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Radial derivatives of the mouse ventral pallium traced with *Dbx1*-LacZ reporters

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ABSTRACT

The progeny of *Dbx1*-expressing progenitors was studied in the developing mouse pallium, using two transgenic mouse lines: (1) $Dbx1^{nlslacZ}$ mice, in which the gene of the β-galactosidase reporter (LacZ) is inserted directly under the control of the *Dbx1* promoter, allowing short-term lineage tracing of *Dbx1*-derived cells; and (2) $Dbx1^{CRE}$ mice crossed with a Cre-dependent reporter strain ($ROSA26^{loxP\text{-}stop\text{-}loxP\text{-}LacZ}$), in which the *Dbx1*-derived cells result permanently labeled (Bielle et al., 2005). We thus examined in detail the derivatives of the postulated longitudinal ventral pallium (VPall) sector, which has been defined among other features by its selective ventricular zone expression of *Dbx1* (the recent ascription by Puelles, 2014 of the whole olfactory cortex primordium to the VPall was tested). Earlier notions about a gradiental caudorostral reduction of *Dbx1* signal were corroborated, so that virtually no signal was found at the olfactory bulb and the anterior olfactory area. The piriform cortex was increasingly labeled caudalwards. The only endopiriform grisea labeled were the ventral endopiriform nucleus and the bed nucleus of the external capsule. Anterior and basolateral parts of the whole pallial amygdala also were densely marked, in contrast to the negative posterior parts of these pallial amygdalar nuclei (leaving apart medial amygdalar parts ascribed to subpallial or extratelencephalic sources of *Dbx1*-derived GABAergic and non-GABAergic neurons). Alternative tentative interpretations are discussed to explain the partial labeling obtained of both olfactory and amygdaloid structures. This includes the hypothesis of an as yet undefined part of the pallium, potentially responsible for the posterior amygdala, or the hypothesis that the VPall may not be wholly characterized by *Dbx1* expression (this gene not being necessary for VPall molecular distinctness and histogenetic potency), which would leave a dorsal *Dbx1*-negative VPall subdomain of variable size that might contribute partially to olfactory and posterior amygdalar structures.

1. Introduction

The ventral pallium (VPall) was first defined as a primary histogenetic subdivision of the mouse and chicken pallial telencephalon by Puelles et al. (1999, 2000), in their original proposal of a tetrapartite pallium model (MPall, DPall, LPall, VPall). The VPall domain lies next to the striatal subpallium and was first distinguished molecularly from the neighboring lateral pallium domain (LPall) on the basis of its minimal expression of *Emx1*, a marker otherwise widespread in the other parts of the pallium (see also Smith-Fernández et al., 1998; Gorski et al., 2002). The VPall division was later further characterized during early embryonic development by its distinct expression of *Dbx1* in the ventricular zone (Yun et al., 2001; Medina et al., 2004) and *Lhx9* in the mantle

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(García-López et al., 2008; Abellán et al., 2009). Comparative neuroanatomical studies had used previously a simpler tripartite model of the pallium, in which VPall and LPall were lumped as 'lateral pallium', supposedly forming an olfactory pallial region that included the olfactory cortical areas and the claustroamygdaloid complex held to lie deep to them (reviewed by Striedter, 1997). Consistently with the new concept of molecularly distinct VPall and LPall sectors within the earlier olfactory 'lateral pallium' region, Puelles et al. (1999, 2000) and Medina et al. (2004) suggested that both divisions might contribute radially migrated cells to adjacent subsets of olfactory, claustral and amygdalar formations, in line with the classic postulates of Holmgren (1925). The cortical areas ascribed to the VPall included the whole olfactory bulb, plus the ventromedial part of the anterior olfactory area and the ventromedial prepiriform/piriform cortex (covered by the lateral olfactory tract), plus the anterior and posteromedial amygdaloid corticoid formations (Medina et al., 2004). The VPall also was thought to produce some deep (so-called hypopallial) nuclear formations, including a ventromedial part of the claustrum-endopiriform nuclear complex and the lateral/basomedial parts of the pallial amygdala (Medina et al., 2004). In contrast, the dorsolateral anterior olfactory area, the dorsolateral prepiriform/piriform cortex, plus the larger dorsolateral part of the claustrum/endopiriform complex, jointly with the basolateral amygdaloid nucleus and the posterolateral cortical part of the amygdala, were ascribed to the LPall (Puelles et al., 1999, 2000, 2007; Puelles, 2001; Medina et al., 2004). This classification was employed in later studies in mouse studying *Lhx9* expression in the VPall mantle (García-López et al., 2008; Abellán et al., 2009), and was used as well in various other reports (e.g., Hirata et al., 2002, 2009; Guirado and Dávila, 2002; Legaz et al., 2005b; Martínez-García et al., 2007, 2012). The proposal of a distinct VPall also received support from data on genetic fate mapping of the Dbx1-positive progenitors (found in the VPall but not in other pallial sectors) using Cre/loxP technology (Bielle et al., 2005; Hirata et al., 2009; Teissier et al., 2010; Waclaw et al., 2010). Nevertheless, such Dbx1 fate mapping data also raised concerns on the description of VPall derivatives, since ventropallial Dbx1-lineage cells appeared to occupy most of the basal amygdalar complex (Waclaw et al., 2010), and not only part of it, as suggested previously (Medina et al., 2004).

Apart of the VPall and LPall, the tetrapartite pallial model of Puelles et al. (1999, 2000) also contained the dorsal pallium (DPall; the isocortex) and the medial pallium (MPall; hippocampal and perihippocampal allocortex), which essentially conformed to earlier conventions (as in the tripartite pallial model; review in Striedter, 1997). Due to corroborating evidence obtained by mappings of pallial *Emx1* expression in a diversity of gnathostome species, the new tetrapartite pallial model –and, accordingly, the new VPall sector- found wide acceptance in recent developmental and comparative neuroanatomical literature (e.g., Smith-Fernández et al., 1998; Pombal and Puelles, 1999; Puelles et al., 1999, 2000, 2007; Redies et al., 2001; Puelles, 2001; Bachy et al., 2002; Brox et al., 2003, 2004; Wullimann and Rink, 2002; Lindsay et al., 2005; Bielle et al., 2005; Medina et al., 2004, 2005; Moreno and González, 2006; Bardet et al., 2008; Causeret et al., 2011; De Carlos et al., 1996; Fan et al.,1996; Ferreiro-Galve et al., 2008; Tole et al., 2005; Remedios et al., 2007; Abellán et al., 2009; Medina and Abellán, 2009; Nomura et al., 2009; García-Calero and Puelles, 2009; Kerwin et al., 2010; Teissier et al., 2010), as well as in various book chapters and reviews (Martínez-García et al., 2007, 2012; Aboitiz et al., 2002; Aboitiz and Montiel, 2007; Aboitiz and Zamorano, 2013).

However, recent expression studies on the developing mouse and chicken claustrum in which the *Nr4a2* marker gene was mapped (Puelles, 2014; Puelles et al., 2015) have suggested a modification of the earlier tetrapartite model: the claustrum, which is rather selectively identified by this marker, is not divided into ventropallial and lateropallial parts, and emerges as an exclusively lateropallial derivative formed deep to the insular cortex (rather than deep to the olfactory cortex, as was assumed previously). If the old tripartite pallium model is used, the new data imply (against Holmgren, 1925) that the claustrum develops outside of the olfactory pallium, and would be associated to the insula within the classic dorsal pallium. However, since the VPall is still evident, and is supported by the Dbx1 genetic fate mapping results mentioned above, there is advantage in reformulating the tetrapartite model (see Puelles, 2014), so that the newly defined LPall contains exclusively the claustrum and the insular cortex (specifically its agranular/dysgranular portion; Palomero-Gallagher and Zilles, 2004).

An 'insular claustrum' concept had been initially conjectured by most pioneering investigators of the mammalian cortex, such as Meynert (1868, 1872a,b) and Brodmann (1909). but had fallen into disuse subsequently, due to what now turns out to be misguided embryologic analysis (de Vries, 1910; Landau, 1923; Holmgren, 1925; Kuhlenbeck, 1927, 1973, 1977; see Puelles, 2014 for details). The newly reformulated nucleo-cortical complex formed by the mammalian claustrum (an early generated nuclear mass, initially present at the brain surface) and the insula (which secondarily adopts a superficial position via radial migration across the pre-existent claustrum), redefines the LPall concept relative to the original definition cited above (Puelles et al., 1999, 2000; Medina et al., 2004). This implies that the agranular insula falls out of the DPall and enters the LPall, and the whole olfactory cortex results ascribed to the VPall. There is no part of the claustrum proper that originates within the VPall. However, there apparently exist migrated claustral populations that secondarily invade tangentially the VPall and settle down deep to the olfactory cortex, forming the dorsal endopiriform nucleus (Puelles, 2014). Another novel aspect in the recently modified pallial model is that the reformulated LPall ends caudally at mid-telencephalic levels, as does the insula; the perirhinal cortex may be considered to represent the caudalmost part of the insular cortex, as it covers the caudalmost claustrum cells (Puelles, 2014). This implies that the reformulated LPall does not contribute to the amygdalar pallial nuclear complex. As a consequence, the earlier argument that adduced the presence of *Emx1* expression at the basolateral amygdaloid nucleus as an index of its lateropallial nature (Puelles et al., 2000; Gorski et al., 2002; Medina et al., 2004) results weakened in the new scenario, and an alternative explanation seems required for this genoarchitectonic peculiarity (no tangential migration has been observed so far that might account for these data).

