucette 1989; Gong and Shipley 1996; Bailey et al. 1999; Au et al. 2002).

Beginning at early embryonic ages, migratory cells (MgC) leave the OP following the extending ORN axons and contribute to the formation of the MM (Tarozzo et al. 1995; Wray et al. 1989; 1994). Several important classes of differentiated cells have an OP/migratory origin: one is the S100+ ensheathing cells, which accompany the

ORN axons along their entire length and provide Schwann-like non-myelin forming protections to the neurites (Astic et al. 1998; Tennent and Chuah 1996). The other is the precursors of the Gonadotropin Releasing Hormone (GnRH) neurons found in the septo-hypothalamic area of the adult brain. These cells begin to express GnRH while migrating from the OP, reach the OB-septum area around E14.5–E15.5 and enter the basal brain together with VNO axons (Dubois et al. 2002; Schwanzel-Fukuda and Pfaff 1989; Fueshko and Wray 1994; Wray et al. 1994). The importance of OP-derived cell migration is highlighted by the severity of the phenotype of Kallmann patients, in which ON–OB connections and the migration of GnRH cells are compromised at various levels (Rugarli et al. 1996; Dode and Hardelin 2004; Gonzàlez-Martinez et al. 2004).

The organization of the peripheral olfactory pathway is the result of sequential interactions between growing axons, MgCs, the nasal mesenchyme, and the FB (Key 1998; Lin and Ngai 1999; Brunjes and Greer 2003; Balmer and LaMantia 2005). Numerous signals and cell population, of both OP and mesenchymal origin, are known to participate, such as the chemoattractant Netrin1 (Astic et al. 2002), semaphorins/neuropilins (Williams-Hogarth et al. 2000), Eph/Ephrins (Gao et al. 2000), Slit/Robo (Li et al. 1999), NCAM (Treloar et al. 1997), Galectin-1 (Puche et al. 1996), p75 NGF receptor (Tisay et al. 2000) and Wnts (Zaghetto et al. 2007). Conversely, little is known about the genetic control of intrinsic axonal properties.

A number of observations indicate that the ability of embryonic ORN axons to connect to anterior FB is mainly due to intrinsic ORN axons properties, hence cell-autonomous, while the role of the FB is thought to be minimal (Nedelec et al. 2005; Levi et al. 2003; Balmer and LaMantia 2005). In adult life ORNs in the OE are constantly replaced and the newly generated neurons extend their axons along the established pathway to the OB, converging to the correct glomerulus (Mombaerts 2006; Treloar et al. 2002). Thus, the adult olfactory pathway possessed unique regeneration properties (Nedelec et al. 2005), that distinguish it from most of the CNS and peripheral nervous system. For these reasons the intrinsic properties of ORN axons and how these interact with OECs acquires high scientific as well as medical importance.

A number of transcription factor genes are known to play important roles: targeted inactivation of *Fez1*, *Klf7*, *Arx* and *Emx2* in the mouse leads to partial or complete loss of ORN axon-to-FB connections (Yoshida et al. 1997; Mallamaci et al. 1998; Yang et al. 1998; Yoshihara et al. 2004; Laub et al. 2005; Hirata et al. 2006). All these phenotypes suggests that the corresponding transcription factors may control guidance and/or connectivity properties solely of extending ORN axons, i.e. a cell autonomous role. However, these genes could also affect ORN axon growth and guidance, migration and differentiation of OP-derived migratory cells, or act to control axon–mesenchyme interactions in a cell-nonautonomous way. We (Levi et al. 2003) and others (Long et al. 2003) have shown that in mice with targeted inactivation of the *distalless*-related homeogene *Dlx5* ORNs extend axons but fail to contact the OB and form a nerve layer. It has also been proposed that *Dlx* and *Msx* genes may also control the differentiation of GnRH+ MCs (Givens et al. 2005).

To understand some of the basic mechanisms of olfactory development and the functions of transcription factors in vivo, we used the *Dlx5* model to clarify the axon-vs.-migratory role of *Dlx5* in the formation of primitive connections, and to re-examine the possible role of *Dlx* genes in the differentiation of MgCs.

Materials and methods

Mouse strains, sample collection

The Institutional Animal Care Committee and the Ministry of Health approved all the animal procedures. Mice with targeted disruption of *Dlx5* have been reported (Acampora et al. 1999). In these, the *lacZ* reporter was inserted to provide a marker for *Dlx5*-expressing cells. *β-actin-eGFP* mice (Okabe et al. 1997) were used as donors in grafting experiments. This strain was maintained heterozygous, crossbred with *Dlx5*+/– mates to obtain double heterozygous; from these we obtained *Dlx5*–/–;*eGFP*+ embryos, at the expected frequency. Embryos were collected between 10.5 and 14.5 day of gestation by caesarean section, or at birth (P0), fixed in 4% PFA ON, washed in PBS, cryoprotected in 30% sucrose, frozen in OCT and sectioned at 12 µm. Newborns were perfused with 4% PFA, then either the brain dissected, post-fixed, washed in PBS, stained with Xgal and processed for cryostatic sections, or the entire head washed in PSB, stained with Xgal, decalcified with 10% EDTA and processed as above. βgal+ nuclei were detected by staining with Xgal (Merlo et al. 2002a, b), or by immunostaining with anti-βgal antibody.

Histochemistry and in situ hybridization

Immunohistochemistry was performed on 10–15 µm cryostatic sections, according to standard protocols. Permeabilization was done with cold 10% Triton X100 at 4°C for 10 min followed by blocking with 10% goat serum, 0.5% Triton for 1 h at R.T. The following antibodies were used: guinea-pig anti-βgal (1:1,000 a gift from Dr. T. Sargent lab.); rabbit anti-βgal (1:5,000; MP Biomedical, Illkirch France); rabbit anti-S100 (1:200; Dako

Cytomation, Denmark); mouse anti-Neuronal Class-III βTubulin (monoclonal TuJ1; 1:1,000; SIGMA, St. Louis MO, USA); mouse anti-PSA-NCAM (monoclonal; 1:1,000; AbCys SA, Paris), goat anti-DCX, (polyclonal; 1:400; C18 SantaCruz CA, USA); rabbit anti-GnRH (1:2,000, kindly provided by Dr. R. Benoit) and rabbit anti-peripherin (1:200, Chemicon). Secondary antibodies were anti-mouse-Cy2, anti-rabbit-Cy3 and anti-guinea-pig-biotin (1:200, 1:600, 1:400; Jackson ImmunoResearch, West Grove PA, USA). Monoclonal antibodies were detected with the ARK kit (Dako). Peroxidase was developed with DAB (Dako).

