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► **To cite this version:**

Frédéric Causeret, Maxime Fayon, Matthieu Moreau, Enrico Ne, Roberto Oleari, et al.. Diversity within olfactory sensory derivatives revealed by the contribution of Dbx1 lineages. *Journal of Comparative Neurology*, 2023, 531 (12), pp.1229-1243. <10.1002/cne.25492>. <inserm-04086579>

HAL Id: inserm-04086579

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Submitted on 2 May 2023

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Diversity within olfactory sensory derivatives revealed by the contribution of *Dbx1* lineages

Running title: *Dbx1* in olfactory neurogenesis

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Abstract

In vertebrates, the embryonic olfactory epithelium contains progenitors that will give rise to distinct classes of neurons, including olfactory sensory neurons (OSN, involved in odor detection), vomeronasal sensory neurons (VSN, responsible for pheromone sensing) and GnRH neurons that control the hypothalamic-pituitary-gonadal axis. Currently, these three neuronal lineages are usually believed to emerge from uniform pools of progenitors. Here we found that the homeodomain transcription factor *Dbx1* is expressed by neurogenic progenitors in the developing and adult mouse olfactory epithelium. We demonstrate that *Dbx1* itself is dispensable for neuronal fate specification and global organization of the olfactory sensory system. Using lineage tracing we characterize the contribution of *Dbx1* lineages to OSN, VSN and GnRH neuron populations and reveal an unexpected degree of diversity. Furthermore, we demonstrate that *Dbx1*-expressing progenitors remain neurogenic in the absence of the proneural gene *Ascl1*. Our work therefore points to the existence of distinct neurogenic programs in *Dbx1*-derived and other olfactory lineages.

Introduction

Vertebrates share an olfactory sensory system specialized in odor detection and pheromone sensing (Poncelet and Shimeld, 2020). Components of the olfactory sensory system emerge from the olfactory placode (OP) that progressively thickens and differentiate into a

morphologically distinct olfactory epithelium (OE) from early steps of embryonic development (around E9.5 in mice). Olfactory sensory neurons (OSN) are the main output of the OE, they extend multiple cilia bearing transmembrane odorant receptors in the nasal cavity, and project their axon to the main olfactory bulb (OB). Vomeronasal sensory neurons (VSN), involved in pheromone sensing, arise from a specialized part of the OE and project to the accessory OB (Katreddi and Forni, 2021). Neurogenesis of OSN and VSN is a continuous process, starting around E10.5 in mice and extending throughout adulthood, therefore allowing constant cell renewal (Sokpor et al., 2018). The distinct cellular states through which OSN and VSN transit during differentiation are relatively well characterized, and include self-renewing progenitors, intermediate progenitors committed to neuronal fate, immature neurons and eventually mature OSN/VSN characterized by the expression of the olfactory marker protein (OMP) (McClintock et al., 2020; Schwob et al., 2017). In adults, the differentiation trajectories leading to the production of OSN and lineage relationship with glial components of the OE have been precisely resolved through scRNAseq (Fletcher et al., 2017; Hanchate et al., 2015).

In addition to OSN and VSN, the embryonic OP also gives rise to gonadotropin-releasing hormone (GnRH) neurons (Duan and Allard, 2020; Wray et al., 1989). Upon specification at the vicinity of the vomeronasal organ (VNO) anlage around E11.5 in mice, GnRH neurons delaminate from the OP and engage a long range migration in the nasal mesenchyme towards the basal forebrain, to eventually settle in the hypothalamus (Cho et al., 2019). These neurons are of critical physiological importance, as GnRH secretion regulates the release of luteinizing and follicle-stimulating hormones from the pituitary gland, and subsequent production of sexual steroids from the gonads thus regulating sexual maturation and reproductive function. Consequently, defects in the specification or migration of GnRH neurons result in hypogonadotropic hypogonadism, as observed in Kallmann syndrome (Oleari et al., 2021). Some OP derivatives remain poorly characterized. This is the case of the so-called “migratory mass” (MM), a heterogeneous population including neurons and glial cells, strategically positioned in between the OE and ventral telencephalon. To date, the precise composition, origin and function of the MM is still poorly defined, although a role as guidepost cells for pioneer OSN axons was proposed (Miller et al., 2010; Valverde et al., 1992).

The molecular logic of neuronal specification in the olfactory sensory system is being increasingly understood and appear similar at embryonic and adult stages (Sokpor et al., 2018). First, progenitors pools express and require the transcription factor *Sox2* for their maintenance and proliferation (Donner et al., 2007). Commitment to neuronal differentiation involves the transition to intermediate progenitor state and is regulated by the successive expression of *Ascl1* and *Neurog1* (Cau et al., 1997; Cau et al., 2002; Guillemot et al., 1993). Finally, lineage-dependent maturation programs unfold and enable appropriate neuronal maturation. Although the main transcription factors expressed in the OE/VNO and involved in fate specification have been described (Chang and Parrilla, 2016; Parrilla et al., 2016), some remain uncharacterized. This is the case of *Dbx1* which is the main focus of this study. *Dbx1* is a homeodomain transcription factor whose expression is largely restricted to the developing nervous system (Lu et al., 1992; Shoji et al., 1996). In the spinal cord, hindbrain and hypothalamus, *Dbx1* is involved in neuronal fate specification (Bouvier et al., 2010; Pierani et al., 2001; Sokolowski et al., 2015). We show that *Dbx1* transcript and protein are

dynamically expressed during embryonic and adult olfactory neurogenesis. Reminiscent of the central nervous system, *Dbx1* expression is spatially and temporally constrained to committed neural progenitors. We found *Dbx1* expression to be dispensable for neuronal production and global organization of the olfactory sensory system. We performed lineage tracing to follow the progeny of *Dbx1*-expressing progenitors and surprisingly found a contribution to OSN, VSN, GnRH and MM neurons, therefore revealing an unanticipated degree of diversity within these populations. Finally, we examined *Ascl1* mutants to reveal that unlike all other known olfactory progenitors, *Dbx1*-expressing cells remain neurogenic in these animals. Our work therefore points to previously unknown mechanism of neuronal specification and diversification in the developing olfactory sensory system.

Material & Methods

Animals

All mouse work was approved by the French Ministry of Higher Education, Research and Innovation as well as the Animal Experimentation Ethical Committee of Paris Descartes University (CEEA-34, licence number: 18011-2018012612027541). The following mouse lines were used: *Dbx1^{LacZ}* (Pierani et al., 2001), *Dbx1^{Cre}* (Bielle et al., 2005), *Rosa26^{LoxP-Stop-LoxP-YFP}* (Srinivas et al., 2001), *Tau^{GFP-NLS-LacZ}* (Hippenmeyer et al., 2005) and *Ascl1* (*Mash1*) knock-out (Guillemot et al., 1993).

Immunostaining and *in situ* hybridization

Samples were fixed by immersion in 4% paraformaldehyde in 120mM phosphate buffer (PB) for 1-4h. They were cryoprotected overnight in 10% sucrose in PB and embedded in 7.5% Gelatin, 10% sucrose in PB. 20 μ -thick cryostat sections were performed and processed for *in situ* hybridization, immunostaining or X-Gal staining (Bielle et al., 2005; Causeret et al., 2011).

In situ hybridization probes for *Dbx1*, *Ascl1* (*Mash1*), *Neurog1* (*Ngn1*), and *Lhx2* were generated by *in vitro* transcription of linearized plasmids described elsewhere (Cau et al., 1997; Guillemot and Joyner, 1993; Lu et al., 1992; Rétaux et al., 1999). Probes for *Stmn2* (*Scg10*), *Gap43*, *Ncam1*, *Omp*, *Gnrh1*, *Vmn1r180* (*V1rd16*) and *Vmn2r121* were obtained from PCR fragments amplified using primers indicated in Supplementary Table 1. Because of sequence homology, both vomeronasal receptor probes are predicted to hybridize with multiple genes of their respective family and were therefore referred to as *Vmn1r* and *Vmn2r* probes in this study. Probes for olfactory receptors were obtained from PCR fragments amplified using previously published primers (Miyamichi et al., 2005).

References and dilutions for primary and secondary antibodies are detailed in Table 1 and Table 2, respectively.