As a result of these changes in the LPall, the VPall is now conjectured to encompass among its radial derivatives the olfactory bulb and the *whole* anterior olfactory area, the *whole* prepiriform and piriform olfactory cortex, and *most* of the pallial amygdala (Waclaw et al., 2010; Puelles, 2014; we will discuss below in the light of present data the possibility that additional regions, such as a part of the entorhinal cortex, may also be VPall derivatives). As regards the hypopallial deep nuclei, the larger part of the endopiriform population, forming the conventional *dorsal endopiriform nucleus* (EPd; present both rostrally and caudally, deep to the olfactory cortex) consists of lateropallial cells apparently migrated tangentially from the claustral hypopallium into the VPall, and only the smaller *ventral endopiriform nucleus*, plus a newly defined *bed nucleus of the external capsule*, are held to be radial ventropallial derivatives, leaving aside the pallial amygdala (EPv, BEC; Puelles, 2014). The BEC corresponds in topography to the *reservoir* of Bayer and Altman (1991) and Altman and Bayer (1995), but seems a definitive structure, rather than a transient cell aggregate as these authors conjectured. These rather

small VPall nuclear formations, which can be easily distinguished from claustral LPall-derived structures, lie mostly rostrally to the crossing of the anterior commissure, deep to the prepiriform cortex. Caudally to this landmark, the larger pallial amygdala in its entirety (hypopallial nuclei deep to corticoid amygdalar domains), plus the piriform cortex proper and associated minor distinct subareas (accompanied throughout by a deep caudal extension of the migrated lateropallial EPd nucleus), may belong likewise to the reformulated VPall (Puelles, 2014; Abellán et al., 2014).

In addition, VPall-derived early-born Cajal–Retzius cells have been shown to migrate tangentially into the cortical marginal stratum at early developmental stages (Bielle et al., 2005), and a VPall population of glutamatergic neurons called Pierani cells by Puelles (2014) invade tangentially the whole isocortex as of E14.5 (Teissier et al., 2010, 2012).

Though this actualized concept of VPall (Puelles, 2014) encompasses a larger territory than the VPall of Puelles et al. (2000) and Medina et al. (2004), the VPall is simpler as newly defined, since ventral and dorsal parts of the olfactory cortex no longer are held to be primarily diverse as regards molecular patterning and histogenesis, and the implication of claustral entities within VPall is limited to the tangentially migrated EPd. In their study of *Emx1* progeny, Gorski et al. (2002) observed strong *Emx1*-labeling of the EPd; this result was not understood at the time (being contradictory with the Puelles et al., 2000 model), but can be seen now to be consistent with the newly suggested LPall origin and subsequent migration of EPd (Puelles, 2014). It remains unclear, however, why *Emx1*-labeled cells appear in the basolateral and basomedial amygdalar nuclei (Gorski et al., 2002).

In principle, the neurons held to form the nuclear and cortical VPall areas should originate from a distinct *Dbx1*-positive ventricular progenitor domain forming a thin longitudinal band intercalated between the lateral angle of the lateral ventricle and the pallio-subpallial boundary (Medina et al., 2004). This band has been visualized in recent times thanks to genetic fate mapping of *Dbx1*-expressing progenitors using the cre-loxP technology (Bielle et al., 2005; Hirata et al., 2009; Teissier et al., 2010; Waclaw et al., 2010). However, a detailed analysis of all VPall derivatives across rostral and caudal levels of the telencephalon is still pending. Based on data reported by various groups, it appears that the VPall progenitor domain typically thickens dorsoventrally from rostral (retrobulbar) levels to caudal (amygdalar) levels along the hemisphere (Yun et al., 2001; Medina et al., 2004; Bielle et al., 2005; Teissier et al., 2010, 2012). The VPall neuroepithelium also expresses other characteristic markers, such as *Sfrp2*,*Nrg*-family members, *Fgf7*, and *Rtn1*, though these various signals do not fully overlap (Kim et al., 2001; Assimacopoulos et al., 2003; Hirata et al., 2002), and in general the corresponding derivatives have not been traced into the mantle.

The expression of *Dbx1* is restricted to the proliferating progenitor cells, while the derived postmitotic neurons down-regulate *Dbx1* expression. This impedes tracing by ISH the locally produced postmitotic cells into their targets in the mantle. Nevertheless, cells derived from *Dbx1*-expressing progenitors can be labeled in mice, using transgenic animals (Bielle et al., 2005; Hirata et al., 2009; Waclaw et al., 2010). Two transgenic mouse lines have been employed to this end: (1) $Dbx1^{nlslacZ}$ mice, in which the gene of the β-galactosidase reporter (LacZ) is inserted directly under the control of the *Dbx1* promoter, allowing short-term lineage tracing of *Dbx1*-derived cells; and (2) $Dbx1^{CRE}$ mice crossed with a Cre-dependent reporter strain ($ROSA26^{loxP\text{-}stop\text{-}loxP\text{-}LacZ}$), in which the *Dbx1*-derived cells result permanently labeled (Bielle et al., 2005; there is also a $Dbx1^{creCC\text{-}EGFP}$ strain employed by Waclaw et al., 2010, which expresses enhanced green fluorescent protein [EGFP]. Other lines of mice employed by Hirata et al. (2009) have Dbx1-cre coupled to tamoxifen-sensitive estrogen receptor [$Dbx1^{CreERT2}$] crossed with R26R-LacZ or R26R-YFP lines; these allow labeling by age groups, depending on the timetable of tamoxifen administration, and lead to permanent expression of LacZ or the yellow fluorescent protein.

Using some of these genetic approaches, Bielle et al. (2005) already showed that the *Dbx1*-positive VPall progenitors give rise to an early-born, tangentially migrating subpial subpopulation of Cajal-Retzius cells, which invades the neighboring cortical plate (DPall). Subsequent studies by Teissier et al. (2010, 2012) revealed that an important set of later VPall derivatives – named Pierani cells by Puelles (2014) – widely migrate tangentially via the subventricular zone, reaching all layers of the isocortex (DPall), totaling about 4–5% of the whole cortical population at birth. Surprisingly, most of these migrated excitatory ventropallial neurons die within the first postnatal month. However, one important effect of their transient presence in the cortical subventricular zone is a sizeable mitogenic influence on the number of pyramidal cells produced by the DPall progenitors (see Teissier et al., 2010, 2012; and reviews by Puelles, 2011; Borello and Pierani, 2010). Hirata et al. (2009) and Waclaw et al. (2010) studied with these methods the amygdalar derivatives of the telencephalic *Dbx1*-expressing progenitors, which include glutamatergic VPall derivatives expressing *Tbr1*, as well as GABAergic preoptic area derivatives expressing subpallial markers. These studies mainly focused on the medial amygdala (Hirata et al., 2009) or the central amygdala (Waclaw et al., 2010). They did not address systematically the fates of *Dbx1*-labelled radially-migrated cells throughout the VPall.

In the present study we used the cited transgenic approach to trace the derived lineage of all *Dbx1*-expressing progenitors in the mouse VPall. As previously observed by Bielle et al. (2005) and Teissier et al. (2010), there is a caudo-rostrally decreasing gradient of *Dbx1* signal along the VPall matrix zone, with hardly any expression at the anterior olfactory area and the olfactory bulb. Indeed, some data suggest that a dorsal part of the VPall progenitor domain may lack *Dbx1* expression throughout the length of the domain (possibly expressing *Emx1* instead). Notwithstanding this limitation as regards demonstrating all VPall derived cells using this approach, our results substantially support the newly revised concept of the VPall (Puelles, 2014).

2. Materials and methods

$Dbx1^{nlslacZ}$ mice, allowing short-term lineage tracing of *Dbx1*-derived cells, and $Dbx1^{CRE/iLacZ}$ mice, in which the *Dbx1*-derived cells result permanently labeled, were described elsewhere (Bielle et al., 2005). The latter strain resulted from crossing $Dbx1^{CRE}$ mice with a Cre-dependent reporter strain ($ROSA26^{loxP-stop-loxP-LacZ}$; Srinivas et al., 2001; Teissier et al., 2010). Screening of the mutant alleles was performed by PCR genotyping as described previously (Bielle et al., 2005). Noon on the day of the vaginal plug was considered as E0.5.Various embryonic (E10.5, E12.5, E14.5, E18.5) and postnatal offspring (P1, P60) were fixed with 4% paraformaldehyde and cut into 50 μm-thick sections on a vibratome, and stained for β-galactosidase activity (X-Gal staining solution: 0.02% Igepal, 0.01% sodium deoxycholate, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ diluted in 0.1 M phosphate buffer [pH 7.5]) under conditions reported elsewhere (Borello et al., 2008; Teissier et al., 2010). Some of the material was counterstained for Tbr1 (see antibody table) or GABA immunoreaction (see antibody table) to check respectively pallial versus subpallial ascription of Dbx1-labeled cells.