In situ hybridization was carried out on cryostatic sections from E13.5 and E14.5 WT and *Dlx5–/–* embryos, with DIG-labeled RNA probes, according to published procedures (Merlo et al. 2002a, b). The following murine cDNAs were used: *Dlx1* (linearized with *Bam*HI/transcribed with T7), *Dlx2* (*Hin*dIII/T3), *Dlx3* (*Not*I/T3), *Dlx5* (*Eco*RI/T7), *Dlx6* (*Spe*I/T7), *p75* (*Eco*RI/T7) and *Bmp4* (*Eco*RI/T7). Probes for *Bmp4* and *p75* were gifts from, respectively, Dr E. Bober and Dr S. Bertuzzi. Signals were developed with NBT/BCIP (Sigma).

Photomicroscopy

Bright Field micrographs were taken with a digital photocamera (Nikon) on an Olympus AX70 microscope. Fluorescence micrographs were taken with a confocal Microscope (Olympus FluoView), using sequential scanning/stacking functions, and digitally merged with pseudocoloring. The images were contrast balanced, color matched, rotated and cropped using Photoshop 7 (Adobe) and assembled into the figures with QuarkXpress 6.1 (Pantone).

Organotypic slice cultures

Embryonic heads were dissected in cold PBS-glucose (0.6%), embedded in warm (42°C) 3% low melting point agarose (SIGMA) which was then set at RT and transferred on ice. The gel blocks were sectioned by vibratome (250 μm) and kept 1 h in PBS-glucose on ice. Slices were transferred to Millicell-CM membranes (Millipore, Billerica MA, USA) floating on DMEM-F12 medium (GIBCO, Invitrogen, Carlsbad CA, USA), 6.5 mg/ml glucose, 0.1 M glutamine, and 10% FBS, at 37°C for 1 h, then changed to Neurobasal medium supplemented with B27 (GIBCO), and maintained at 100% humidity, 5% CO_2 at 37°C.

Tracing of the migratory pathway

Sections of the nasal region of E10.5 WT embryos were cut and cultured as illustrated above. Small crystals of Cell Tracker Green (CTG, Invitrogen) were placed on the exposed surface of the OP with a fine needle, the cultures continued for 24 h, fixed, photographed at low magnification, and processed for cryopreservation. Slices were then re-sectioned (11 μm thickness), immunostained for βgal and examined by confocal microscopy.

Grafting of OP

For the grafting experiments, vibratome sliced (250 μm) of the olfactory region of E11.5 WT embryos were used as host; these sections were maintained in organotypic cultures for 48 h, as described above. The donor tissue was derived from *Dlx5+/–* or *–/–* E11.5 embryos, that were also heterozygous for the *βactin-eGFP* reporter transgene, to allow for visualization of donor cells. Donor heads were sectioned as for the host tissues, kept in cold PBS-glucose and further dissected to separate OP fragments. These were transferred onto the host slice using a glass capillary tube. Cultures were changed to Neurobasal/B27, maintained for 48 h, as above, then fixed in 4% PFA and examined by fluorescence microscopy, or stained with Xgal.

Results

Expression of *Dlx5* and *Dlx6* in the OP and OE

Whole-mount staining of E10.5 $Dlx5^{+/lacZ}$ embryos with Xgal shows a strong signal in the OP (Fig. 1A, B), and in E14.5 $Dlx5^{+/lacZ}$ embryos a strong signal in the VNO, the OE and along the ON (Fig. 1C).

We carried out in situ hybridizations for *Dlx1*, *Dlx2*, *Dlx3*, *Dlx5* and *Dlx6* on sagittal sections of WT E14.5 embryos. The hybridization signal for *Dlx5* and *Dlx6* is identical to that observed with Xgal staining (compare Fig. 1C, D, E). In the periphery, *Dlx5* expression is observed in the VNO and OE but not the respiratory epithelium (Fig. 1R–T). In the CNS, *Dlx5* expression is observed in the ganglionic eminences, the OB and the cortex (tangentially migrating interneuron precursors), as expected (Fig. 1R–T). *Dlx6* was expressed in the same territories as *Dlx5*, although expression in the OB is weaker (Fig. 1U–W). *Dlx3* expression is observed in the VNO, ectoderm epithelium of the head, and in the respiratory epithelium lining the nasal cavities, is weak in the OE, and absent in the CNS (Fig. 1N–Q). No expression of *Dlx1* or *Dlx2* can be detected in the VNO and OE at this stage, while signal for *Dlx1* and *Dlx2* is observed in the ganglionic eminences, OB and cortex (Fig. 1G–M). All *Dlx* genes are also expressed in the tooth buds. Thus, *Dlx5-Dlx6* are expressed in similar patterns in the peripheral olfactory system, and we can assume that the anti-dll (pan-Dlx) antibody would recognizes mainly *Dlx5* and to a lower extent *Dlx6* and *Dlx3* in these regions.