Antibody characterization

The GFP antibody has been analyzed by western blot and immunohistochemistry using transgenic mice expressing GFP (manufacturer data).

The β -Galactosidase antibody has been analyzed by immunohistochemistry using transgenic mice expressing β -Galactosidase (manufacturer data).

The Phospho-Histone H3 (Ser10) monoclonal antibody recognizes phosphorylated histone H3 at Ser10 and does not cross-react with histone H3 phosphorylated at Ser28. The phosphor specificity was confirmed by Western blots on HeLa cell lysates that have a band at 17 kDa which is abolished by treatment of lysates with λ phosphatase (manufacturer data).

The Cleaved Caspase-3 (Asp175) monoclonal antibody detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175 and does not recognize full-length caspase-3 or other cleaved caspases (manufacturer data).

The Dbx1 antibody specificity was previously assessed by immunostaining on mouse spinal cord sections. Staining of wild-type tissue was consistent with *Dbx1* mRNA expression, and no staining was observed in *Dbx1* knock-out mice (Pierani et al., 1999; Pierani et al., 2001).

The Isl1/2 antibody was previously characterized by immunostaining on mouse spinal cord sections. (Dasen et al., 2005). Our staining in the olfactory system concurred with previous observations (Shan et al., 2020; Taroc et al., 2020b).

The GnRH antibody produces a strong positive labeling of LHRH/GnRH in rat hypothalamus (median eminence) and the staining is completely abolished by pretreatment of the diluted antibody with GnRH (manufacturer data). It is widely employed for the detection of mouse GnRH neurons in embryonic and adult tissues and recognizes both the processed and the pre-hormone and mature peptide (Cariboni et al., 2015; Macchi et al., 2017; Taroc et al., 2017; Whittaker et al., 2021).

The Sox2 antibody detects a specific band at approximately 36 kDa in Western blots multiple mouse cell lines and labels murine stem cells by immunocytochemistry (manufacturer data). It recognizes neural progenitors in the developing OE (Engel et al., 2016) and produced a pattern consistent with previously published data (Donner et al., 2007; Tucker et al., 2010).

The OMP antiserum is from a goat that received multiple immunizations with purified rodent Olfactory Marker Protein (OMP). It recognizes a single band at 19 kDa on a western blot of mouse brain and pre-incubation with the immunogen peptide blocks all staining (Baker et al., 1989; Koo et al., 2004). It is widely used to stain mature OSNs in mouse developing and postnatal olfactory epithelium as well as cells in the migratory mass (Cariboni et al., 2015; Guillemot et al., 1993; Miller et al., 2010; Taroc et al., 2020b).

The CTIP2/Bcl11b monoclonal antibody detects 2 bands representing CTIP2 at about 120 kDa in human Jurkat cells and mouse brain lysates (manufacturer data). The detection of mature OSNs is consistent with previous reports (Enomoto et al., 2011) and its specificity was assessed in *Ctip2/Bcl11b* knock out mouse tissues (Enomoto et al., 2019).

The TUBB3 antibody is a monoclonal antibody that detects a single 50kDa band on Western blots of mouse brain lysates (manufacturer data) and has been widely used for the detection of neuronal cells and processes, including in the mouse olfactory system (Murdoch and Roskams, 2008; Tucker et al., 2010).

The Peripherin antibody stains a single 57 kDa band on a Western Blot on PC12 cell lysates and does not stain vimentin, GFAP, alpha-internexin or any of the neurofilament subunits (manufacturer data). The expression on vomeronasal/terminal axons in the nasal parenchyma of mouse embryos is similar to what has been previously shown (Cariboni et al., 2015; Casoni et al., 2016).

The Lhx2 antibody generates a band of 43 kDa on Western blots of mouse brain lysates. The staining pattern observed in the olfactory system was consistent with published data (Ikeda et al., 2007; Miller et al., 2010).

The mouse anti-HuC/D antibody labels mature and immature neurons (Saito et al., 2018) and its specificity was confirmed by blocking peptide with the HuD peptide (Marusich et al., 1994). HuC/D staining was previously observed in in post-mitotic neurons within the OE (Wittmann et al., 2014).

Imaging

Visible staining was acquired either on a Zeiss Axiovert microscope, a Keyence VHX-2000 microscope or a Hamamatsu Nanozoomer 2.0 slide scanner. Fluorescent images were obtained with Zeiss LSM710 or Leica SP5 confocal microscopes.

Results

Dbx1 is expressed in the olfactory epithelium

Dbx1 was previously reported to be expressed in the olfactory placode at E9.5 in mouse (Causeret et al., 2011). Given its well-characterized function in neuronal fate specification (Bouvier et al., 2010; Pierani et al., 2001; Sokolowski et al., 2015), we thought to evaluate its putative involvement in olfactory neurogenesis. We performed *in situ* hybridization experiments on coronal cryosections of mouse embryos at E10.5 and E11.5, stages corresponding to the onset of neuronal production. We found *Dbx1* to be expressed by scattered cells that preferentially occupied medial or dorso-lateral positions within the olfactory epithelium (OE) at E10.5 (Figure 1a). A similar distribution was found at E11.5 with most *dbx1*-expressing cells being found in the anterior and medial aspect of the OE and only few in dorso-lateral regions (Figure 1a). At this stage, *Dbx1* expression was not detected in the caudal half of the OE (not shown). Immunostaining confirmed that the Dbx1 protein is expressed in the OE (Figure 1b). We then used the *Dbx1^{LacZ/+}* mice in which expression of β -galactosidase (β Gal) was previously shown to faithfully recapitulate that of *Dbx1* (Pierani et al., 2001). X-Gal staining indicated that Dbx1 expression decreased from E12.5 to E14.5, with only few cells found within the developing OE, and increased again at E18.5 (Figure 1c). Together, these data indicate that *Dbx1* is dynamically expressed in the embryonic olfactory epithelium.

In the mouse developing brain and spinal cord, Dbx1 is expressed by neurogenic progenitors and subsequently downregulated in postmitotic neurons (Bielle et al., 2005; Lu et al., 1992; Pierani et al., 2001). In order to test whether this also applies to the olfactory system, we analyzed *Dbx1* expression together with a set of genes which are sequentially upregulated during olfactory neurogenesis. Thus, early progenitors committed to a neuronal fate express the proneural gene *Ascl1* (formerly known as *Mash1*), they generate immediate neuronal precursors (INPs) identified by *Neurog1* expression, which undergo terminal differentiation in postmitotic neurons expressing *Stmn2* (*Scg10*), *Gap43* and finally *Ncam1* according to their maturation state (Calof et al., 2002). Double fluorescent *in situ* hybridization experiments performed at E11.5 indicated that *Dbx1* mRNA never colocalized with either *Ascl1*, *Stmn2*,

Gap43 or *Ncam1* (Figure 1d). By contrast, we found all *Dbx1*-expressing cells to be *Neurog1*⁺ (Figure 1d). We therefore concluded that *Dbx1* expression is temporally regulated during the process of olfactory neurogenesis, restricted to a subset of INPs. We then took advantage of the fact that in *Dbx1*^{LacZ/+} embryos, βGal persists longer than the *Dbx1* protein, resulting in short term tracing (Pierani et al., 2001). Immunostaining on these embryos indicated that β-Gal colocalizes with *Lhx2*, which is expressed by INPs and maintained in neurons, *HuC/D*, an early neuronal marker, and *CTIP2*, an early marker of OSN, but is almost never found in *Sox2*⁺ progenitor cells (Figure 1e). Taken together, these results indicate that *Dbx1* is expressed by INPs just prior to neuronal differentiation (Figure 1f).