Primary antibody	Source	Catalog. ref/ Lot. no.	Immunogen	Dilution
Rabbit anti GABA		Cat. No. A2052,	Synthetic peptide corresponding to	1:4000

(*Continued*)

Primary antibody	Source	Catalog. ref/ Lot. no.	Immunogen	Dilution
	Sigma; Saint Louis, Missouri, USA.	lot no045K4757	γ-aminobutyric acid (GABA) conjugated to BSA as immunogen	
Rabbit anti Tbr1	Millipore (Chemicon); Billerica, USA.	Cat. No. AB9616, lot noLV1504425	Synthetic peptide from mouse Tbr1	1:4000
Chick anti-GFP	AvesLabs, Inc.; Oregon, USA.	Cat. No.GFP-1020, lot no1223FP03	IgY fraction purified from chicken yolk	1:2000
Cy3 donkey anti-rabbit	Jackson Immunoresearch, Laboratories Inc.	Cat. No.711-165-152, lot no80791		1:700
Alexa Fluor 488 goat anti-chicken IgG (H+L)	Invitrogen (Molecular Probe), Oregon, USA.	Cat. No. A11039, lot no41658A		1:1000
Zenon Alexa Fluor Rabbit IgG labelling Kit 647	Invitrogen, Oregon, USA.	Cat. No. Z25308, lot no466585	contain affinity-purified Fab fragment a goat-anti Fc antibody conjugated to Alexa Fluor 647 dye	

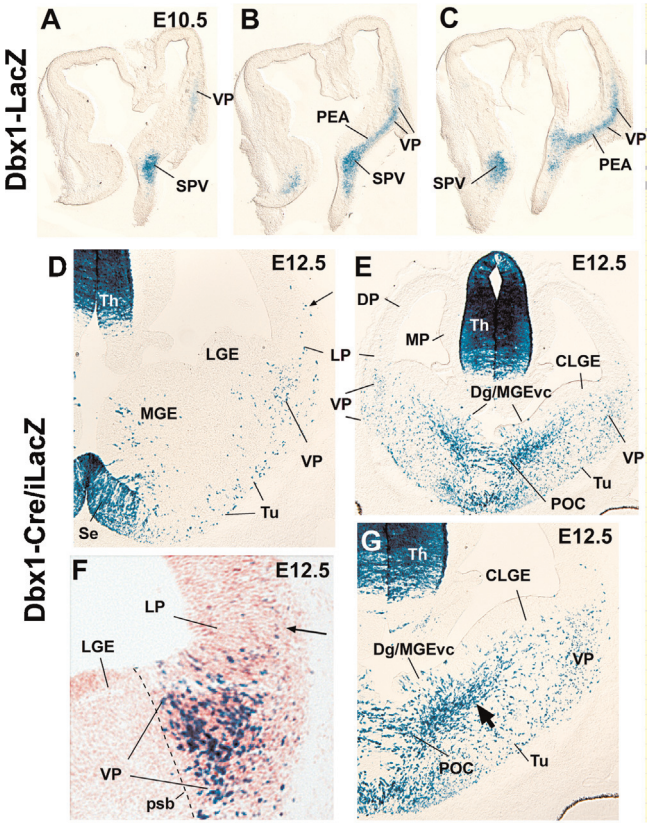

Fig. 1. Horizontal (A–C) or coronal (D–G) sections through the telencephalon of Dbx1-LacZ or Dbx1-Cre/βactin-indolyl-LacZ mice at E10.5 or E12.5. Dbx1-derived cells showing β-galactosidase activity are labeled in blue. Numerous Dbx1-derived cells in the ventral pallium, septum, preoptic area in the subpallium (a few are also present in the anterior peduncular area), and supraopto-paraventricular domain of the alar hypothalamus (SPV). Note the cell corridor of Dbx1-derived cells extending from the ventral pallium into the hypothalamic SPV (panels D, C; this cell corridor represents the pallial extended amygdala o PEA). During early embryonic stages (E10.5–E12.5), some of the Dbx1-derived cells are located in the ventricular zone of the ventral pallium (where the progenitor cells reside), while the majority are observed in the periventricular stratum (panel F). A few Dbx1-derived cells also appear to extend from the ventral pallium into the lateral pallium (arrows in D, F), and some cell even reach the dorsal pallium primordium.

3. Results

In the first part of the Results we will describe the distribution of cells derived from *Dbx1*-expressing progenitors during early (Fig. 1), intermediate (Fig. 2) and late (Figs. 3–5) prenatal stages in the brains of $Dbx1^{nlslacZ}$ or $Dbx1^{CRE}$; $ROSA26^{loxP\text{-}stop\text{-}loxP\text{-}LacZ}$ mice. Later we will describe the distribution of these cells in young adult animals, using the brains of $Dbx1^{CRE}$; $ROSA26^{loxP\text{-}stop\text{-}loxP\text{-}LacZ}$ mice (Figs. 6 and 7), and comment on relevant marker co-localization patterns (Fig. 8).

3.1. Distribution of Dbx1*-derived (βgal⁺) cells in the telencephalon at early stages (E10.5-E12.5)*

In agreement with earlier data (Bielle et al., 2005), the ventricular zone of the VPall, the septum, the diagonal area (previously known as anterior peduncular area [AEP], and also known as the ventrocaudal part of the medial ganglionic eminence) and the preoptic area contains abundant cells positive for β-galactosidase (βgal⁺) in early $Dbx1^{-nlslacZ}$ mouse embryos (E10.5–E11; Fig. 1A–C); this result is consistent with *Dbx1* RNA expression in wild-type mice (Shoji et al., 1996; Lu et al., 1996; Yun et al., 2001; Medina et al., 2004). The βgal⁺ cells in the VPall mantle have a clearcut rostrocaudal gradiental distribution (Fig. 1A–C). Rostral parts of the hemisphere lack, or have very few, βgal+ cells (Fig. 1A, left side). Labeled cells start to increase in number at intermediate levels (Fig. 1A, right side; 1D, E, G), and become relatively abundant in sections cut at the caudal pole of the hemisphere, where they correspond to the pallial amygdala (Fig. 1B,C). This caudal periventricular βgal⁺ cell population reaches the caudomedial portion of the hemisphere after turning around the caudal ganglionic eminence (subpallial amygdala). Its cells are rostromedially continuous with similarly labeled elements lying at the supraopto-paraventricular area of the alar hypothalamus, and possibly also are continuous with its dorsal neighbour, the labeled preoptic area (SPV; Fig. 1A–C); the connection of these domains across the hemispheric stalk occurs precisely under the terminal sulcus. This pallio-hypothalamo-preoptic link appears to represent the primordium of the pallial extended amygdala (PEA), a previously postulated glutamatergic cell corridor connecting the pallial amygdala with the hypothalamic SPV (Puelles et al., 2007; Abellán and Medina, 2008); note this cell corridor does not belong to the subpallium, since it expresses typical pallium markers such as *Pax6* in the ventricular zone and *Tbr1* and *Sim1* in the mantle zone (Bulfone et al., 1995; Fan et al., 1996; Puelles et al., 2000; Puelles and Rubenstein, 2003; Medina et al., 2004; see also *Sfrp2* in Kim et al., 2001). The SPV-PEA cell corridor partially encompasses at its caudal end the primordium of the medial amygdala, in the caudomedial hemispheric region (García-López et al., 2008; Abellán and Medina, 2008). Some of the βgal⁺ SPV-PEA cells establish contact across the upper paraventricular region with βgal⁺ cells located in the prethalamic eminence, which lies in the dorsal part of diencephalic prosomere 3, and therefore lies topologically caudal to the SPV (Puelles and Rubenstein, 2003; PThE; Fig. 1C).

At E10.5 and E12.5, using both $Dbx1^{nlslacZ}$ embryos (Fig. 1A–C,F) and permanent tracing with $Dbx1^{CRE}$; $ROSA26^{loxP\text{-}stop\text{-}loxP\text{-}LacZ}$ (Fig. 1D,E, G) βgal⁺ cells in the VPall are more abundant in the

subventricular zone and adjacent intermediate mantle than in the ventricular zone (SVZ; Fig. 1A–C, D–G). Moreover, some *Dbx1*-derived cells have translocated radially to superficial (subpial) positions within the ventral pallium, and are observed at the primordium of the piriform cortex (Fig. 1F). Sparse βgal$^+$ cells were found within the adjacent lateral ganglionic eminence, or within the olfactory tuberculum (LGE; OTu; Fig. 1D). In addition, a few labeled cells seem to have moved tangentially away from the VP into the overlying sectors of the pallium, i.e., lateral and dorsal pallium (arrow in Fig. 1D), consistent with previous reports (Bielle et al., 2005; Griveau et al., 2010).

The septocommissural preoptic area, a thin dorsal and median part of the preoptic region that extends into the *Dbx1*-positive septum at the median crossing of the anterior commissure, clearly represents a source of numerous βgal$^+$ cells that invade the telencephalon (POC; Fig. 1E,G; see about POC in Bardet et al., 2008, Flames et al., 2007, García-López et al., 2008; Hirata et al., 2009; Griveau et al., 2010; Bardet et al., 2008). At intermediate coronal section levels through the medial and lateral ganglionic eminences, few cells from the septum proper seem to disperse towards the adjacent medial ganglionic eminence (Fig. 1D); contrarily, at section levels through the caudal ganglionic eminence and the POC, numerous labeled cells disperse tangentially across the diagonal area (Dg) into adjacent parts of the amygdalar subpallium (POC, Dg; Fig. 1E,G). The density of these cells decreases significantly near the palliosubpallial boundary, suggesting scarce intermixing at these early stages with the group of labeled neurons streaming radially within the VPall at intermediate and caudal sectors of the telencephalon (see the clearcut medial limit of ventropallial βgal$^+$ cells at the pallio-subpallial boundary −psb- in Fig. 1F). However, this image, which is characteristic of *Dbx1*^{nlslacZ} material, due to the temporary labeling obtained, changes with permanently labeled *Dbx1*^{CRE} preparations, which show labeled POA derivatives reaching as far as the PSB (see Teissier et al., 2010).