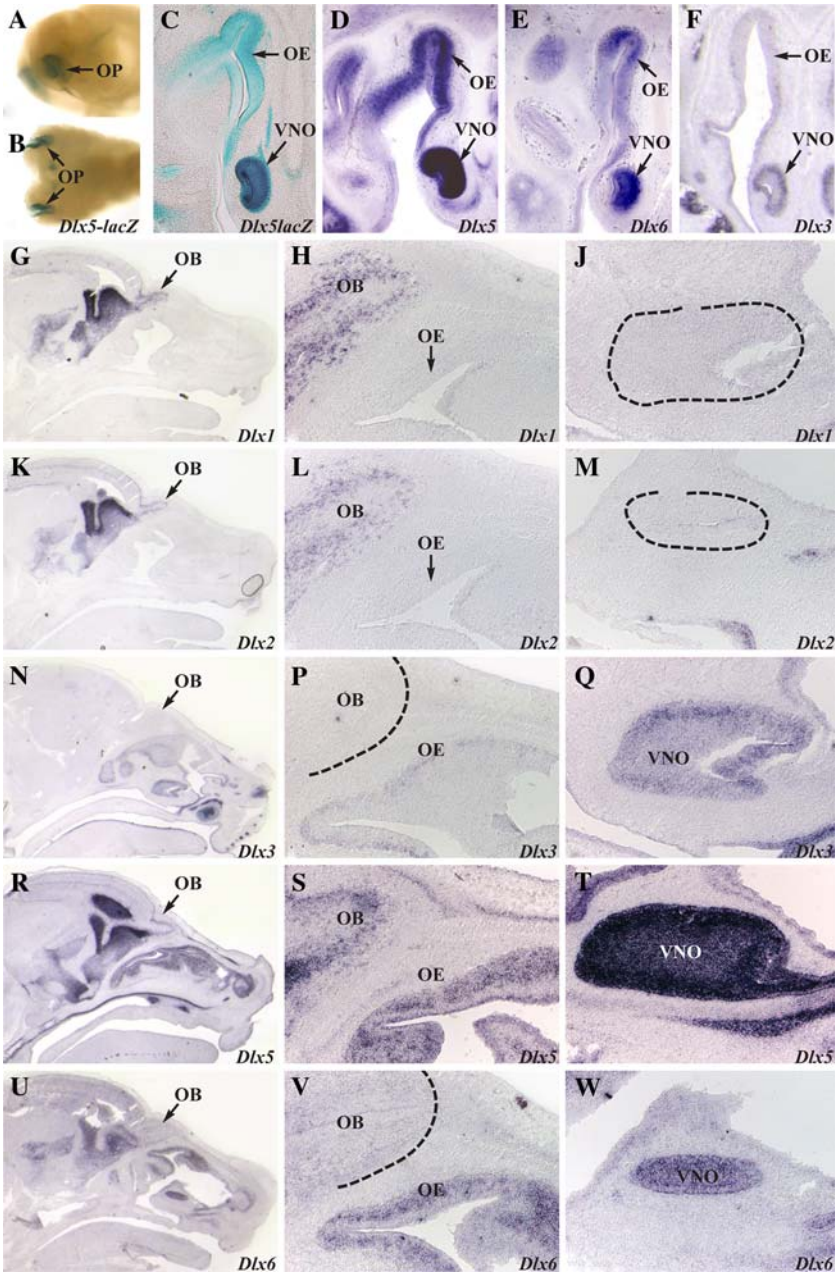

Fig. 1 Expression of *Dlx* genes in the peripheral olfactory system**. (A, B)** Whole mount Xgal staining of E10.5 $Dlx5^{lacZ/+}$ embryos, lateral (**A**) and ventral (**B**) views. (**C**) Xgal staining of frontal section of E14.5 $Dlx5^{lacZ/+}$ embryo. (**D–F**) In situ hybridization for *Dlx5* (**D**), *Dlx6* (**E**) and *Dlx3* (**F**) on frontal sections of E14.5 WT embryos. (**G–W**), In situ hybridization for *Dlx1* (**G–J**), *Dlx2* (**K–M**), *Dlx3* (**N–Q**), *Dlx5* (**R–T**) and *Dlx6* (**U–W**) on longitudinal sections of E14.5 WT embryos. Micrographs on the left are lower magnification; on the middle and on the right, higher magnification of, respectively, the OB-OE and the VNO region. *Dlx5* and *Dlx6* (and to a much lesser extent *Dlx3*) are expressed in the OB, OE and VNO. *Dlx1* and *Dlx2* are expressed in the OB but not in the OE and VNO. Abbreviations: OB, olfactory bulb; OE, olfactory epithelium; OP, olfactory placode; VNO, vomero-nasal organ

In *Dlx5* mutant embryos ORN axons extend normally but fail to reach the FB

In late (E15-E16) *Dlx5–/–* embryos ORN axons fail to form a nerve layer at the surface of the OB, and appear to be misoriented (Levi et al. 2003; Long et al. 2003). However, in these studies early stages were not examined. To clarify whether olfactory connections are absent from the earliest stages and whether ORN axons express the appropriate early markers, we stained frontal sections of WT and *Dlx5–/–* E10.5 embryos for βIII-tubulin and peripherin. ORN neurites and MgCs are present both in the normal and in the mutant heads, with no apparent differences (Fig. 2A–D). Furthermore, a migratory mass (MM) is visible in both cases. At this stage most neurites have not reached the FB. Staining of slightly older embryos (E10.75) show that in normal heads axon bundles grew from the MM and contacted the FB, but in the absence of *Dlx5* these contacts are absent (Fig. 2).

To further characterize early ORN axons, and to discriminate the neurites from the MgCs (many of which are βIII-tubulin+), we carried out double immunostaining for βIII-tubulin and peripherin (markers for peripheral axons at early developmental ages, Chien et al. 1998; Wray et al. 1994). Early ORN neurites were double positive (merge color, Fig 2A, B) for both markers, both in the WT and in

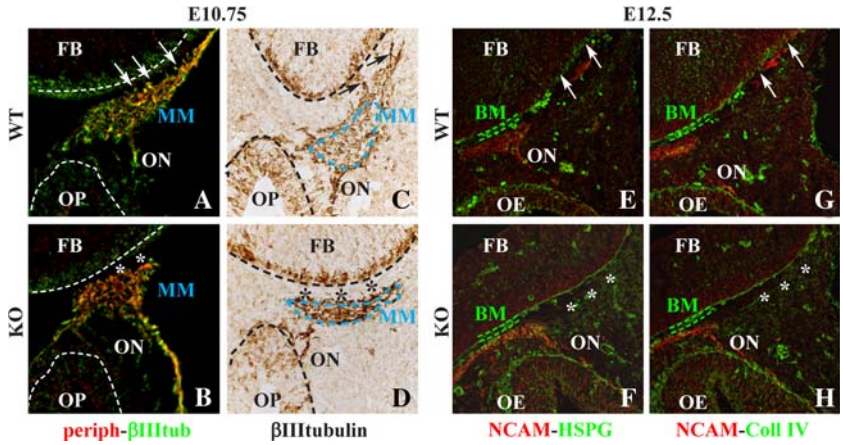

Fig. 2 Early defects in the peripheral olfactory system of *Dlx5* mutant embryos**.** (**A–D**), Frontal sections of E10.75 normal (top) and *Dlx5–/–* (bottom) embryos, immunostained for peripherin (red) and *β*III-tubulin (green) (**A, B**), or for *β*III-tubulin alone (brown) (**C, D**). The ''merge'' pseudocolor (yellow) in **A** and **B** indicates areas or colocalization. The OP, ON and FB are indicated. The migratory mass (MM) is outlined (dashed blue line). Note the absence of axon-FB contact in the *Dlx5–/–* embryos (asterisks). (**E–H**), Organization of the basement membrane (BM) in the region ventral/anterior to the FB of WT (top) and *Dlx5–/–* (bottom) embryos. Double immunostaining for NCAM (red fluorescence) and either HSPG (green in **E**, **F**) or Collagen-IV (green in **G**, **H**). The BM (outlined with dashed green lines) is fenestrated (white arrows) in WT, but is intact in *Dlx5* mutant (white asterisks)

the *Dlx5* null embryos. Thus, the absence of *Dlx5* results in a condition of failure of the ORN axons to contact the FB, from the earliest stages.