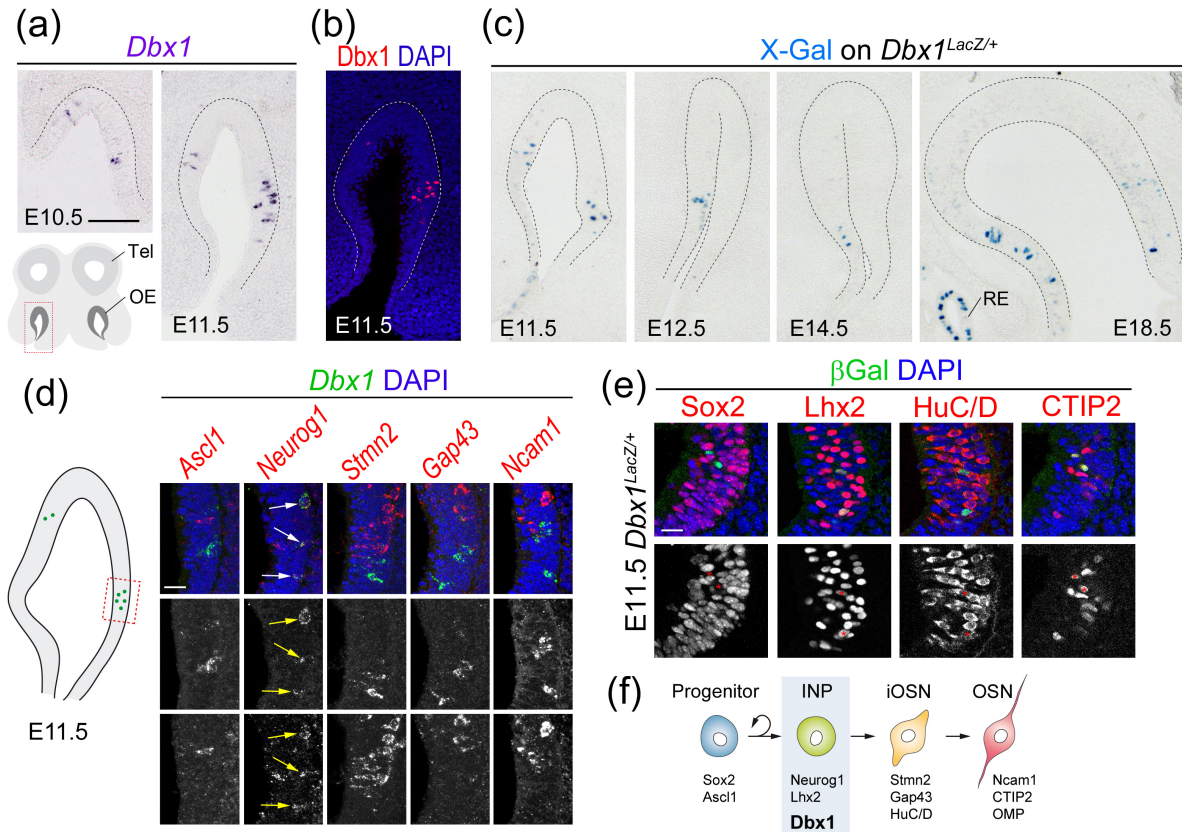


Figure 1. Expression of *Dbx1* during olfactory development

(a) *In situ* hybridization using a probe against *Dbx1* on coronal sections of the E10.5 and E11.5 mouse olfactory pit. The bottom left drawing indicates the level at which sections were collected. (b) Immunostaining for *Dbx1* (red) on sections similar to (a). Nuclei are stained using DAPI (blue). (c) X-Gal staining on coronal sections from E11.5 to E18.5 *Dbx1*^{LacZ/+} embryos. (d) High magnification of a region of the E11.5 olfactory epithelium containing *Dbx1*-expressing cells (as indicated on the drawing) following double fluorescent *in situ* hybridization with probes against *Dbx1* (green) and the indicated genes (red). Nuclei are shown in blue using DAPI. (e) Immunofluorescent staining on E11.5 *Dbx1*^{LacZ/+} embryos using antibodies against βGalactosidase (green) and the indicated proteins (red) and DAPI (blue) to stain nuclei. (f) Drawing summarizing the findings: *Dbx1* is expressed by neurogenic progenitors. OE: olfactory epithelium, Tel: telencephalon, RE: respiratory epithelium. INP: intermediate neural progenitor, OSN: olfactory sensory neuron, iOSN: immature OSN. Scale bars: 100μm in a, 20μm in d, e.

A strong *Dbx1* expression was also found in adults as illustrated by X-Gal staining of *Dbx1*^{LacZ/+} animals (Figure 2a,b). The pattern of staining, with sharp boundaries between positive and negative regions in the OE, indicated that *Dbx1* expression is zonally restricted at this stage (Figure 2b). In order to identify the cells that express *Dbx1*, we stained the olfactory epithelium with antibodies against *Sox2* and *CTIP2* to distinguish between glial

sustentacular (SUS) cells (Sox2⁺) on the apical side and the neuronal compartment (CTIP2⁺) on the basal side (Figure 2c). We found that 71% ± 6% βGal⁺ cells are neurons and the remaining are SUS cells (Figure 2e). In addition, immunostaining revealed that approximately half of βGal⁺ cells (46% ± 5%) are mature olfactory sensory neurons (OSNs) expressing the olfactory marker protein (OMP) (Figure 2d,e). We therefore concluded that *Dbx1* is expressed during both embryonic and adult olfactory neurogenesis as well as during adult gliogenesis.

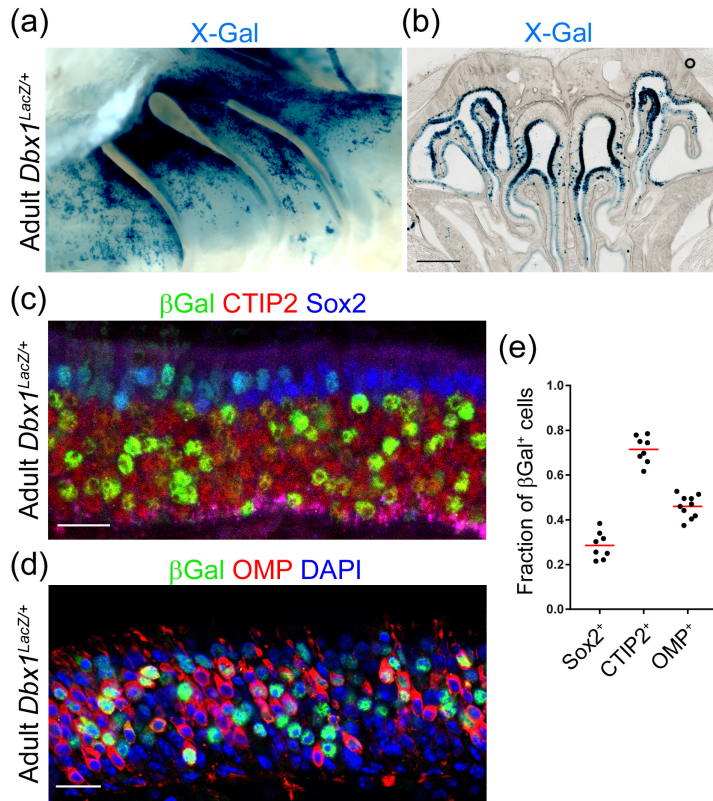


Figure 2. *Dbx1* expression in the adult olfactory epithelium

(a) Whole-mount X-Gal staining of the olfactory epithelium of an adult *Dbx1*^{LacZ/+} mouse. (b) X-Gal staining on a coronal section through the olfactory epithelium of an adult *Dbx1*^{LacZ/+} mouse. (c) Immunostaining on the olfactory epithelium of an adult *Dbx1*^{LacZ/+} mouse using antibodies against βGalactosidase (green), CTIP2 (red) and Sox2 (blue). (d) Immunostaining on the olfactory epithelium of an adult *Dbx1*^{LacZ/+} mouse using antibodies against βGalactosidase (green) and OMP (red), as well as DAPI to stain nuclei (blue). (e) Quantification of the percentage of marker-positive cells among βGalactosidase-positive cells. Scale bars: 1mm in b, 20μm in c, d.

***Dbx1* is dispensable for olfactory neurogenesis**

In order to investigate the consequences of *Dbx1* loss on olfactory neurogenesis, we analyzed *Dbx1*^{LacZ/LacZ} animals and compared them to *Dbx1*^{LacZ/+} counterparts. Because mutant animals die at birth (Pierani et al., 2001), analyses could only be conducted up to late embryonic stages. In mutants, the spatio-temporal pattern of βGal expression in the developing OE appeared similar to heterozygous littermates (compare Figures 3a and 1c), although the intensity of βGal staining as well as the number of βGal⁺ cells was increased (see also Figures 3b, c, e and data not shown). This could result from (i) increased proliferation of βGal⁺ cells, (ii) decreased cell death or (iii) increased βGal accumulation due to the presence of a second LacZ allele. We first performed PH3 immunostaining to label mitotic cells, and found no significant differences between control and mutants, either in the distribution or number of positive cells (57 ± 9 per mm of OE and 54 ± 13 respectively; Figure 3c). To detect apoptosis, we performed immunostaining against activated Caspase3. In both mutant and heterozygous animals, we found very few apoptotic cells (9 ± 3 per mm of OE and 7 ± 4 respectively) indicating that the loss of *Dbx1* does not impair cell viability (Figure 3d).