3.2. Distribution of Dbx1-derived βgal$^+$ cells in the ventral pallium at intermediate stages (E14.5)

At E14.5, the ventral pallium progenitor zone continues to produce new βgal$^+$ neurons that follow radially the ventropallial migratory stream (vms; Fig. 2A,B). The vms seems to have expanded somewhat rostralward, but still does not reach the olfactory bulb region (not shown). This migratory formation is now strikingly elongated radially towards the brain surface, appearing sharply delimited with regard to the adjacent pallio-subpallial boundary and subpallium; this change coincides with a marked thickening of the local telencephalic wall (vms, VP; Fig. 2A,B; Medina et al., 2004). In some earlier publications, the vms was referred to as the 'lateral migratory stream', a name based on the misunderstanding that this stream is oriented tangentially, orthogonally to the radial texture of the cortical primordium (e.g., Bayer and Altman, 1991; Nieuwenhuys et al., 1998; Hirata et al., 2002, 2009). However, tight fascicles of radial glial processes co-localize with the vms, and these end subpially at the olfactory cortex, as detected with appropriate immunochemical markers or DiI-fluorescent labelling, and therefore the complex must be understood as being topologically radial (see Misson et al., 1991; Valverde and Santacana, 1984; De Carlos et al., 1996; Nieuwenhuys et al., 1998; Hirata et al., 2002; Bupesh et al., 2011a,b). This implies that the *Dbx1*-derived, βgal$^+$ ventropallial neurons migrate radially into more superficial strata of the ventral pallium, as defined molecularly (Puelles et al., 2000; Hirata et al., 2002; Puelles, 2014). However, while most βgal$^+$ cells follow the ventropallial migratory stream, some βgal$^+$ cells move tangentially and dorsally away from the vms into the deep parts of the overlying lateral and dorsal pallium (LP; DP; arrow in Fig. 2B,C), as was described by Bielle et al. (2005) and Teissier et al. (2010).

The largest number of superficial βgal$^+$ neurons was found caudally. They fan out of the vms into the ventropallial amygdala

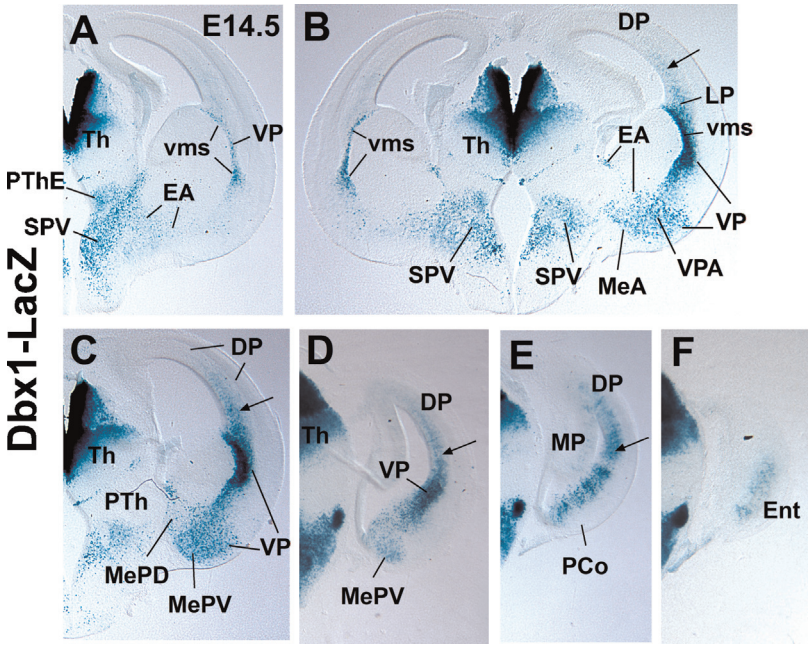

Fig. 2. Frontal sections through the telencephalon of Dbx1-LacZ mice at E14.5. Dbx1-derived cells showing β-galactosidase activity are labeled in blue. Note the high number of Dbx1-derived cells in the ventral pallium (VP). Many of these cells migrate in the ventropallial migratory stream (vms), and many have reached the mantle, and occupy the primordium of ventropallial amygdalar nuclei/cortical areas (VPA), and other olfactory areas. Numerous Dbx1-derived cells from the ventral pallium also invade the medial amygdala (MeA, MePV). However, the medial amygdala also appears to receive Dbx1-derived cells from the subpallium (peduncular/preoptic areas) and the supraopto-paraventricular domain of the hypothalamus (SPV). Caudally, the Dbx1-derived cells of the ventral pallium appear to populate the entorhinal cortex (panel D).

and the ventral part of the nearby piriform cortex (VPA, Pir; Fig. 2B; Bielle et al., 2005). The former was previously held to encompass mainly the primordia of the lateral and basomedial amygdalar nuclei, as well as part of the amygdaloid cortical areas (see Medina et al., 2004). However, our present observations are in accordance with the new definition of VPall and LPall proposed by Puelles (2014). Our data show that the whole anterior part of the basal amygdalar complex (including the primordia of the lateral, basolateral and basomedial nuclei), plus the anterior cortical amygdalar area, are densely populated by βgal$^+$ neurons and, thus, appear to derive from the *Dbx1*-expressing VPall progenitors (Fig. 2B; note that these groups are also aligned with the radially-oriented vms). In contrast, the posterior pole of the basal complex and the posterior cortical amygdalar areas (primordia of the posterolateral and posteromedial cortical areas) are poor in βgal$^+$ neurons (PCo; Fig. 2E; see Discussion). Additional βgal$^+$ cells were observed in the anterior and posteroventral parts of the medial amygdalar nucleus, as well as in other parts of the subpallial extended amygdala (MeA, MePV, EA; Fig. 2B,C). In contrast, the posterodorsal medial amygdala contains only isolated βgal$^+$ cells (MePD; Fig. 2C). The βgal$^+$ cell population of the supracapsular pallial extended amygdala extends to the hemispheric stalk, continuing over the medial amygdala along the periventricular stratum of the terminal sulcus, medial to the caudal and medial ganglionic eminences, and converging finally upon the hypothalamic supraopto-paraventricular area and the prethalamic eminence (SPV; PThE; Fig. 2A–C). This is the pallial amygdalo-hypothalamic corridor mentioned above, which connected with the large extratelencephalic population of βgal$^+$ cells found in the supraopto-paraventricular area already at E10.5 (EA; SPV; Fig. 1A, B). It is difficult to delimit this labeled corridor from the separate βgal$^+$ cell stream that interconnects the medial amygdala across the subcapsular *subpallial* hemispheric region with the periventricular diagonal and preoptic commissural areas of the subpallium (MeA; Fig. 2B; see also data for E18.5 in Fig. 3C). This suggests that the medial amygdalar region may have diverse origins of its *Dbx1*-derived cells, including the ventral pallium, the subpallial diagonal and preoptic-commissural areas, and the hypothalamic SPV area (as previously concluded by García-López et al., 2008; and corroborated using genetic or experimental fate mapping by Hirata et al., 2009; Carney et al., 2010; García-Moreno et al., 2010; and Bupesh et al., 2011a,b). Caudal to the amygdala and the piriform cortex, where the strongly labeled ventricular VPall zone ends (Fig. 2D), the labeled βgal$^+$ mantle territory extends into the entorhinal area, which shows a series of radial clumps of βgal$^+$ cells (Fig. 2E), ventrally to the tangential stream of labeled cells migrating into dorsal pallium (arrow in Fig. 2C–E). Close analysis shows that the primordium of the medial entorhinal cortex (MEC) is nearly empty of βgal$^+$ neurons (Fig. 2F), in agreement with its recently suggested medial pallial origin (Abellán et al., 2014), while the primordium of the lateral entorhinal cortex (LEC; Ent in Fig. 2F) shows caudally a relatively low density of βgal$^+$ neurons (not higher than that observed in the temporal part of dorsal pallium invaded by tangentially migrating neurons – arrows in Fig. 2A–E –, in agreement with a possible tangential migration of neurons originated at the ventral pallium (Bielle et al., 2005; Teissier et al., 2010).

3.3. Distribution of Dbx1-derived, βgal$^+$ cells in the ventral pallium at late prenatal stages (E18.5)

At this stage of development a thin trail of βgal$^+$ cells delineates the ventropallial migratory stream, which is much thinner than before, but still interconnects at mid-telencephalic levels the ventropallial periventricular stratum (adjacent to the pallio-subpallial boundary, near the lateral angle of the ventricle) with the nuclear and cortical derivatives now clearly established within the superficial mantle (Figs. 3–5). The fate of the residual βgal$^+$ vms still observed at E18.5 is unclear; in any case, no such deep trail of βgal$^+$ cells was observed in the juvenile or adult brain (P60; Fig. 7B; see next section). Some of these cells possibly continue to translocate radially until they incorporate into one of the derived ventropallial nuclear or cortical primordia. The closest labelled cell aggregate appears at the locus where the fibers of the anterior commissure bend dorsalward into the external capsule (ac; ec; Fig. 3A,B); a cell aggregate found here in embryos was denominated 'reservoir' by Bayer and Altman (1991); Altman and Bayer, 1995), who considered it a transient formation. It corresponds to what we previously called ventromedial claustrum (Puelles et al., 1999, 2000; Medina et al., 2004), but Puelles (2014) reinterpreted it as a non-claustral permanent VPall derivative, and named it 'bed nucleus of the external capsule (BEC; Figs. 3B, C). This term describes appropriately its final interstitial topography relative to the external capsule, and allows restriction of the term "*claustrum*" to the larger "subinsular" aggregate that derives from the lateral pallium (Puelles et al., 2000; Medina et al., 2004; Puelles, 2014). The BEC extends some distance caudally into rostral amygdalar section levels, where it characteristically appears lateral to the lateral amygdaloid nucleus (BEC; L; Fig. 3C). A different VPall derivative is the ventral endopiriform nucleus (EPv), a superficial neighbor of the BEC found next to the ventral striatum, which is represented by cells distributed deep to layer III of the medialmost olfactory cortex, in the space next to the pallio-subpallial boundary that is left free by the larger lateropallial EPd nucleus, which remains unlabeled in present material (VEn; EPd occupies the unlabeled space lateral to VEn; Fig. 3C; Puelles, 2014).