Forebrain basement membrane is unfenestrated in *Dlx5–/–* embryos

At approximately E14.5 during mouse embryo development the BM of the OB region is fenestrated and then eliminated to allow the MM to form the OB nerve layer (Gong and Shipley 1996; Treloar et al. 1996). We have examined the organization of the BM (see Julliard and Hartmann 1998) in *Dlx5–/–* mice by immunostaining with antibodies that recognize laminin, collagen-IV, heparan-sulfate proteoglycan (HSPG), chondroitin-sulfate proteoglycan (CSPG), on sections of WT and $Dlx5^{lacZ}$*–/–* embryonic noses at E12.5, E14.5 and P0. ORN axons were visualized by immunostaining for NCAM. Already at E12.5 the WT BM appeared discontinuous and fenestrated. Instead, in *Dlx5–/–* embryos the BM appeared uninterrupted (Fig. 2F, H). Similar results were obtained at E14.5 and P0 where fenestration of the BM was easily seen in the WT embryo, while an uninterrupted BM envelops the *Dlx5–/–* OB (data not shown). At P0, the OBs of *Dlx5–/–* animals often appear detached from the meninges, and all the ECM markers examined were found to be expressed as a continuous layer (data not shown).

Misguidance and lack of connection are cell-autonomous defects

We previously established that WT axons can contact and form glomeruli-like structures in *Dlx5–/–* OBs grafted in vivo (Levi et al. 2003). Thus the lack of connectivity is not due to OB-related impediments. Since *Dlx5* and *Dlx6* are expressed in the OP cells and its migratory derivatives, one logical hypothesis is that *Dlx5* acts cell-autonomously to provide specific guidance/connectivity properties to the early ORN neurites. To investigate this, we compared the behavior of *Dlx5* mutant and WT ORN axons in the presence of a normal extracellular environment and FB, in conditions we have shown to support development of the olfactory pathway (Zaghetto et al. 2007). We excised fragments of the OP from E11.5 WT or *Dlx5–/–;eGFP* embryos, and grafted these onto the OP of cultured slices from normal embryos, of the same age. After 48 h in organotypic culture, WT/eGFP+ OPs consistently yielded neurites and MgCs directed towards the FB and contacting its surface (29 or 34 informative grafts; Fig. 3A–D). On the contrary, neurites from *Dlx5–/–*/eGFP+ OPs inevitably adopted abnormal trajectories and in no case reached the FB surface (0 of 12 informative grafts; Fig. 3). These results indicate that the connectivity defect of *Dlx5–/–* embryos is likely intrinsic to the ORN or the OP-derived MgCs, and exclude a role for mesenchyme and FB in this defect.

Expression of *Dlx* genes in early olfactory MgCs

In *Dlx1;Dlx2* mice fewer GnRH+ cells are observed reaching the basal FB (Givens et al. 2005). Based on this observation and the presence of putative Dlx homeodomain binding sites in the GnRH promoter, a role for *Dlx* genes in the differentiation of the GnRH subpopulation MgCs has been proposed. However, the coexpression of Dlx and

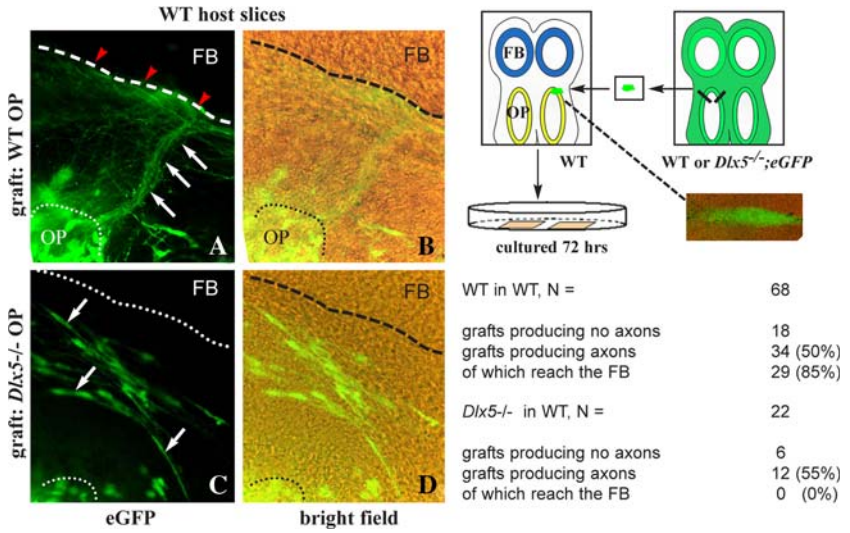

Fig. 3 The olfactory defect in *Dlx5–/–* embryos is cell-autonomous Grafting of *Dlx5–/–* OPs onto cultured slices of WT embryonic olfactory region. Donor tissues were obtained from either *Dlx5–/–;eGFP* or WT;*eGFP* (control) E10.5 embryos, derived from cross-breeding of *Dlx5+/–* and *Dlx5+/–;β-actin-eGFP* parents. Donor fragments corresponding to the dorsal-medial portion of the OP were dissected and grafted, as indicated. (**A, B**) Graft of a WT OP onto WT slices. (**C, D**) Graft of a *Dlx5–/–* OP onto WT slices. Micrographs on the right are a bright field of the fluorescent ones on the left. Results from several independent experiments are summarized in the table (bottom right)

GnRH has not been fully defined. Furthermore, the proposed role of *Dlx1-Dlx2* genes is surprising, since *Dlx5* and *Dlx6* (and to a much lower extent *Dlx3*) are the only *Dlx* genes expressed in the OE (Fig. 1). Therefore we examined expression of *Dlx5* in MgCs and the consequence of *Dlx5* inactivation. Staining of frontal sections of E10.5 WT embryos with the anti-dll (pan-Dlx) antibody reveals streams of Dlx+ cells in the frontonasal mesenchyme, between the medial-dorsal aspect of the OP and the anterior-basal FB (Fig. 4A, B). The same result was obtained by staining sections of $Dlx5^{+/lacZ}$ embryos for βgal (Fig. 4C–E). We verified that anti-dll and anti-βgal antibodies recognize the same cells, by double immunostaining of frontal sections of $Dlx5^{+/lacZ}$ embryos with the two reagents; the results indicate a perfect colocalization of the Dlx+ (nuclear) signal and the βgal+ (cytoplasmic) staining in the OP and in MgCs (Fig. 4C, D). We then carried out double immunostaining of sections from E11.5 $Dlx5^{+/lacZ}$ embryos to detect βgal and PSA-NCAM (a surface marker for immature neurites). βgal+ cells are seen exiting the OP and invading the mesenchyme between the OP and the FB, surrounded by PSA-NCAM+ neurites (Fig. 4E). Thus, the earliest OP derived MgCs belong to a Dlx5+ cell lineage.