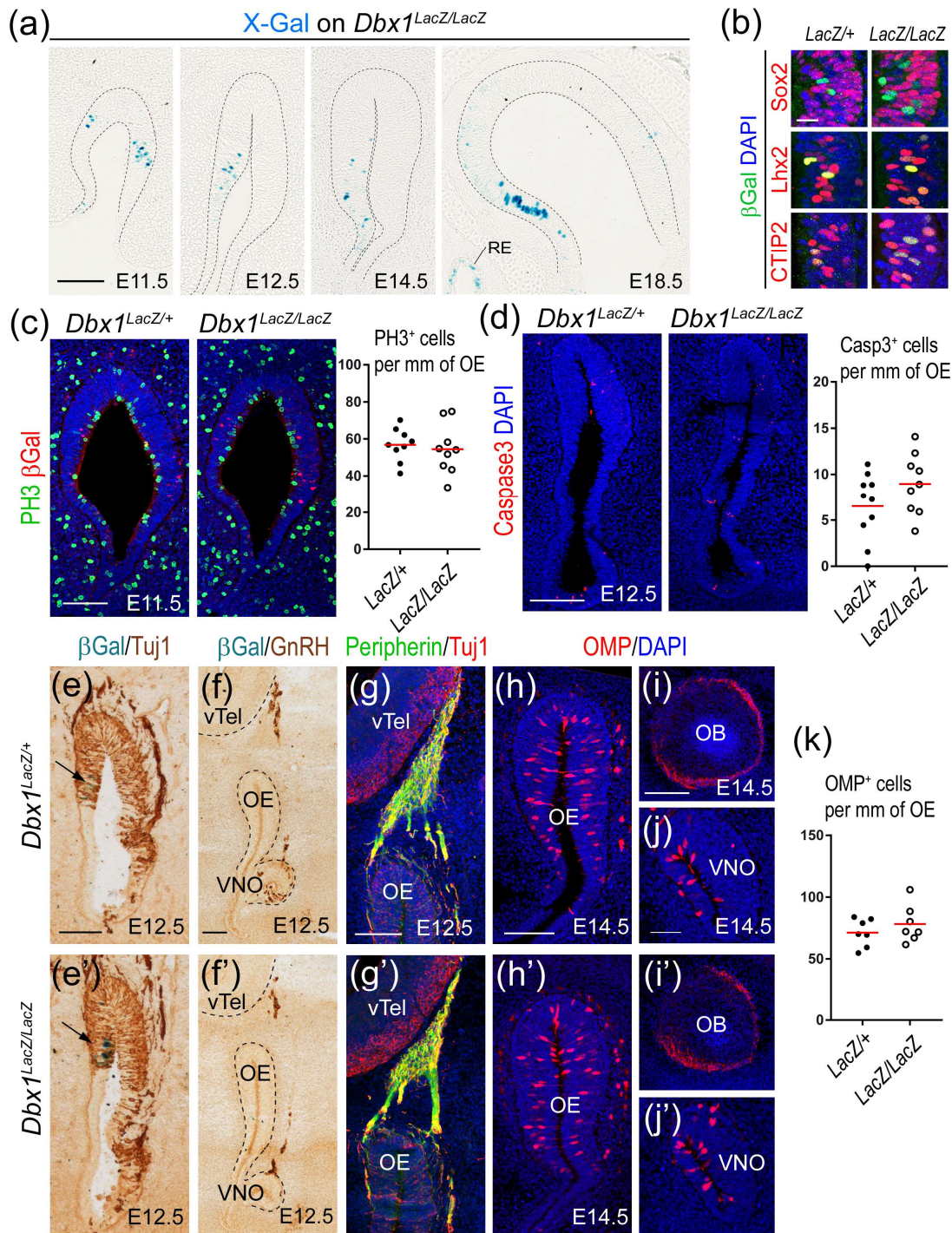


Figure 3. Consequences of *Dbx1* loss during olfactory neurogenesis

(a) X-Gal staining on coronal sections from E11.5 to E18.5 *Dbx1^{LacZ/LacZ}* embryos. (b) Immunostaining for β Galactosidase (green) and either Sox2, Lhx2 or CTIP2 (red) in *Dbx1^{LacZ/+}* and *Dbx1^{LacZ/LacZ}* embryos. (c) Immunostaining against phospho-histone 3 (green) and β Galactosidase (red) on coronal cryosections of the olfactory pit in control *Dbx1^{LacZ/+}* and mutant *Dbx1^{LacZ/LacZ}* E11.5 embryos. Quantification of mitotic PH3⁺ cells are shown on the right. (d) Immunofluorescence against activated caspase 3 (red) in control *Dbx1^{LacZ/+}* and mutant *Dbx1^{LacZ/LacZ}* E12.5 embryos. Quantification of apoptotic Caspase 3⁺ cells are shown on the right. (e, e') DAB immunostaining against Tuj1 (brown) combined with X-Gal staining (cyan) in *Dbx1^{LacZ/+}* (e) and *Dbx1^{LacZ/LacZ}* (e') E12.5 embryos. (f, f') Same as e, e' using GnRH immunostaining. (g, g') Immunofluorescence against Peripherin (green) and Tuj1 (red) in control (g) and mutant (g') embryos. (h-j, h'-j') OMP immunostaining showing the E14.5 OE (h, h'), OB (i, i'), and VNO (j, j'). (k) Quantification of OMP⁺ cells. vTel: ventral telencephalon, OE: olfactory epithelium, VNO: vomeronasal organ, OB: olfactory bulb. Scale bars: 100 μ in a, c, d, e, f, g and h, 20 μ m in b, 200 μ m in i, 50 μ m in j.

We then tested whether the loss of *Dbx1* could impact cell identity. Similar to heterozygous controls, we found that mutant βGal^+ cells were *Sox2*⁻, *Lhx2*⁺ and *CTIP2*⁺ (Figure 3b), indicating that they retain their identity of neurogenic progenitor despite the loss of *Dbx1*. We further analyzed olfactory neurogenesis in mutant and control animals. Tuj1 staining appeared similar in control and mutant OE, including in the area of βGal^+ cells (Figure 3e, e'). The number and trajectory of migrating GnRH⁺ neurons was also unchanged at E12.5 (Figure 3f, f'). Bundles of sensory axons growing from the OE to the brain, identified by Peripherin or Tuj1 staining, appeared normal in thickness and position in *Dbx1*^{LacZ/LacZ} animals (Figure 3g, g') suggesting that OSN/VSN axon guidance does not require *Dbx1*. This was confirmed by OMP staining at E14.5, which allowed to visualize cell bodies of mature OSN (Figure 3h, h') and VSN (Figure 3j, j') in the OE and VNO, respectively, as well their axons reaching the olfactory bulb (Figure 3-i, i'). No qualitative or quantitative difference (Figure 3k) was found between control and mutants.

In order to assess terminal neuronal differentiation, we monitored the expression of vomeronasal and olfactory receptor genes in control and mutant animals at E18.5 (Figure 4a). In the VNO, neurons expressing receptors from the V1R family (Dulac and Axel, 1995) occupy apical positions whereas those expressing V2R genes (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997) are located more basally. *In situ* hybridization experiments using probes targeting multiple genes of either family indicated that such an organization is maintained in *Dbx1* mutants (Figure 4b). In the OE, the expression of olfactory receptor genes is subjected to zonal restriction such that OSN expressing the same receptor are confined to the same region (Ressler et al., 1993; Vassar et al., 1993; Zapiec and Mombaerts, 2020). At E18.5, *Omp* expression indicates that the number and distribution of mature OSN are comparable in mutants and control littermates (Figure 4c), consistent with our findings at E14.5. In addition, the zonal organization of the OE appears unaffected by *Dbx1* loss as illustrated by the consecutive expression of *Olfir247*, *Olfir1395*, *Olfir449* and *Olfir1507* in consecutive dorsomedial to ventrolateral domains (Figure 4c).