The vms continues to show at E18.5 the gradiental rostrocaudal distribution of βgal$^+$ cells described at earlier stages, being barely β – galactosidase-positive at the rostral third of the hemisphere (Fig. 3A), while this signal progressively increases caudalwards (Fig. 3B–D; Fig. 4A–C). Caudally, the βgal$^+$ cells of the amygdalar VPall lie closer to the ventricle than more rostrally, in the neighborhood of the claustrum. A caution to keep in mind in this context is that the radial dimension defined by the vms (and the consequent direction of neuronal migration) possibly changes progressively caudalwards with respect to the coronal sections used here. A changing topology of the radial dimension at the caudal pole of the hemisphere, in which the relevant ventricular cavity may lie *at the back of the amygdala* (i.e., in a different section) rather than dorsally in the same section, would cause the caudal end of the VPall to be cut tangentially in coronal sections, whereas its vms would appear in more rostral sections (Fig. 4C,E; Fig. 5A–D).

At the most rostral levels, where few ventropallial βgal$^+$ cells are visible, their β – galactosidase signal is distinctly weaker than at more caudal regions (Fig. 3A–D; these sections were all processed together under identical conditions). In slightly more caudal sections, the local picture changes, due to transition of the BEC into the lateral nucleus of the amygdala and the ventral endopiriform nucleus, both of which contain abundant βgal$^+$ cells (L; VEn; Fig. 3C). In contrast to the BEC, the EPv/VEn is a more dispersed population, which typically lies outside and underneath the external capsule, and approaches the deep stratum of the piriform cortex. There is at this stage a dispersed field of βgal$^+$ cells interspersed between the EPv and the piriform cortex, possibly indicating radial migration still in progress into the latter (Pir; Figs. 3A-C). The piriform cortex tends to show a bilayered structure in *Dbx1^{nlsLacZ}* material, particularly close to the amygdala; this seems to be caused by separate aggregation of βgal$^+$ cells at the developing pyramidal and multiform cell layers (II, III; Fig. 3D,E). This distinction is blurred at other section levels (Fig. 3A–C and Fig. 4A,B). Sections rostral to the amygdala nevertheless clearly illustrate that the olfactory VPall βgal$^+$ population is more

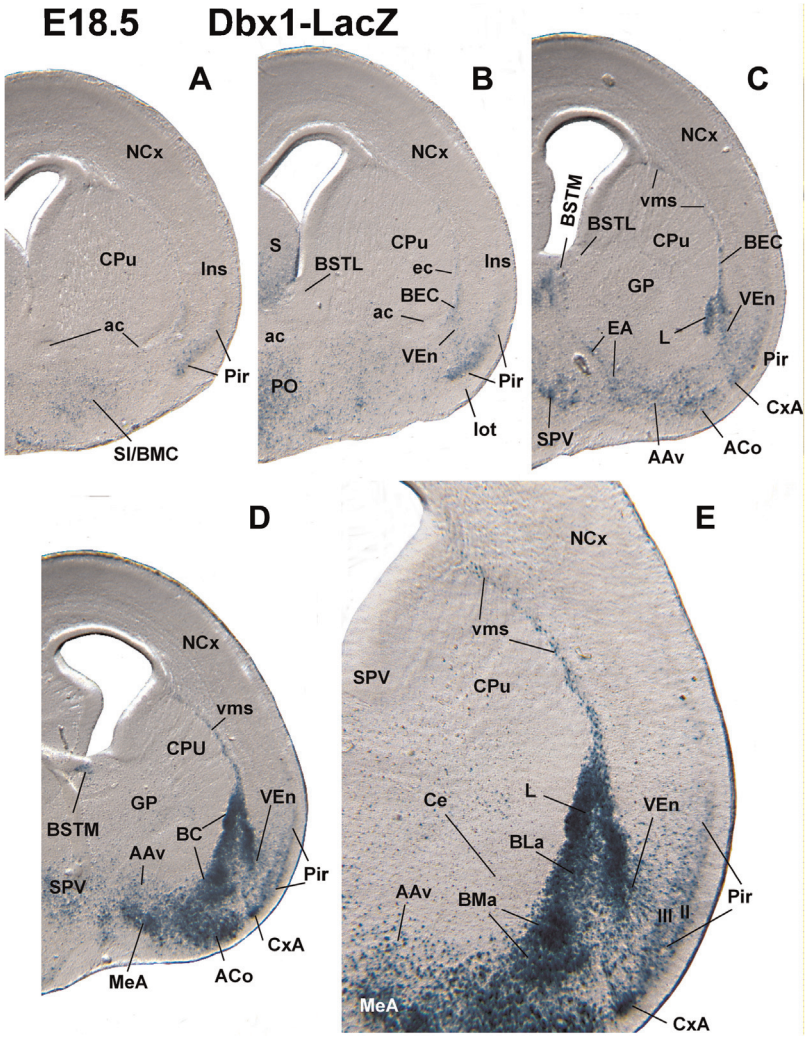

Fig. 3. Coronal sections through the telencephalon of Dbx1-LacZ mice at E18.5, at the levels of the anterior commissure (A, B) or rostral amygdala (C–E). Panel E is a detail of the hemisection shown in (D). Dbx1-derived cells showing β-galactosidase activity are labeled in blue. Note the rostrocaudal gradiental distribution of Dbx1-derived cells in the ventral pallium, with the majority of them observed at caudal hemisphere levels. At caudal, amygdalar levels (caudal to the anterior commissure), many Dbx1-derived cells are observed in the ventropallial migratory stream. Many Dbx1-derived cells of ventral pallial origin are also observed in specific nuclei and cortical areas of the mantle, including the ventral part of the piriform cortex (Pir), a ventral part of the claustral complex (including the bed nucleus of the external capsule [BEC]-previously known as ventromedial claustrum- and the ventral endopiriform nucleus [VEn]), the cortico-amygdaloid transition cortex (CxA), the anterior parts of the lateral (L), basolateral (BLa) and basomedial (BMa) amygdalar nuclei, and the anterior cortical amygdalar area (ACo). In addition, numerous Dbx1-derived cells are observed in the medial extended amygdala, being particularly abundant in the anterior medial amygdala (MeA). The Dbx1-derived cells of the MeA and other parts of the medial extended amygdala appear to have several origins, in Dbx1 expressing progenitors of the ventral pallium, subpallial peduncular/preoptic areas and hypothalamic SPV. See text for more details.

abundant at the ventral sector of the piriform primordium, which covers the lateral olfactory tract (Pir; Fig. 3A,B; Puelles et al., 2000). More caudally, labeled neurons also occupy partially the dorsally contiguous part of the piriform cortex, their number decreasing towards the LPall (Pir; Fig. 3A–E). Medially adjacent to the piriform cortex, the distinct cortico-amygdaloid transition area also shows abundant βgal$^+$ cells, which are connected radially, first with the EPv (CxA; VEn; Fig. 3C–E) and more caudally with the posterior basolateral amygdaloid nucleus (CxA; BLp; Fig. 4A). At even more caudal levels lying behind the amygdala, the lateral entorhinal cortex primordium contains some βgal$^+$ cells in a patchy distribution within its thin periventricular zone, suggestive of positive neuroepithelial clones intermixed with negative ones (VPall; LEC; Fig. 5B,C). Just in front of these levels the caudal ventropallial neuroepithelium appears wider and more distinctly positive (Fig. 5A). This difference in thickness of the labelled periventricular stratum may indicate that the entorhinal elements may have migrated tangentially into this position from the adjacent VPall, rather than being born in the neuroepithelium immediately related to this pallial sector. Tangentially migrating cells that distribute to more dorsally place cortical areas are still visible at these levels (arrows, Fig. 5A,B).

As noted above, the whole anterior part of the basal amygdalar complex is heavily populated with numerous βgal$^+$ cells at E18.5. This includes the lateral nucleus of the amygdala, the anterior basolateral nucleus and the anterior basomedial nucleus (L; BLa; BMa; Fig. 3C–E; Fig. 4A,B). The rostral tip of this basal nuclear complex appears just deep to the rostral pole of the anterior amygdalar cortical area, at which level only a moderate number of βgal$^+$ cells is visible (ACo; Fig. 3C). However, the latter area displays numerous, densely packed βgal$^+$ cells at more caudal levels (ACo; Fig. 3D; Fig. 4A). The intermediate parts of the lateral, basolateral and basomedial amygdalar nuclei also contain abundant βgal$^+$ cells (L; BMp; Fig. 4B–E; Fig. 5A). In contrast, the caudal pole of the basal complex, and the posterolateral and posteromedial amygdalar cortical area only contain few dispersed βgal$^+$ cells (BLp, BLv, PLCo; Fig. 4A–C; Fig. 5B–D). The possible origin of this posterior part of the pallial amygdala will be treated in the Discussion.