Dlx5 is expressed by OP-derived MgCs

The location of Dlx5+ cells in the nasal mesenchyme and their association with PSA-NCAM+ axons strongly suggests they are OP-derived MgCs; however it is not a conclusive demonstration. To demonstrate their OP origin, we placed a crystal of the vital fluorescent dye CTG on the OP of thick coronal slices of $Dlx5^{+/lacZ}$ E10.5 embryos, and maintained these slices in organotypic cultures for 48 h. Under these conditions, CTG-marked cells leave the OP and migrate towards the FB (Fig. 5A). Immunostaining for βgal colocalized CTG and *Dlx5-lacZ* expression and revealed that all CTG+ cells found along these migratory streams were also βgal+, hence Dlx5+ (Fig. 5B–D). These data conclusively show that in early embryos $Dlx5^{lacZ}$ expressing cells are OP-derived MgCs.

Expression of *Dlx5* in specific subpopulation of migratory cells

We also examined E14.5 embryos for *Dlx* expression in MgCs. Based on several marker, subtypes of MgCs can be recognized at this stage. In $Dlx5^{+/lacZ}$ embryos few Xgal+ cells detach from the VNO and organize in clusters along the nerve pathway, most of them can be found near the medial-ventral aspect of the OB (Fig. 4J, K). To confirm expression of endogenous *Dlx5*, we carried out in situ hybridization with all *Dlx* probes on sagittal sections of WT E14.5 embryos. *Dlx5*-expressing cells were observed in the submucosal layer of the septum, along the VNO nerve pathway (Fig. 4F). Notably, no *Dlx3* and *Dlx6* expression was detected in corresponding sections (Fig. 4G, H).

We then examined the expression of the $Dlx5^{lacZ}$ reporter in various sub-populations of MgCs in E14.5 normal embryos, by performing double immunostaining

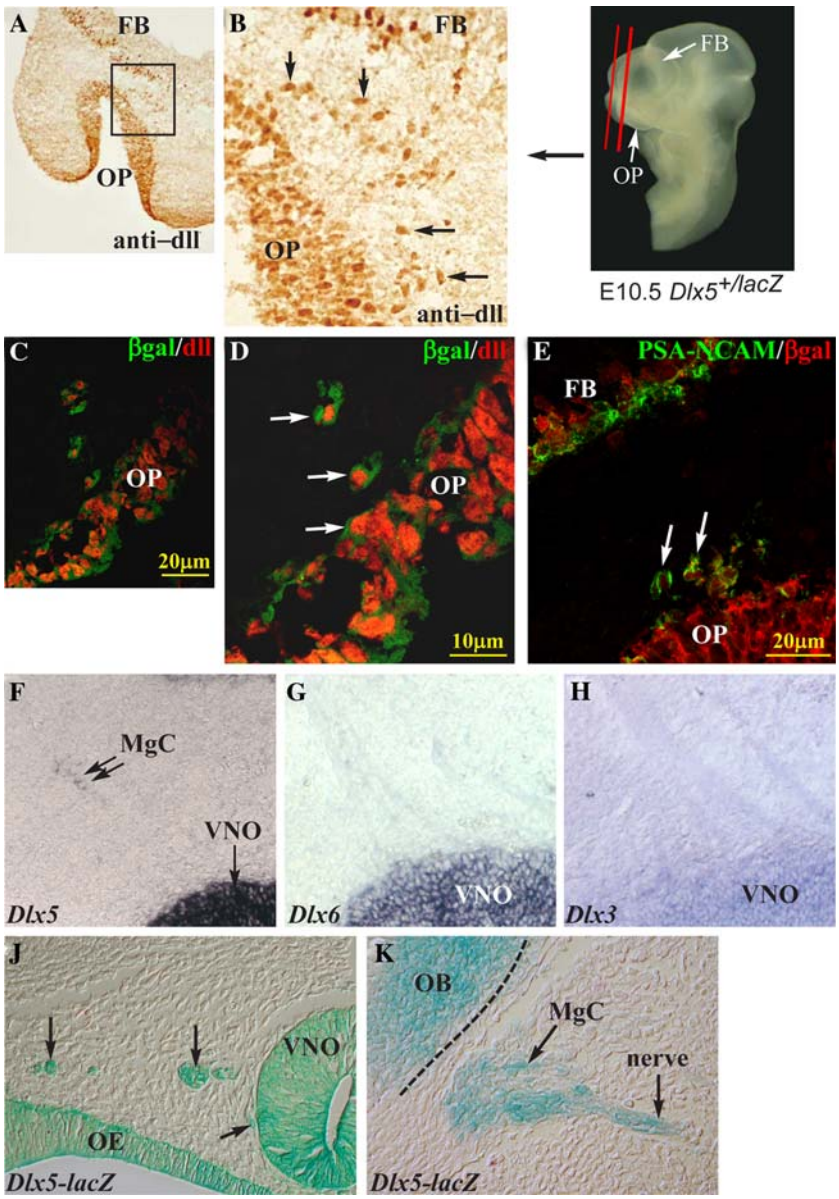

Fig. 4 Expression of *Dlx5* in migratory cells. The section planes are indicated on the right. (**A, B**) Immunostaining with anti-dll on frontal sections of E10.5 WT embryos. In (**B**) higher magnification of the area shown in **A**. Black arrows indicate Dlx+ cells in the nasal mesenchyme. (**C, D**) Double immunostaining for βgal and Dlx in similar sections from $Dlx5^{+/lacZ}$ embryos. White arrows indicate double-positive cells in the nasal mesenchyme. (**E**) Double immunostaining for βgal and PSA-NCAM in similar sections of $Dlx5^{+/lacZ}$ embryos. White arrows indicate axons closely associated to βgal+ cells. (**F–H**), In situ hybridization for *Dlx5* (**F**), *Dlx6* (**G**) and *Dlx3* (**H**) on sagittal sections of E14.5 WT embryos, through the VNO region. Black arrows indicate positive cells in the septum mesenchyme. (**J, K**) Xgal staining of frontal sections of E14.5 $Dlx5^{+/lacZ}$ embryos to show migratory cells (MgC) in the septum mesenchyme (**J**) and near the medial aspect of the OB (**K**). Abbreviations as in the text