Taken together our data suggest that the production and maturation of the various neuronal subtypes in the developing olfactory system is not dramatically affected in the absence of *Dbx1*.

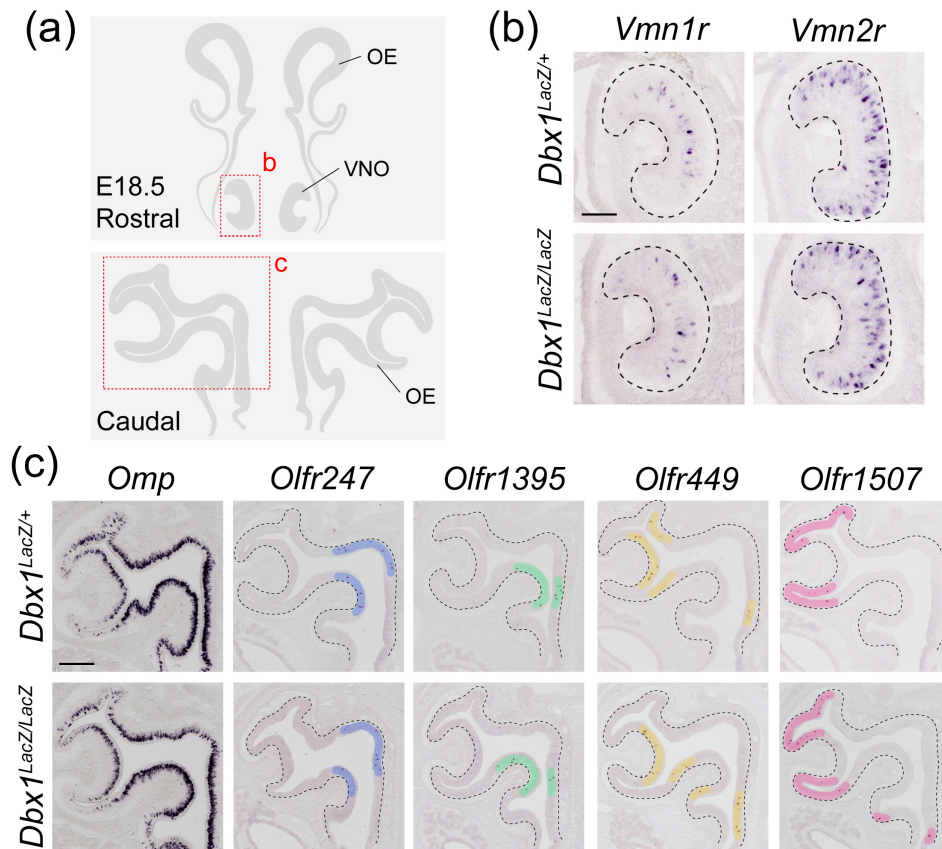


Figure 4. Consequences of *Dbx1* loss on the zonal organization of the olfactory system

(a) Drawing of the position corresponding to the distinct panels of the figure. (b) *In situ* hybridization using probes against *Vmn1r* and *Vmn2r* genes in *Dbx1^{LacZ/+}* and *Dbx1^{LacZ/LacZ}* embryos. (c) *In situ* hybridization using probes against *Omp*, *Olfr247*, *Olfr1395*, *Olfr449* and *Olfr1507* in *Dbx1^{LacZ/+}* and *Dbx1^{LacZ/LacZ}* embryos. OE: olfactory epithelium, VNO: vomeronasal organ. Scale bars: 100 μ m in b, 200 μ m in c.

***Dbx1* lineages contribute to multiple components of the olfactory system**

Given *Dbx1* expression in olfactory neural progenitors, we decided to explore the contribution of the *Dbx1* lineage to the various components of the olfactory system. We performed genetic tracing using *Dbx1^{Cre};R26^{YFP}* animals (Griveau et al., 2010) in order to permanently label the progeny of *Dbx1*-expressing progenitor cells. At E10.5, consistent with the early onset of *Dbx1* expression, scattered GFP⁺ cells were found in the developing OE (Figure 5a,b). In addition, GFP⁺ cells were detected in the mesenchyme between the OE and the ventral telencephalon, an area containing a group of cells referred to as the migratory mass (MM) (Miller et al., 2010; Valverde et al., 1992). The MM was previously described as a heterogeneous population, containing both neuronal and glial cells, and proposed to act as a guidepost for sensory axons growing towards the brain (Miller et al., 2010). We found that the *Dbx1* lineage exclusively contributes to the neuronal content of the developing MM as GFP⁺ cells are also positive for Tuj1, which labels neurons, but never for BLBP, a marker for the glial olfactory ensheathing cells (Figure 5b). Consistently, most GFP⁺ cells in the MM were found to be Lhx2⁺ whereas a fraction expressed OMP (Figure 5b). At E11.5 GFP⁺ cells were found in both the OE and the respiratory epithelium (Figure 5c). Consistent with our *Dbx1* expression data, most *Dbx1*-derived cells in the OE were found to express Lhx2 and CTIP2 but rarely Sox2, indicative of their neuronal (rather than progenitor) identity. At E12.5, *Dbx1*-derived cells were found in the vomeronasal organ (VNO) (Figure 5d). In addition,

GFP⁺ cells were observed in the mesenchyme medial to the OE, forming streams of cells displaying a morphology highly reminiscent of neurons migrating from the VNO towards the brain. Most but not all of these cells could be identified as GnRH⁺ neurons (Figure 5e). We found that the Dbx1-lineage accounts for approximately 28% ± 9% of all GnRH neurons. Recently, the transcription factor *Isl1* was shown to be specifically expressed by most developing GnRH neurons and proposed to discriminate two distinct subpopulations (Shan et al., 2020; Taroc et al., 2020b). To determine whether Dbx1-related heterogeneity correlates with *Isl1* expression, we performed immunostaining at E12.5. We found that at this stage almost all GnRH⁺ cells express *Isl1/2*, regardless of their belonging to the Dbx1 lineage or not (Figure 3f, 97% ± 3% of GFP⁺ and 98% ± 1% of GFP⁻ cells).