In addition, as in previous stages, the medial amygdala, anterior ventral amygdala and neighboring areas -corresponding to medial parts of the medial extended amygdala- contain abundant βgal$^+$ cells (AAv, Me; Fig. 3D,E; Fig. 4A–C). In the medial amygdala, βgal$^+$ cells are more numerous in the anterior and posteroventral parts (MeA; MePV), appearing scarce and dispersed in the posterodorsal subnucleus (MePD) (Fig. 3D; Fig. 4A,B). As noted above, the βgal$^+$ cells in the medial amygdala, and perhaps also those of the AAv and medial extended amygdala, may have a subpallial origin, namely at the diagonal/preoptic subpallial areas, from where they may have reached the amygdala via migration through the subpallial EA (Hirata et al., 2009; Bupesh et al., 2011a,b). Our control results corroborating this possibility will be described below (see Fig. 8). As before, a bridge population of βgal$^+$ cells connecting the ventropallial amygdala with the extratelencephalic supraopto-paraventricular area along the bottom of the terminal sulcus is still visible at this stage (Fig. 3C,D; Fig. 4A–E).

3.4. Distribution of Dbx1-derived cells in the ventral pallium in young adult mice (2 months) using Cre fate mapping

In order to analyze the *Dbx1*-derived cells in the ventral pallium at juvenile stages, we used *Dbx1^{CRE}*; *ROSA26$^{loxP\text{-}stop\text{-}loxP\text{-}LacZ}$* mice, in which these cells are permanently labeled (Bielle et al., 2005) (Figs. 6 and 7). Basically, the postnatal distribution of βgal$^+$ cells observed using this method is almost identical to that of βgal$^+$ cells labeled by *Dbx1^{nlslacZ}* short-term expression at E18.5, although the cells are now more dispersed due to interstitial neuropile growth during postnatal development. The βgal$^+$ cells derived from the ventral pallium are abundantly found in the BEC, EPv, PirCx, CxA, LA, BLa, BMa, and ACo pallial formations, where they represent a subpopulation of the TBR1-positive elements (Figs. 6 and 7; see counterstained material at E18.5 and P1 in Fig. 8A,D), as well as in the subpallial areas populated by *Dbx1*-lineage neurons during early development (such as the medial extended amygdala). Dispersed cells expressing both Dbx1^{CreLacz} label and GABA

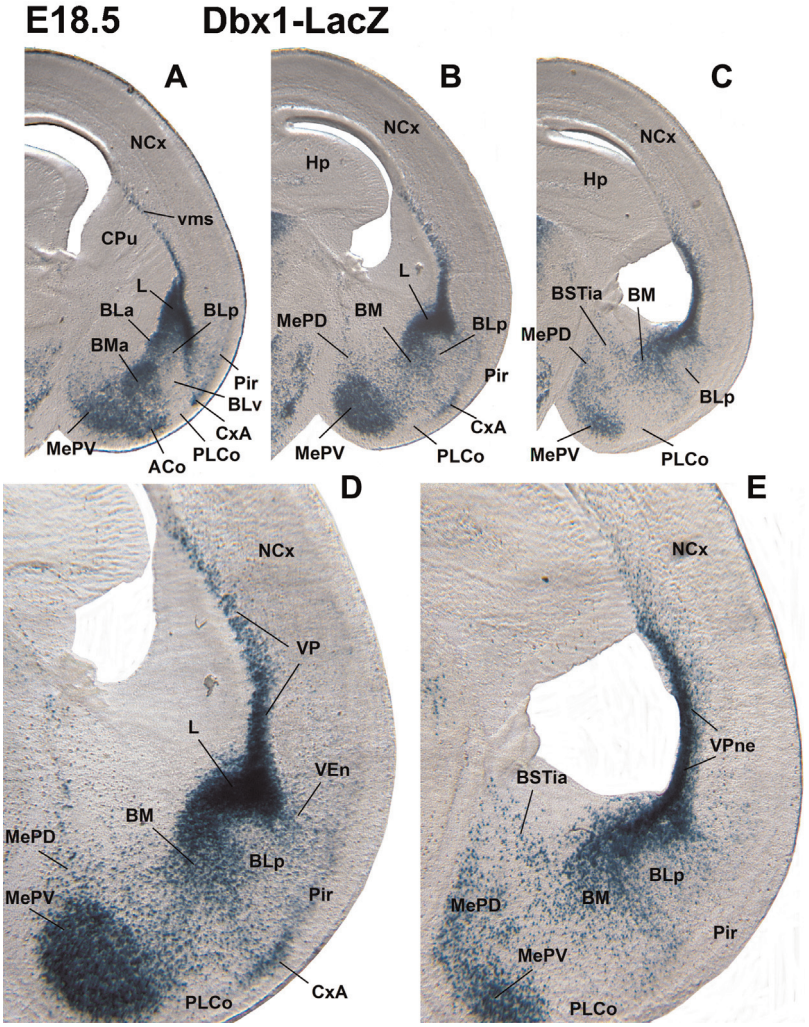

Fig. 4. Coronal sections through the telencephalon of Dbx1-LacZ mice at E18.5, at the levels of the intermediate (A) and caudal amygdala (B–E). Panels D and E are details of the hemisections shown in B and C, respectively. Dbx1-derived cells showing β-galactosidase activity are labeled in blue. As more rostrally (see Fig. 3), many Dbx1-derived cells are observed in the ventropallial migratory stream. Many Dbx1-derived cells of ventral pallial origin are also observed in specific nuclei and cortical areas of the mantle, including the ventral part of the piriform cortex (Pir), the ventral endopiriform nucleus (VEn), the cortico-amygdaloid transition cortex (CxA), the the lateral (L), anterior basolateral (BLa) and basomedial (BM) amygdalar nuclei, and the anterior cortical amygdalar area (ACo). In contrast, the posterior and ventral parts of the basolateral amygdalar nucleus (BLp, BLv) and the posterolateral cortical amygdalar area (PLCo) only contain a few, dispersed Dbx1-derived cells, and the majority of their cells may originate in the lateral pallium. As more rostrally, numerous Dbx1-derived cells are observed in the medial extended amygdala, being particularly abundant in the posteroventral medial amygdala (MePV), although many cells are also present in the posterodorsal medial amygdala (MePD). As those of the anterior medial amygdala, the Dbx1-derived cells of the MePD/MePV appear to have several origins, in Dbx1 expressing progenitors of the ventral pallium, subpallial peduncular/preoptic areas and hypothalamic SPV. See text for more details.

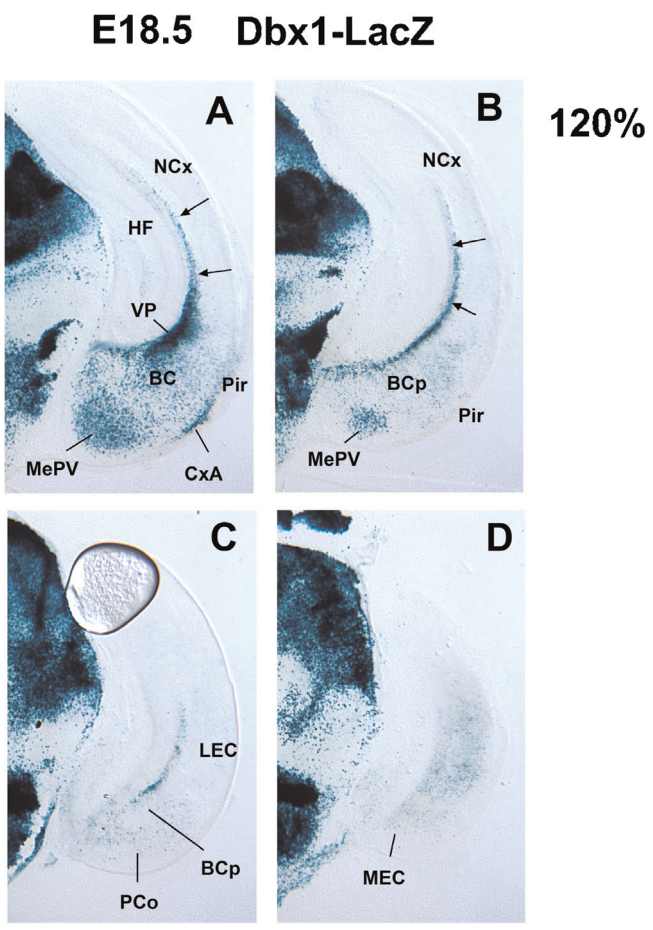

Fig. 5. Coronal sections through the telencephalon of Dbx1-LacZ mice at E18.5, at the very caudal telencephalic levels, showing the very caudal parts of the amygdala and the entorhinal cortex. Panel D is a details of the hemisection shown in C. Dbx1-derived cells showing β-galactosidase activity are labeled in blue. The ventropallial neuroepithelium is heavily labeled with numerous Dbx1-derived cells. Many Dbx1-derived cells of ventral pallial origin are also observed in the posteromedial cortical amygdalar area (PMCo), the entorhinal cortex and the amygdalo-hippocampal transition area. Note the patchy distribution of Dbx1-derived cells in the ventricular zone at the most caudomedial levels (panel D). See text for more details.