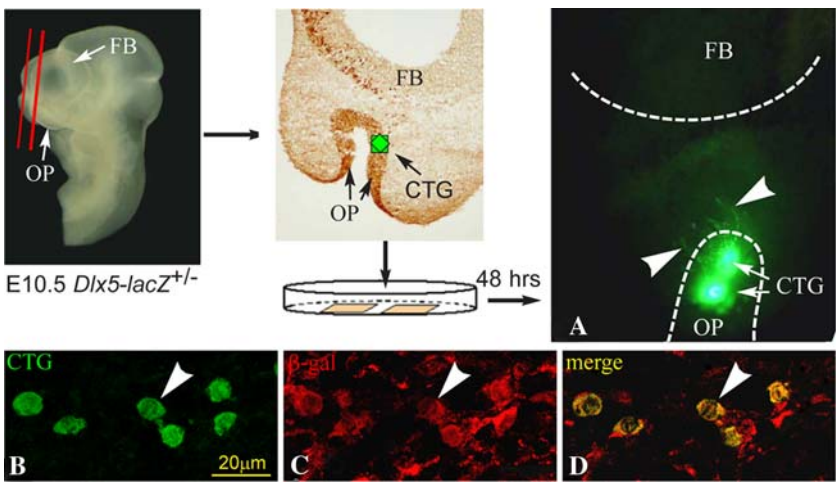

Fig. 5 *Dlx5* expressing cells are MgC derived from the OP. The experimental scheme is illustrated on the top-left. (**A**) CTG-crystals were applied onto the OP of $Dlx5^{+/lacZ}$ (normal) E11.5 embryos; after 48 h in culture streams of CTG+ (green) cells are observed (top right, low magnification). (**B–D**), Resectioning of the cultured slices followed by immunostaining for βgal, reveals double CTG+ (green) βgal+ (red) cells in the nasal mesenchyme (**D**). These cells are necessarily derived from the OP

for βgal (*Dlx5*) and either DCX (Gleeson et al. 1999), GnRH (Romanelli et al. 2004), or S100 (Astic et al. 1998; Tennent and Chuah 1996) on frontal sections of $Dlx5^{+/lacZ}$ embryos. As seen with Xgal staining, abundant βgal+ cells were found near the medial surface of the OB (Fig. 6H, K). In these cells, βgal immunoreactivity did not colocalize with DCX (data not shown), suggesting that Dlx5 is not a general marker for MgCs in this brain region.

GnRH is the best characterized marker for OP-derived neuroendocrine MgCs, destined to the septum-hypothalamic area of the CNS (Romanelli et al. 2004). In normal E14.5 embryos GnRH+ cells were observed near the OB, as expected. In $Dlx5^{+/lacZ}$ E14.5 embryos βgal immunoreactivity was found to colocalize with GnRH in approximately 70% of MgCs. Indeed, numerous βgal+ cells were negative for GnRH (Fig. 6K). Thus, the Dlx5+ cells comprise at least two subpopulations of MgCs, based on the expression of GnRH.

S100 is a marker for olfactory ensheathing cells (Astic et al. 1998). Very few S100+ cells were observed along the olfactory pathway of normal E14.5 embryos. However, abundant S100+ cells were observed in newborn animals in the OB nerve layer and associated with the olfactory nerve; however no colocalization with βgal staining was observed. Together, these results indicate that *Dlx5* expression marks a population of early MgCs rapidly migrating to the OB, a fraction of which is GnRH+ while the remainder is negative for GnRH and other markers. The fate of the Dlx+;GnRH– cells is currently unknown.

MgCs are present and differentiated in *Dlx5–/–* embryos

A detailed examination of the olfactory and migratory phenotype in *Dlx5–/–* embryos may clarify the developmental role of this transcription factor. First, we determined if there is a reduction in the number of ORNs in *Dlx5–/–* OP compared to WT. We immunostained serial sections of E11.5 and E14.5 WT and *Dlx5–/–* embryos for Emx2, which is expressed in early ORN nuclei (Mallamaci et al. 1998). Cell counts indicate a 50% and a 75% reduction in Emx2+ ORN in, respectively, E11.5 and E14.5 embryos (Fig. 6A). We expect that such numerical difference should results in a reduced number of ORN axons and MgCs. We immunostained serial sections of E10.5/E11 $Dlx5^{lacZ}$**+/–** (normal) and *Dlx5–/–* embryos for βgal and fewer axons were observed in the *Dlx5* mutant compared to WT (Fig. 6B, C), however with considerable variability between specimens.

To determine whether in *Dlx5–/–* embryos MgCs were present we stained serial sections of E14.5 *Dlx5+/–* and *Dlx5–/–* embryos for βgal. At this age early MgCs should be located near the OB while later formed MgCs should be found along VNO axons. In WT we observed streams of βgal+ cells associated to PSA-NCAM+ neurites, approaching the medial aspect of the OB (Fig. 6H). In the *Dlx5* mutant specimen βgal+ cells were found in corresponding regions associated to PSA-NCAM+ axons, although they never came into close proximity with the OB (Fig. 6J). We counted the βgal+ MgCs located distal to the VNO, and estimated a reduction of 60% in the *Dlx5–/–* embryos, compared to WT.

To further characterize the MgCs that are present in the *Dlx5* mutant embryo, we performed in situ hybridization on coronal sections of E13.5 normal and *Dlx5–/–* embryos, to detect *Bone Morphogenetic Protein-4* (*Bmp4*), which are expressed in MgCs (Peretto et al. 2002) and low-affinity NGF receptor *p75*, a marker for ensheathing cells (Ubink and Hokfelt 2000). *Bmp4* and *p75* expressing MgCs were detected in normal specimens along the migratory route (Fig. 6D, F). In the mutant, clusters of *Bmp4* and *p75* expressing cells were associated with misoriented axon bundles, in the submucosa of the nasal cavity (Fig. 6E, G).

We then examined the presence and migration of GnRH+ cells in E14.5 embryos. In normal embryos GnRH+ cells approached the OB near the midline, associated to ORN fibers (Fig. 6K). In *Dlx5* mutants abundant GnRH+ cells were observed associated with misguided and tangled OMP+ ORN axons in the nasal submucosa (Fig. 6L, M). At birth no GnRH+ cells were found in the septum-hypothalamic area of the FB in *Dlx5–/–* animals (Fig. 6P). This is consistent with the lack of olfactory connections. Instead GnRH+ cells were ectopically found in the nasal submucosa and connective tissue between the OE and the OB, associated with tangles of ORN axons (Fig. 6R, R'). In WT animals GnRH+ cells were absent from the nose and always located in the appropriate FB regions (Fig. 6N, Q).

Finally, at birth S100+ cells were abundantly present along the olfactory and VNO axon bundles, both in WT and in *Dlx5–/–* mice (data not shown).

In conclusion, the disruption of *Dlx5* results in a reduced number of MgCs, that correlates to a reduced number of ORN in the neuroepithelium, but does not appear to affect cell migration per se. Furthermore, MgC types defined by the expression of specific markers, including GnRH+ neurons, were all present. These data suggest that *Dlx5* may not be essential for GnRH differentiation, contrary to what has been previously reported in *Dlx1-Dlx2* double mutant mice (Givens et al. 2005).