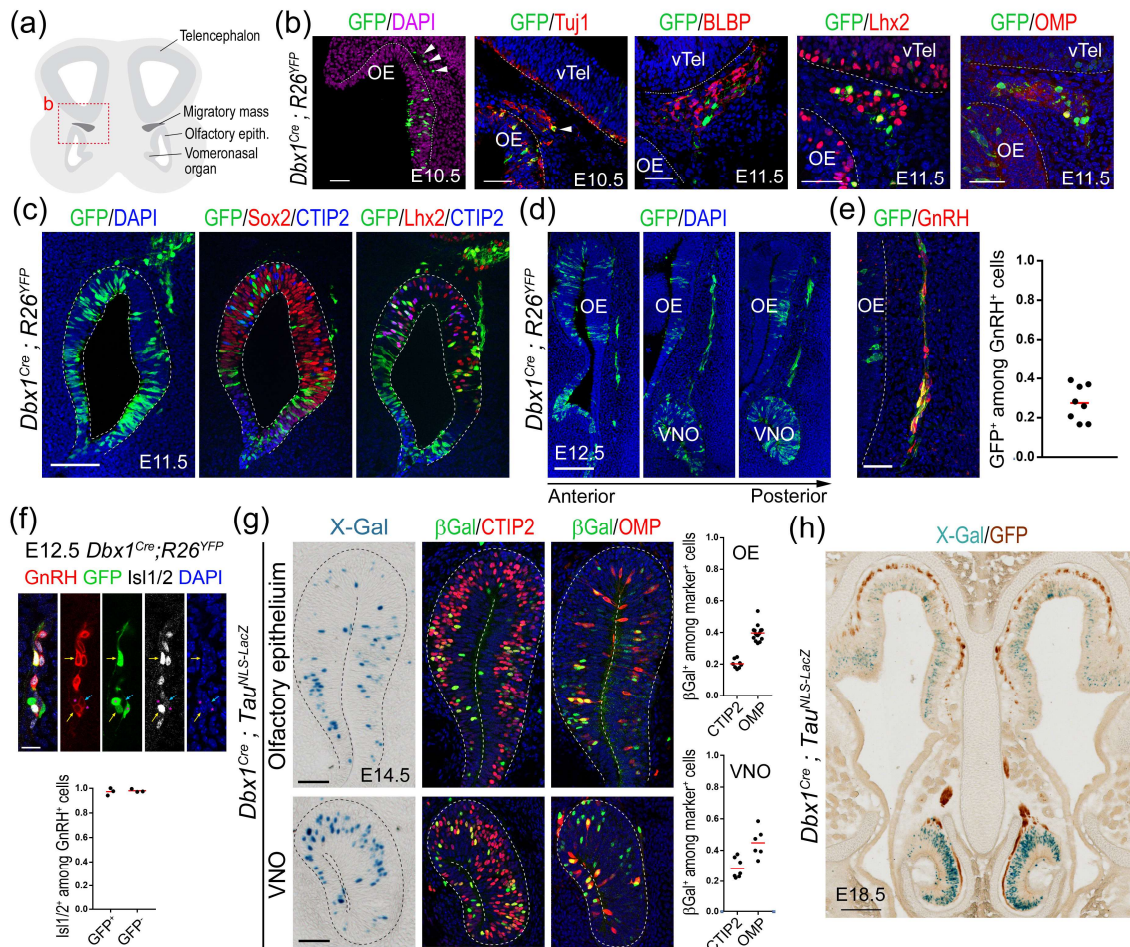


Figure 5. Genetic tracing of the Dbx1 lineage in olfactory epithelium derivatives

(a) Drawing of the region of the migratory mass shown in (b). (b) Immunofluorescence against GFP (green) and either Tuj1, BLBP, Lhx2 or OMP (red) in *Dbx1^{Cre};Rosa26^{YFP}* embryos of the indicated stage. DAPI is shown in blue or purple. (c) Immunofluorescence for GFP (green), Sox2 or Lhx2 (red) and DAPI or CTIP2 (blue) at E11.5. (d) Immunostaining of GFP along the antero-posterior axis of the olfactory system at E12.5. (e) Co-immunostaining for GFP (green) and GnRH (red), and quantification of the overlap. (f) Immunofluorescence for GFP (green), GnRH (red) and *Isl1/2* (white) at E12.5. Yellow arrows point to cells of the Dbx1 lineage expressing both GnRH and *Isl1/2*, pink asterisks indicate cells of the Dbx1-lineage double-negative for GnRH and *Isl1/2*. The blue arrow shows one of the very few GnRH neurons we classified as negative for *Isl1/2* staining. Quantification is shown at the bottom. (g) X-Gal staining (cyan) and immunofluorescence for β Galactosidase (green) and CTIP2 or OMP (red) in the OE (top) and VNO (bottom) of E14.5 *Dbx1^{Cre};Tau^{YFPNLS-LacZ}* embryos. Quantification of the colocalisation is shown. (h) X-Gal staining (cyan) combined with DAB staining for GFP (brown) at E18.5 in *Dbx1^{Cre};Tau^{YFPNLS-LacZ}* embryos. vTel: ventral telencephalon, OE: olfactory epithelium, VNO: vomeronasal organ. Scale bars: 50 μ m in b, e and g, 100 μ m in c and d, 20 μ m in f, 200 μ m in h.

From E14.5 onwards, it is possible to genetically label specifically the neuronal progeny of Dbx1-expressing cells using *Dbx1^{Cre};Tau^{NLS-LacZ}* animals. Dbx1-derived neurons are evident in both the OE and VNO at this stage (Figure 5g). Quantifications indicated that in the OE, the Dbx1 lineage accounts for 20% ± 3% of immature CTIP2⁺ neurons and 40% ± 5% of fully differentiated OMP⁺ OSNs, suggesting a decrease with time in the contribution of the Dbx1 lineage to olfactory neurogenesis. Similarly, in the VNO, 28% ± 7% of immature neurons and 45% ± 9% of mature vomeronasal sensory neurons (VSNs) derive from Dbx1-expressing progenitors (Figure 5g). At E18.5 the distribution of Dbx1-derived neurons within the OE and VNO was found to be homogenous (Figure 5h).

Taken together, these data indicate that Dbx1-expressing progenitors contribute significantly to the various neuronal components of the peripheral olfactory system, and support the idea that OSNs, VSNs and GnRH neurons are heterogeneous populations.

Dbx1⁺ progenitors remain neurogenic in *Ascl1* mutants

Previous studies concur to the idea that the proneural transcription factor *Ascl1* is a key player in olfactory neurogenesis (Sokpor et al., 2018). In the OE of *Ascl1* mutants, the expression of *Neurog1* is almost completely lost and that of *Lhx2* severely reduced (Cau et al., 1997; Cau et al., 2002). This results in a dramatic reduction of OMP⁺ OSN at later stages (Guillemot et al., 1993). To date, the identity of *Ascl1*-independent OSN remain elusive. We therefore thought to analyze *Dbx1* expression in *Ascl1* mutants. Surprisingly, the number and distribution of *Dbx1*⁺ cells appeared unaffected by the loss of *Ascl1* (Figure 6a). Furthermore, double *in situ* hybridization experiments indicated that *Dbx1*⁺ cells remain *Neurog1*⁺ and *Lhx2*⁺ in *Ascl1*^{-/-} embryos (Figure 6b,c), indicating they retain an identity of neurogenic progenitor. Immunostaining confirmed this result at the protein level, and further indicated that *Lhx2*⁺ cells remaining in *Ascl1* mutants are located nearby *Dbx1*-expressing regions (Figure 6d). Therefore, opposite to the rest of the developing OE, the expression of *Neurog1* in *Dbx1*⁺ cells is independent of *Ascl1*, and *Dbx1*-expressing progenitors appear able to undergo *Ascl1*-independent neurogenesis. Finally, since *Dbx1*-expressing progenitors also contribute to the GnRH lineage, we investigated GnRH neuron production in *Ascl1* mutants and found it is severely reduced, but not completely abolished (Figure 6e), confirming previously published data (Taroc et al., 2020a; Tucker et al., 2010) and reminiscent of the ~1/3 contribution of the *Dbx1* lineage to GnRH neurons. This strongly suggests that *Dbx1*⁺ progenitors account for the *Ascl1*-independent specification of GnRH neurons and further underlines the extent of diversity among progenitors involved in olfactory neurogenesis.