immunoreaction are intermixed in most of these areas (see counterstained material at E18.5 and P1 in Fig. 8B,C), though there are practically none within the lateral amygdaloid nucleus (not shown). The part of the piriform cortex closest to the pallio-subpallial boundary and the cortico-amygdaloid transition area (CxA) also contain numerous βgal$^+$ cells (Figs. 6A-D; 7A,B). In the piriform cortex, the positive cells are present in the three layers, but are more concentrated in layer II. In contrast, there are fewer labeled cells in the part of the Pir next to the insular cortex (Pir; Fig. 6A; right leading line), as well as in the posterior part of the basal amygdalar complex (including the ventral part of the basolateral nucleus, BLv, and the posterior poles of L, BL, and BM; Fig. 7A–C, where TBR1-positive cells nevertheless predominate; see counterstained material at E18.5 and P1 in Fig. 8D), and the posterolateral and posteromedial cortical amygdalar areas (PLCo, PMCo; Fig. 7A–C). After considering Dbx^{CreLacz} preparations counterstained with antibodies to either TBR1 (glutamatergic pallial cells) or GABA (subpallium-originated interneurons or projection cells) (Fig. 8), we believe that most of the βgal$^+$ pallial amygdalar cells likely derive from the sizeable *Dbx1*-expressing caudal VPall progenitor domain; the contribution of subpallial GABAergic Dbx1-derived cells to these areas may be equivalent in proportions to the 20% of GABAergic interneurons that invade tangentially the isocortex (DPall) and the claustro-insular complex (LPall). Regarding the entorhinal cortex, the MEC was nearly free of βgal$^+$ cells, as observed at late embryonic developmental stages, while the LEC contained a few dispersed βgal$^+$ cells (Fig. 7C). The area occupied by the nucleus of the lateral olfactory tract was also largely devoid of βgal$^+$ label (LOT1/2/3; Fig. 6B; Fig. 8C).

In addition, the medial amygdala and neighboring subpallial parts of the medial extended amygdala also contain βgal+ cells, which are most abundant in the anterior and posteromedial parts of the medial amygdala (MeA, MePV; Fig. 6C,D; Fig. 7A,B). These nuclei do not show TBR1 immunoreaction (Fig. 8D), so that a pallial origin can be discarded. A subpallial source (preoptic and diagonal domains) is also unlikely, due to the scarcity of local GABAergic cells (not shown). This pattern suggests that these Dbx1-derived cells originate in an extratelencephalic *Dbx1*-positive progenitor domain, most probably the hypothalamic SPV area, whose cells are glutamatergic (Puelles et al., 2012) and are known to produce a subset of cells that migrate supra- and subcapsularly into the medial amygdala (Wang and Lufkin, 2000; González-Moreno et al., 2010; Morales-Delgado et al., 2011).

4. Discussion

After attending to technical considerations, we will discuss first our results on radially migrated VP derivatives. Next we will reexamine under the light of present data the contrast between ventropallial and lateropallial parts of the pallium, as recently reformulated (Puelles, 2014). Finally we will consider the issue of medial and extended amygdala regions as sites of convergence of cell populations of diverse origins.

4.1. Technical considerations and comparison with Dbx1 RNA expression

We noted some differences in the labeling obtained in *Dbx1$^{nlslacZ/+}$* versus *Dbx1$^{cre/+}$; ROSA26$^{loxP\text{-}stop\text{-}loxP\text{-}LacZ}$* embryos. These are likely due to a delay in the recombination and expression of the reporter gene in *Dbx1$^{cre/+}$*; *ROSA26$^{loxP-stop-loxP-LacZ}$* embryos, such that the earliest *Dbx1*-derived cells apparently are not labelled in these animals, although those that do get labelled, remain so permanently (Bielle et al., 2005). Nevertheless, the βgal$^+$ labelling obtained in *Dbx1$^{nlslacZ/+}$* animals does show labelled ventricular zone progenitors (mainly from early until intermediate stages, but also at E18.5, at caudal amygdalar levels), which supposedly continue expressing the *Dbx1* gene apart of producing migrating and differentiating neuronal progeny. Comparatively, the ventricular zone expression obtained with *Dbx1* mRNA in situ hybridization was much stronger, but is soon downregulated, and is strongly reduced at E14.5 (Yun et al., 2001; Medina et al., 2004; Nomura et al., 2009). Otherwise, the labelling obtained with the reporter coincides in position with that obtained using Dbx1 RNA in situ hybridization (Yun et al., 2001; Medina et al., 2004; Flames et al., 2007). The stability of the nuclear β-galactosidase protein allows to visualize the postmitotic VPall derivatives for at least 24 h after the Dbx1 gene has been downregulated in postmitotic cells. This suggests that the relative scarcity of labelled cells found at subpial levels of the VPall mantle at E10.5 and E12.5 (Fig. 1A–C,F) may be due to the slightly earlier birthdates of such neurons. The data obtained with both reporters regarding Dbx1-lineage derivatives in the endopiriform, amygdalar, septal and preoptic domains agrees substantially in topography with previous descriptions using other transgenic mice with Dbx1-lineage fate mapping (Bielle et al., 2005; Hirata et al., 2009; Teissier et al., 2010; Waclaw et al., 2010).

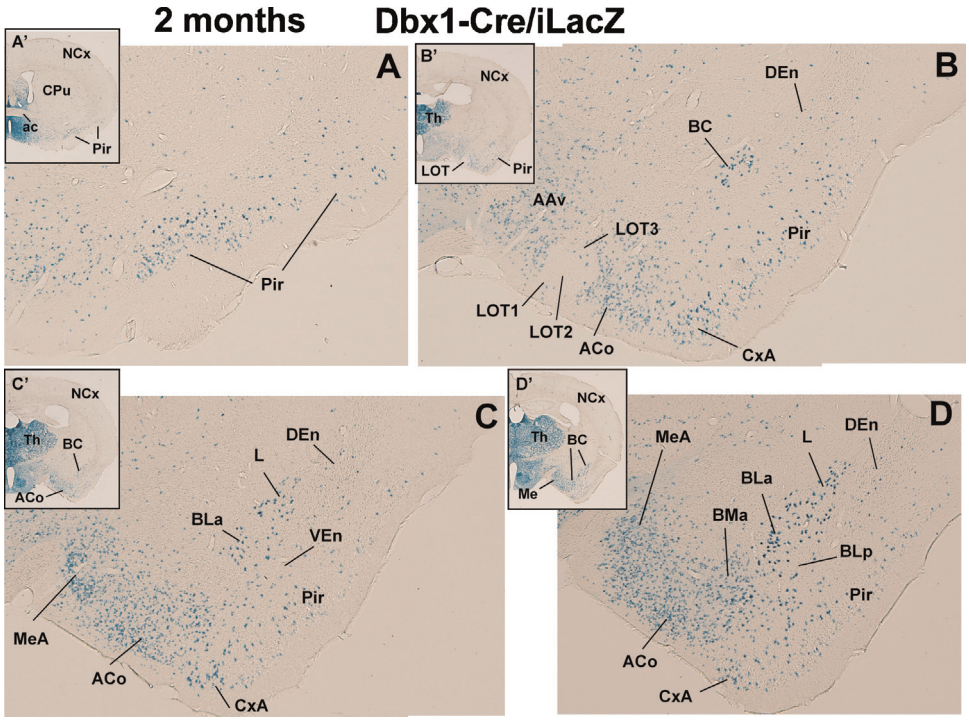

Fig. 6. Coronal sections through the telencephalon of 2 months-old Dbx1-Cre; β-actin-indolyl-LacZ mice, at rostral amygdala levels. Panels A, B, C and D are details of the hemisections shown in A′, B′, C′ and D′, respectively. The distribution of Dbx1-derived cells in the piriform cortex and amygdala is similar to that shown in previous stages, although the cells now show a more loosely organization. See text for more details.

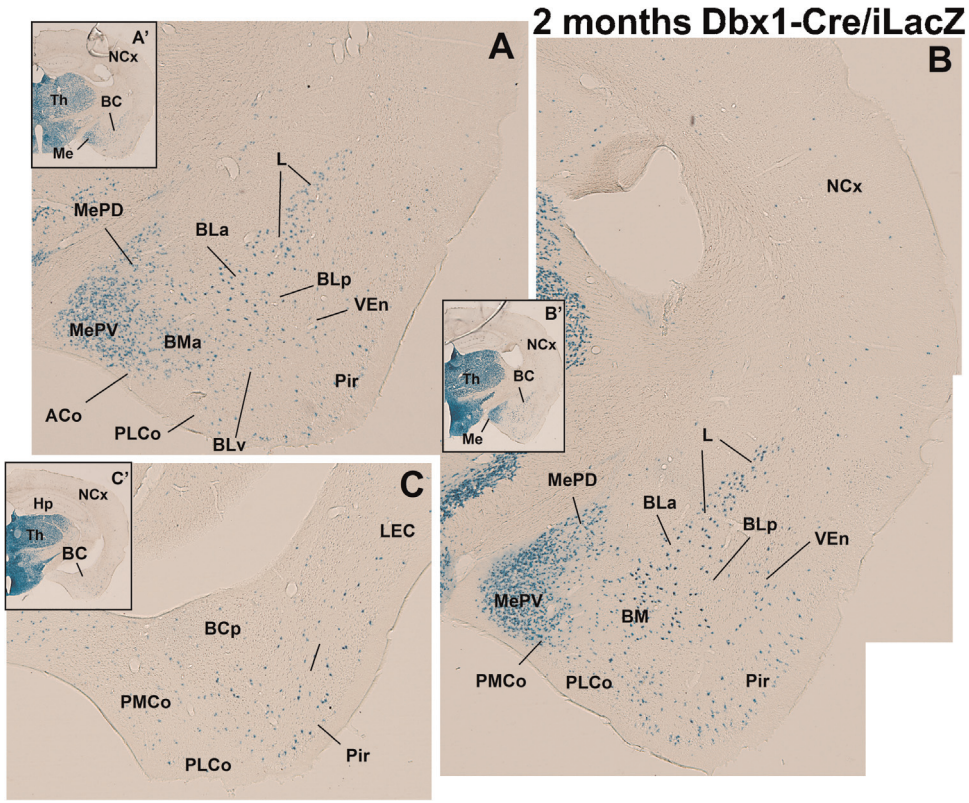

Fig. 7. Coronal sections through the telencephalon of 2 months-old Dbx1-Cre; β-actin-indolyl-LacZ mice, at caudal amygdala levels. Panels A, B, and C are details of the hemisections shown in A', B', and C', respectively. The distribution of Dbx1-derived cells in the ventral piriform cortex, ventral claustral complex, and amygdala is similar to that shown in previous stages, although the cells now show a more loosely organization. See text for more details.