Discussion

Morphogenesis and wiring of the embryonic olfactory system requires interactions between cells and signals of

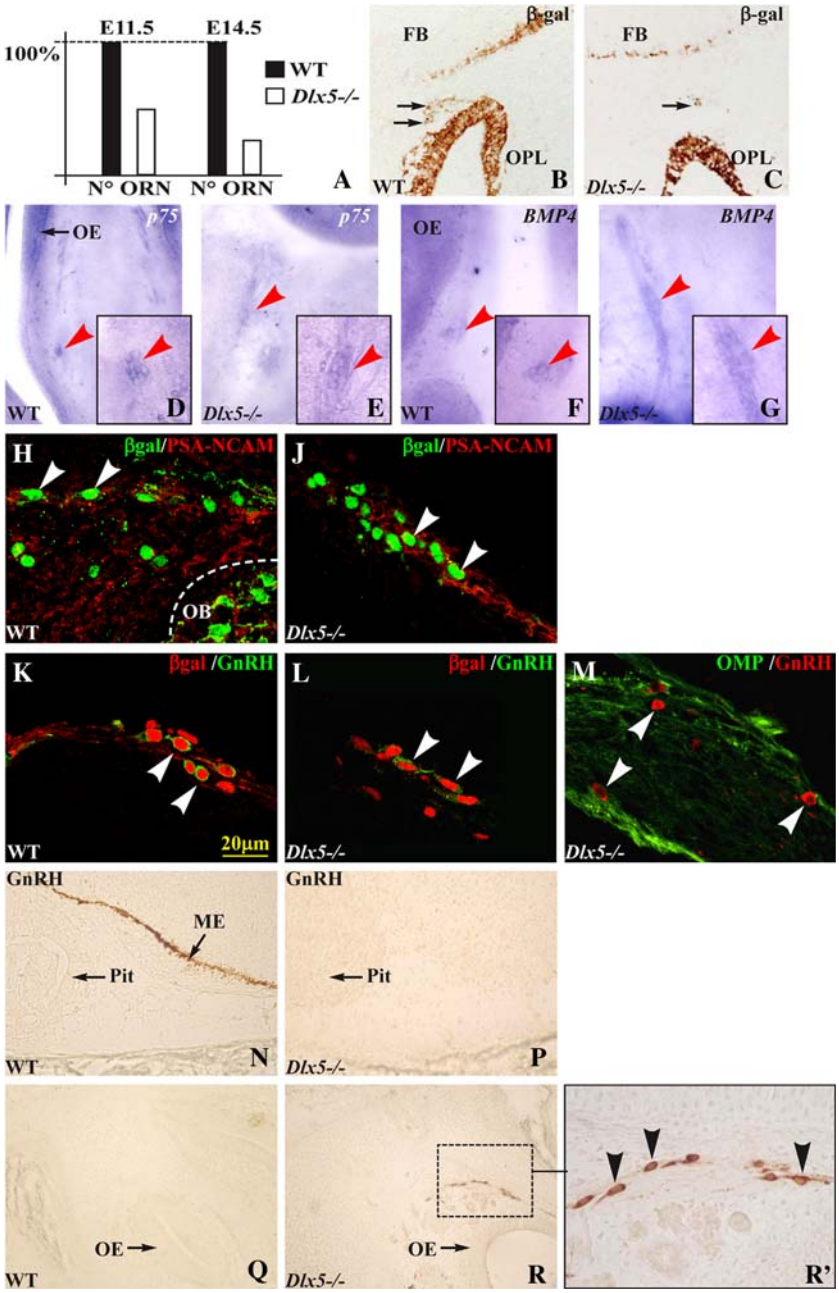

Fig. 6 MgCs in the *Dlx5–/–* embryos. (**A**) Estimation of the number of (Emx2+) ORN in WT (black bars) and *Dlx5–/–* (white bars) OPs, at E11.5 (left) and E14.5 (right). (**B, C**) Immunostaining for *β*gal on serial sections of E11 OP. Black arrows indicate tangles of axons. (**D–G**) In situ hybridization on frontal sections through the nasal region of E14.5 WT (**D, F**) and *Dlx5–/–* (**E, G**) embryos, to detect *p75* (**D, E**) and *Bmp4* (**F, G**) mRNA. Clusters of expressing cells (red arrowheads) are found in both cases, along the olfactory pathway. (**H, J**) Double immunostaining for *β*gal (green) and PSA-NCAM (red) in WT (H) and *Dlx5–/–* (**J**) E14.5 embryos, demonstrating *β*gal+ cells in both cases (white arrowheads) associated to tangles of axons. (**K, L**) Double immunostaining for *β*gal (red fluorescence) and GnRH (green) in WT (K) and *Dlx5–/–* (**L**) E14.5 embryos, showing double positive cells (white arrowheads) along the migratory pathway. (**M**) Double immunostaining for OMP (green) and GnRH (red) in a *Dlx5–/–* E14.5 specimen, revealing the presence of GnRH+ cells (white arrowheads) associated to tangled (OMP+) olfactory axons. (**N–R**) Staining for GnRH of the hypothalamic region of the FB (**N, P**) and the nasal septum (**Q, R, R'**) on longitudinal sections of WT (**N, Q**) and *Dlx5–/–* (**P, R, R'**) newborn animals. In *Dlx5–/–* animals GnRH signal is absent in the Median Eminence (ME) of the hypothalamus, while ectopic GnRH+ cells are found around the nasal septum. A higher magnification is shown in **R'**. In the WT specimen, GnRH+ cells are absent from the nasal septum but present in the hypothalamus, as expected. Genotypes (bottom left) and detected antigens (top right) are indicated for each micrograph

different origin, including the OP, MgCs, nasal mesenchyme and FB-OB (Lin and Ngai 1999; Bailey et al. 1999; St.John et al. 2002; Brunjes and Greer 2003; Bashin et al. 2003; Nedelec et al. 2005). Although the importance of FB in ORN innervation process appears to be modest (Levi et al. 2003; Hirata et al. 2006), several transcription factor genes, expressed by mature and immature ORN in the OP and OE appear to be essential for guidance and connectivity. In this report we use the *Dlx5* mutant embryos as an informative model for similar phenotypes caused by disruption of OP-expressed transcription factors. We show that all the components to organize the olfactory pathway are present but connections are invariably absent in Dlx mutants. We also show that this defect is principally cell-autonomous, supporting the notion that Dlx5 is a component of an intrinsic ORN program that confers guidance and connectivity properties to the olfactory and VNO axons.