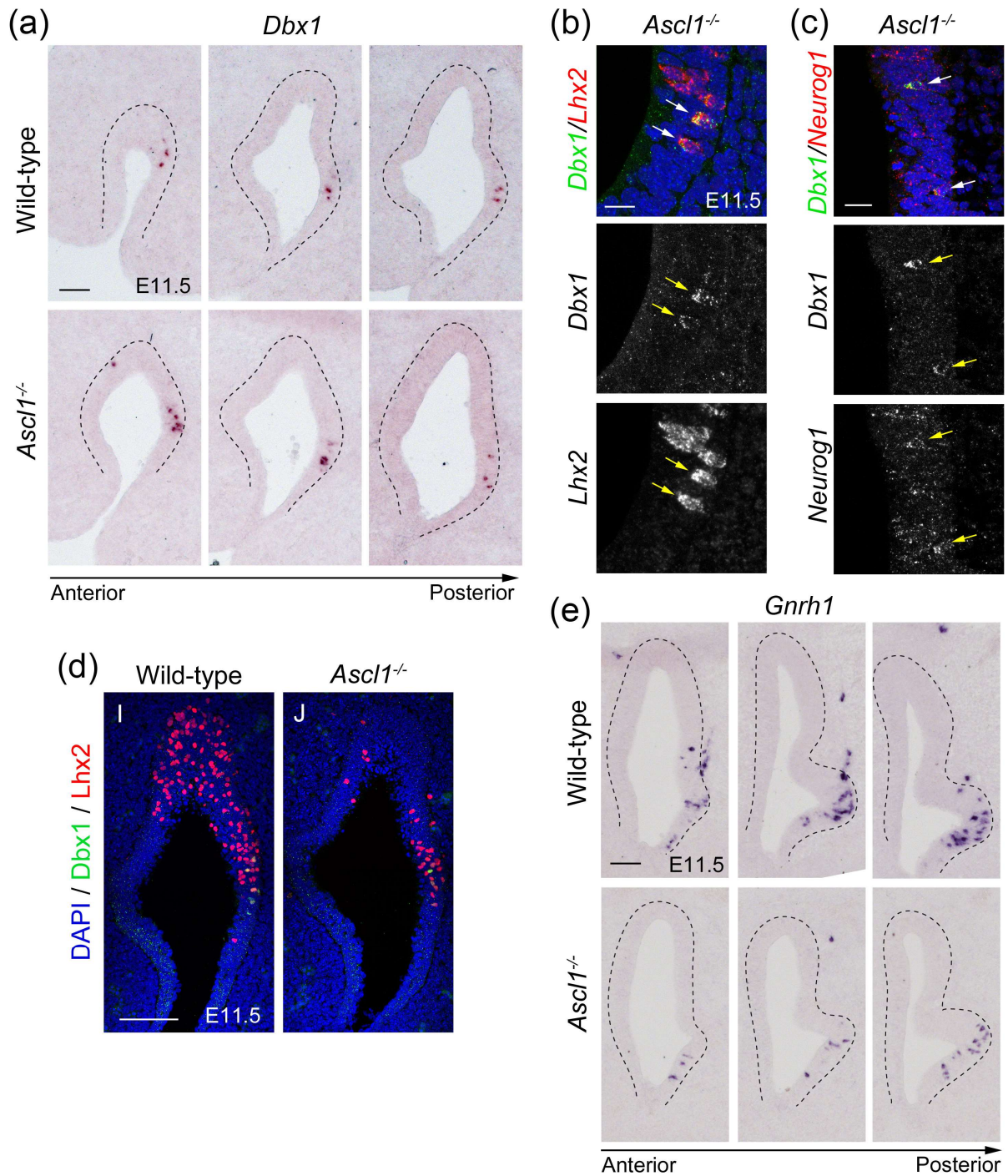


Figure 6. Consequences of *Ascl1* loss for *Dbx1*-related olfactory neurogenesis

(a) *In situ* hybridization for *Dbx1* along the antero-posterior axis of the E11.5 olfactory epithelium in control and *Ascl1*^{-/-} embryos. (b) Double fluorescent *in situ* hybridization for *Dbx1* (green) and *Lhx2* (red) in the olfactory epithelium of E11.5 *Ascl1*^{-/-} embryos. (c) Double fluorescent *in situ* hybridization for *Dbx1* (green) and *Neurog1* (red) in E11.5 *Ascl1*^{-/-} embryos. (d) Immunostaining for *Dbx1* (green) and *Lhx2* (red) in control and *Ascl1*^{-/-} E11.5 embryos. (e) *In situ* hybridization for *Gnrh1* along the antero-posterior axis of the E11.5 olfactory epithelium in control and *Ascl1*^{-/-} embryos. Scale bars: 50µm in a and e, 20µm in b and c, 100µm in d.

Discussion

Dbx1 in the sensory olfactory epithelium

In the spinal cord, *Dbx1* acts as a fate selector, expressed in progenitors and imposing V0 interneuron identity : in its absence, neurons fail to acquire their normal identity and adopt the alternative V1 fate (Pierani et al., 2001). By contrast, our data suggest that in the olfactory sensory system, *Dbx1* does not act as a strong fate inducer. Although we cannot formally exclude the possibility that neurons derived from *Dbx1^{LacZ/LacZ}* progenitors display some degree of misspecification, we found that expression of cardinal markers, global organization of the OE/VNO, axon targeting of OSN/VSN and GnRH neuron migration appeared normal. The only noticeable difference concerned the increased number of β Gal⁺ cells observed in knock-outs. One possible explanation is that two LacZ alleles allow increased accumulation of β Gal. An interesting alternative hypothesis is that *Dbx1* is involved in the cycling behavior of progenitors. Consistently, *Dbx1* gain-of-function was proposed to induce terminal differentiation (García-Moreno et al., 2018).

In the developing spinal cord and forebrain, inductive signals restrict *Dbx1* expression at borders between domains (Bielle et al., 2005; Gribble et al., 2007; Lu et al., 1992; Mangale et al., 2008; Novitch et al., 2003; Pierani et al., 1999). Similarly, in the developing OE, *Dbx1* is expressed at the interface between the respiratory and neurogenic domains. Tissue patterning within the OE was shown to occur in response to BMPs and *Fgf8* morphogens (Maier et al., 2010), resulting in the graded expression of transcription factors. Perturbation of such gradients was shown to affect progenitor proliferation properties and precursor identities (Tucker et al., 2010). Our data therefore support a model in which progenitors of the olfactory epithelium form a continuum reflecting morphogenic gradients, from which distinct classes of neurons emerge, similar to what we have recently described along the dorso-ventral axis of the mouse pallium (Moreau et al., 2021). Accordingly, *Dbx1* expression would be constrained at specific coordinates within this gradient, corresponding to transition zones, and the corresponding progeny participate in multiple lineages, thus revealing an otherwise hidden degree of progenitor diversity. Whether such progenitor diversity translates into distinct neuronal identities awaits further studies.

Diversity among sensory olfactory derivatives

Distinct levels of heterogeneity within OSN, VNO and GnRH have already been documented. One of the best example relates to the zonal expression of odorant receptors (Zapiec and Mombaerts, 2020). Currently, the molecular rationale allowing to confine the expression of odorant receptor genes to specific zones of the OE remains unclear. Adult expression of *Dbx1* appear to follow such a zonal organisation, with relatively sharp boundaries between regions of high and low/absent expression. Lineage tracing using Nestin-Cre mice was also shown to result in zonal pattern of recombination (Murdoch and Roskams, 2008), and multiple homeodomain transcription factors are expressed in a zonally restricted manner (Parrilla et al., 2016). This point to the idea that the zonal organization of the OE could reflect OSN ontogeny.

Diversity among GnRH neurons has also been demonstrated at the morphological level (Cottrell et al., 2006; Wray and Hoffman, 1986), on molecular bases (Constantin et al., 2009;

Jasoni et al., 2005; Klenke et al., 2010), or related to the birthdate and final position of the cells (Jasoni et al., 2009). In accordance with our observations, studies based on *Wnt1-Cre*, *Pax7-Cre*, *P0-Cre* lineage showed high-level heterogeneity in the genetic lineage of the olfactory, vomeronasal and GnRH neurons (Forni et al., 2011; Katoh et al., 2011; Murdoch et al., 2010; Shan et al., 2020). The correlation between these degrees of heterogeneity and the one we describe in this study remain to be established. In this perspective, it is worth mentioning that we previously described a partial overlap between *Wnt1* and *Dbx1* lineages (Causeret et al., 2011) as well as a regulatory loop between *Pax7* and *Dbx1* (Gard et al., 2017). Furthermore, a recent preprint investigating the cellular diversity and connectivity patterns in the accessory olfactory system reported that *Dbx1*-driven diversity is not only observed in the VNO but also in the accessory olfactory bulb and medial amygdala (Prakash et al., 2022). This work points to the idea of a molecular code involved in the assembly of olfactory circuits that would be conserved from the sensory epithelia to the central processing structures.

***Ascl1*-independent neurogenesis in the olfactory epithelium**

Ascl1 is a proneural transcription factor and, as such, promotes neuronal differentiation in the olfactory epithelium (Cau et al., 1997; Cau et al., 2002; Guillemot et al., 1993; Murray et al., 2003). Upon *Ascl1* loss, OSN, VSN and GnRH differentiation is severely affected, with only few neurons reaching a mature state (Guillemot et al., 1993; Murray et al., 2003; Taroc et al., 2020a). The origin of these few neurons so far remained elusive. We show that *Dbx1*-expressing cells in the olfactory epithelium of *Ascl1* mutants retain a neurogenic potential, as they maintain *Ngn1* expression. We therefore postulate that neurons produced independently of *Ascl1* belong to the *Dbx1* lineage. This hypothesis is supported by the observation that the remaining OMP⁺ cells in *Ascl1* mutants are early born (Tucker et al., 2010). In addition, the same authors calculated that 29% of Tuj1⁺ neurons are maintained in the E11.5 mutant OE, matching the contribution of the *Dbx1* lineage we describe.