Dlx5 codes for a transcription activator (Panganiban and Rubenstein 2002) expressed in the peripheral and central olfactory areas of the mammalian embryo. *Dlx5* expression is the earliest marker of the OP presumptive territory, before OP formation (Yang et al. 1998). As a transcription factor Dlx5 should exert its function by regulating transcription of target genes, mediating its developmental functions. It is also likely the target genes will be tissue and time-specific, as *Dlx5* is known to carry out several different embryonic functions, in the otic vesicle, limbs, pharyngeal arches and FB interneurons (Acampora et al. 2000; Levi et al. 2003; Merlo et al. 2002a, b). Although the molecular basis for the olfactory defects caused by the absence of *Dlx5* is unknown, most likely Dlx5 regulates expression of molecules essential for enabling the growth cone of ORN axons to sense the environment and engage contact with the FB. Recently we have reported that OP-derived Wnt7b is one of the signaling molecules essential for olfactory connectivity (Zaghetto et al. 2007). One logical possibility is that *Dlx5* may control *Wnt7b* expression in the OP, and/or its release by the ORN axons. However, expression of *Wnt7b* in the *Dlx5* mutant OP was not significantly changed (unpublished data). A transcription profiling approach is under way to identify *Dlx5* targets in the olfactory system.

Of the *Dlx* gene family, *Dlx5* and *Dlx6* (and to a much lesser extent *Dlx3*) are expressed in the OP and OE of the developing mouse, but not *Dlx1* and *Dlx2* (Givens et al. 2006; this article). In MgCs, *Dlx5* is the only mRNA that can be clearly detected. Therefore, it is logical to expected phenotypes affecting the peripheral olfactory system (whether axonal or migratory or both) in *Dlx5* mutant mice, rather then *Dlx1-Dlx2* mutants. Surprisingly, Givens et al. (2005) report differences in the number of GnRH+ cells in *Dlx1-Dlx2* mutant mice. Binding of Dlx and Msx protein on the *GnRH* promoter was estimated in immortalized cultured cells, and the differences in the number of MgC that reached the basal FB in *Dlx1-Dlx2* mutants is small. The reduced number of GnRH cells reaching the basal FB in *Dlx1;Dlx2* mutant animals could be explained as the consequence of the craniofacial anomalies in these mice (which consist of smaller size, dysmorphologies of skeletal elements, and general distorsion of hard and soft tissues; Qiu et al. 1997), resulting in fewer ORN cells in the OP, but maybe not to a cell-autonomous function of the genes. Here we have examined the *Dlx5–/–* animals, and demonstrated that Dlx5 marks a subset of MgCs, some of which are GnRH+. In the *Dlx5* mutant mice the reduction in the number of MCs is concomitant to a similar reduction of ORN in the OP. In spite of this, DCX+, p75+, Bmp+, S100+ and GnRH+ cells all present in the absence of *Dlx5*. As a consequence of the lack of ORN-to-FB connections (a Kallmann-like phenotype), migration of GnRH+ into the CNS is logically interrupted, but nevertheless these cells reach a certain (variable) distance, associated to misguided ORN nerve bundles. Thus there is no evidence of a function of *Dlx5* in the genesis and migration of GnRH+ cells. Although a role for *Dlx5* in GnRH+ neuron differentiation remains possible, considering that in the GnRH promoter homeodomain binding sites have been predicted (Kelley et al. 2002; Nelson et al. 2000) and GnRH expression seems to be reduced in *Dlx5–/–* MgC (this article). This important possibility needs to be further investigated.

OB innervation is thought to be coordinated by local interactions between ORN axons, MgC, FB and BM surrounding the FB. The molecular events leading to BM fenestration and formation of the nerve layer are largely unknown. ORN axons arrive near the BM of anterior FB, and organize in the MM, days prior to axonal contact with the FB, with the exception of a small number of pioneer fibers whose function for connectivity remains elusive. Abundant innervation of the presumptive OB occurs around E13–E14, when the BM separating the MM from the FB parenchyma fenestrates extensively and allows the MM to wrap on the surface of the OB (Doucette 1989). In *Dlx5–/–* embryos the BM remains intact and Wnt-βcatenin responses in the same region are strongly reduced, in spite of initial close proximity of mutant ORN axons with the FB. Recently, we have implicated OP-derived Wnt signaling in the formation of olfactory connections (Zaghetto et al. 2007). One hypothesis is that Wnt signaling on the surface of the OB activates ECM remodeling functions of resident mesenchymal cells. How *Dlx5* (and other transcription factors with similar functions, see below) might infringe on this regulation remains to be verified.

Several murine models with targeted gene inactivation display a phenotype similar to that of *Dlx5–/–*. The transcription factors *Emx2*, *Arx*, *Klf7* and *Fez1*, expressed in

the OP and OE, appear to be essential for the initial contact between the ORN axon and the FB (Yoshida et al. 1997; Mallamaci et al. 1998; Yang et al. 1998; Bishop et al. 2003; Yoshihara et al. 2004; Laub et al. 2005; Hirata et al. 2006). *Arx* is known to be regulated by *Dlx* genes in the embryonic FB (Cobos et al. 2005), and likewise might be regulated in the developing OP. *Emx2* is expressed in the OP and OE, as well as in the OB and FB. Due to the similarity of the olfactory phenotype we hypothesized that *Dlx5* might regulate expression of *Emx2*, or vice versa. However in situ hybridization and immunostaining to detect *Emx2* in the OE of *Dlx5–/–* embryos did not reveal any significant difference (unpublished data). Likewise, a phenotype similar to that of *Dlx5–/–* mice has been reported in *Klf7–/–* mice (Laub et al. 2005). We have examined expression of *Klf7* in *Dlx5–/–* embryo, and conversely the expression of *Dlx5* in *Klf7–/–* embryos, but detected no significant differences (unpublished data). Unlike the other transcription factors, the zinc-finger *Fez* is expressed only in the peripheral system (OP and OE) and not in the FB, suggesting essential involvement of the ORN axons in the establishment of olfactory connections. The targeted inactivation of *Fez* leads to a phenotype nearly identical to that of *Dlx5–/–* (Hirata et al. 2006). The similarity points to the possibility that *Fez* and *Dlx5* might participate in a signaling cascade, however we have not detected significant difference in *Fez* expression in *Dlx5–/–* embryos (unpublished data).

From these partial observations, it appears that the processes controlled by *Dlx5*, *Klf7*, *Emx2* and *Fez* transcription factors might be genetically independent. If so, the formation of initial olfactory connections is most likely the result of the coordinated action of several genes, possibly converging on the control of few key molecular events on the growth cone of extending ORN axons. Thus, the identification of the molecules regulated by these transcription factors will be the next essential step.

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