One remaining open question is the identity of *Dbx1*⁺ precursors. They could either derive from *Ascl1*-expressing progenitors, in which case one could postulate that *Dbx1* is able to compensate for the loss of *Ascl1*. An alternative possibility is that *Dbx1*⁺ progenitors represent a previously uncharacterized progenitor pool, distinct from the *Ascl1* lineage, and therefore insensitive to *Ascl1* loss.

Overall, our work points to the diversity that exists in the olfactory sensory system, and raises new questions regarding how progenitor and neuron diversity are generated.

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Acknowledgements

The authors wish to thank the animal facility of the Institut Jacques Monod. We acknowledge the ImagoSeine facility, member of the France BioImaging infrastructure supported by the French National Research Agency (ANR-10- INSB-04, “Investments for the future”) for help with confocal microscopy. We are grateful to Vanessa Ribes for the kind gift of antibodies, Adrien Calvairoly for help with collecting *Ascl1* mutants and members of the Pierani laboratory for helpful discussions. FC is an Inserm researcher. AP is a CNRS investigator. This work was supported by grants from Agence Nationale de la Recherche (ANR-2011-BSV4-023-01), FRM (Fondation pour la Recherche Médicale) (INE20060306503 and «Equipe FRM DEQ 20130326521») to AP.

Authors contribution

Conceptualization: FC

Methodology: FC, AC, AP

Investigation: FC, MF, MXM, EN, RO, AC

Resources: CP

Writing – original draft preparation: FC

Writing – review and editing: FC, RO and AC

Visualization: FC, MF, MXM, EN

Supervision: FC

Project administration: FC

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Conflict of interest statement The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

All data are available in the main text or the supplementary materials.

Tables

Table 1. List of primary antibodies.

Antibody	Immunogen	Source	Dilution
anti-GFP	Recombinant GFP purified from <i>E. coli</i>	Chicken polyclonal Aves Lab, cat. GFP-1020 RRID: AB_10000240	1:2000
anti- β Galactosidase	Full length native protein purified from <i>E. coli</i>	Chicken polyclonal Abcam, cat. ab9361 RRID: AB_307210	1:1000
anti-Phospho Histone 3 (clone D2C8)	Synthetic phosphopeptide corresponding to residues surrounding Ser10 of human histone H3	Rabbit monoclonal Cell Signaling Technology, cat. 3377 RRID: AB_1549592	1:500
anti-Cleaved Caspase-3 (clone 5A1E)	Synthetic peptide corresponding to residues adjacent to Asp175 of human caspase-3	Rabbit monoclonal Cell Signaling Technology, cat. 9664 RRID: AB_2070042	1:1000
anti-Dbx1	C-terminal peptides of mouse Dbx1 coupled to KLH injected into rabbits and affinity purified	Rabbit antiserum (Pierani et al., 1999) RRID: N/A	1:10000
anti-Isl1/2	Islet mouse	Polyclonal antibody (Dasen et al., 2005) RRID: AB_2801512	1:10000
anti-GnRH	Synthetic LHRH coupled to KHL with CDI linker	Rabbit polyclonal Immunostar, cat. 20075 RRID: AB_572248	1:400
anti-Sox2	<i>E. coli</i> -derived recombinant human SOX2 Gly135-Met317	Goat polyclonal R&D Systems, cat. AF2018 RRID: AB_355110	1:100
anti-Omp	Rodent OMP Protein	Goat monoclonal Wako, cat. 019-22291 RRID: AB_664696	1:1000
anti-Ctip2 (clone 25B6)	Recombinant fragment corresponding to human CTIP2	Rat monoclonal Abcam, cat. ab18465 RRID: AB_2064130	1:300
anti-Tubulin β 3 (clone TUJ1)	Last 15 C-terminal residues of neuron specific Class III β -tubulin derived from rat	Mouse monoclonal BioLegend, cat. 801201 RRID: AB_2313773	1:1000

	brain		
anti-Peripherin	Trp-E-peripherin fusion protein containing all but the 4 N terminal amino acids of rat peripherin	Rabbit polyclonal Sigma-Aldrich, cat. AB1530 RRID: AB_90725	1:500
anti-Lhx2 (clone C-20)	Human C-terminus of LHX2 protein	Goat polyclonal Santa-Cruz, cat. sc-19344 RRID: AB_2135660	1:250
anti-HuC/D (clone 16A11)	Human HuC/HuD neuronal protein	Mouse monoclonal ThermoFisher, cat. A-21271 RRID: AB_221448	1:1000

Table 2. List of secondary antibodies.

Antibody	Conjugated	Source	Dilution
Anti-Rabbit IgG (H+L)	Alexa488	Donkey polyclonal Jackson ImmunoResearch, cat. 711-545-152 RRID: AB_2313584	1:500
Anti-Rabbit IgG (H+L)	Cy3	Donkey polyclonal Jackson ImmunoResearch, cat. 711-165-152 RRID: AB_2307443	1:350
Anti-Rabbit IgG (H+L)	Biotin	Donkey polyclonal Jackson ImmunoResearch, cat. 711-065-152 RRID: AB_2340593	1:500
Anti-Chicken IgY (H+L)	Alexa488	Donkey polyclonal Jackson ImmunoResearch, cat. 703-545-155 RRID: AB_2340375	1:500
Anti-Goat IgG (H+L)	Cy3	Donkey polyclonal Jackson ImmunoResearch, cat. 705-165-147 RRID: AB_2307351	1:350
Anti-Goat IgG (H+L)	Cy5	Donkey polyclonal Jackson ImmunoResearch, cat. 705-175-147 RRID: AB_2340415	1: 250
Anti-Mouse IgG (H+L)	Cy3	Donkey polyclonal Jackson ImmunoResearch, cat. 715-165-151 RRID: AB_2315777	1:350
Anti-Mouse IgG (H+L)	Cy5	Donkey polyclonal Jackson ImmunoResearch, cat. 715-175-151 RRID: AB_2340820	1:250
Anti-Mouse IgG (H+L)	Biotin	Goat polyclonal Jackson ImmunoResearch, cat. 115-065-146 RRID: AB_2338567	1:500
Anti-Rat IgG (H+L)	Cy3	Donkey polyclonal Jackson ImmunoResearch, cat. 712-165-153 RRID: AB_2340667	1:350
Anti-Rat IgG (H+L)	Cy5	Donkey polyclonal Jackson ImmunoResearch, cat. 712-175-153 RRID: AB_2340672	1: 250

Supplementary Table 1 – Related to Material & Methods - Primers used for ISH probes.

Gene	Alias	Forward primer	Reverse primer
<i>Stmn2</i>	Scg10	TCCAACCGAAAAATGAGGTC	TAATGCAGCAACCAGCTCAC
<i>Gap43</i>		CCTGAACCCCTGTCTCTCCTG	CCTTTTTGTTTCATTCCATCACA
<i>Ncam1</i>		CACGTGTTCCCTTTCCCAAGT	CAAATGTGTGGCCAGTGTTTC
<i>Omp</i>		CTAGCTGCTCCTCCTGTGCT	ACCTCCACCAAGTCCACAAG
<i>Gnrh1</i>	LHRH	CTCAACCTACCAACGGAAGC	CGCTGCTGGGTATAAAAACG
<i>Vmn1r</i>		GGCCCAGACAGGTGATTTTA	GCTGCATTTGCTGTTGATGT
<i>Vmn2r</i>		CTCTGGGGAGCTTCACTTTG	CAAGCGGGAGAATGTTGATT
<i>Olfr449</i>	mOR 103-1	TCTTCCTTGCCAACCTGTCT	GGCTTTCTGCTTTCCTGTTG
<i>Olfr41</i>	mOR 103-15	ACCCTCCACAAACCCATGTA	GAAGCCCCAGTGACAGAGAG
<i>Olfr1507</i>	mOR 244-1	TTTTAAATTGTCCTGACAAACTGG	TCTGATTCTCTCAGTCCCTTCA
<i>Olfr247</i>	mOR 265-1	CACCTGCTGGTACAGCTCAA	ATGAGAGGAGCAGGTGGAGA
<i>Olfr1395</i>	mOR 277-1	TGTCTGCTGGCTCATGGTAG	TCCGGTCACTTCTTTGTTC