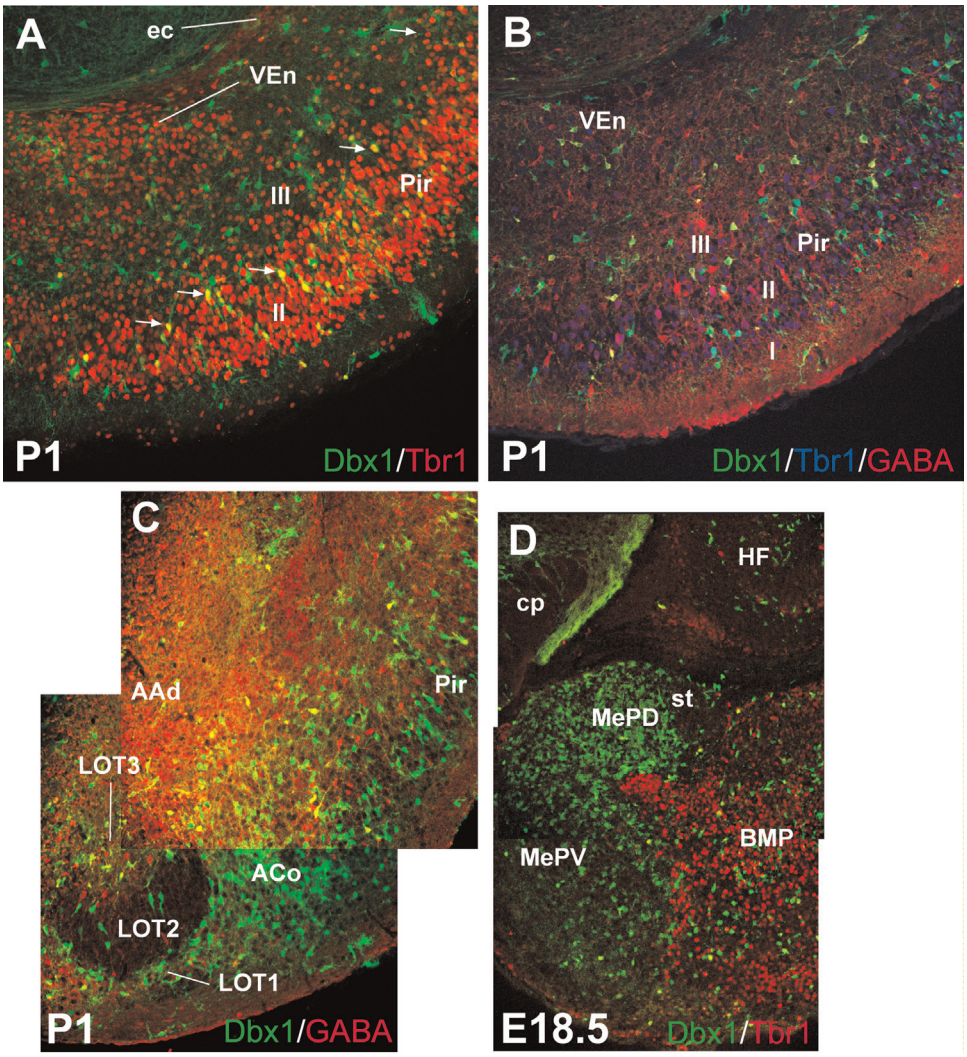

Fig. 8. Coronal sections through the telencephalon of P1 (A–C) and E18.5 (D) Dbx1-Cre/EGFP mice with fluorescent Dbx1 signal (green), counterstained with immunofluorescence for Tbr1 (A,B,D) and/or GABA (B,C) for the characterization of pallial (Tbr1) versus subpallial (GABA) ascription of Dbx1-labeled or unlabeled neurons in the olfactory cortex (A,B) and in the amygdala (C,D). Note in (A) that double-labeled cells (yellow) are present in the olfactory cortex (Pir) and ventral endopiriform nucleus (VEn), albeit representing a subpopulation of all Tbr1-positive (pallial) elements. Subpallium-originated Dbx1 cells (green) are also present. The proportion of this component can be judged as well in (B) (red and yellow cells). (C) shows practical lack of Dbx1-labeled cells at the LOT2, while their number is considerable at the neighbouring ACo and Pir areas, with little overlap of GABA immunoreaction, indicating pallial adscription. (D) illustrates a clearcut border between subpallium- or extratelencephalic-originated Dbx1-labeled cells (green) at the medial amygdala (MePD, MePV) and the Tbr1-labeled pallial amygdala, which contains a subpopulation of Dbx1-labeled cells (BMP).

4.2. The ventral pallium in the mouse and other vertebrates

Using genetic cell tracing with the LacZ reporter gene, our results provide additional evidence that the ventral pallium represents a distinct radial histogenetic subdivision of the telencephalon, and that *Dbx1*-expressing progenitor cells of this pallial subdivision produce specific cell populations of the olfactory cortical areas and the pallial amygdala. This result is consistent with the data on piriform cortex of Bielle et al. (2005), the analysis of Hirata et al. (2009), using Dbx1-Cre-ERT2; R26R-LacZ and Dbx1-Cre-ERT2; R26R-YFP transgenic mice, and the study of Waclaw et al. (2010), using Dbx1-Cre; CC-EGFP transgenic mice. Apart of piriform cortex neurons, the VPall derivatives clearly include as well the bed nucleus of the external capsule (BEC; this is the 'reservoir' of Bayer and Altman, 1991), the ventral endopiriform nucleus, a large anterior part of the basal amygdalar complex (anterior parts of lateral, basolateral and basomedial nuclei), the anterior cortical amygdalar area, and the cortex-piriform transition area. In contrast, the posterior part of the basal complex (including the ventral part of the basolateral nucleus or BLv, and the posterior pole of L, BL, BM) as well as the posterior cortical amygdalar areas (posterolateral and posteromedial cortical areas) contain few Dbx1-derived cells. This pattern may be interpreted as excluding these grisea from being primarily a part of the VPall radial domain as defined by Dbx1 lineage, although these areas apparently do contain some immigrant VPall-derived cells, as well as tangentially immigrated subpallial Dbx1-derived cells. This consideration would force us to the odd conclusion that the classic L, BL and BM cytoarchitectonic entities are implicitly wrongly defined, if they lump together VPall derivatives (anteriorly) with non-VPall derivatives (posteriorly). A large number of amygdalar connectivity and functional studies would need to be reexamined with this idea in mind. However, before jumping to this extreme conclusion, we feel that additional lines of possible interpretation and corresponding test studies are needed to determine whether the classic subdivision can be saved. Firstly, these posterior amygdalar formations may be straightforward caudal VPall derivatives that are invaded by particularly abundant contingents of subpallial, MPall and/or extratelencephalic non-Dbx1-derived cell types, so that locally the Dbx1 marker is diluted (Hirata et al., 2009; Bupesh

et al., 2011a,b); however, it seems strange that this phenomenon affects so selectively the caudal parts of three nuclei (L, BL, BM). Secondly, there might exist a separate pallial sector distinct from both VPall and LPall at this caudal location, as was recently proposed by Abellán et al. (2014); a participation of MPall in amygdalar structure was also considered by these authors, but thought to be unlikely. In support of this concept, the posterior pallial amygdala areas (including BLv, BLp and PLCo) claimed here to show relatively few radial VPall derivatives appear to be rich in *Emx1* expression and Emx1 lineage derivatives (Gorski et al., 2002; Medina et al., 2004). However, this hypothesis does not explain why the derivatives of this extra pallial domain should build the caudal parts of the L, BL and BM nuclei, complementing the rostral VPall ones, rather than producing a separate set of amygdalar nuclei. Finally, a third possibility that needs to be considered is that these molecularly heterogeneous amygdalar structures seem cytoarchitectonically unitary because they are in fact histogenetically unitary, i.e., because their differences may be superficial; their molecular heterogeneity may be a feature of the VPall domain as a whole, if the latter has *Dbx1*-positive and *Dbx1*-negative subdomains. This implies that *Dbx1* expression and Dbx1 lineage would not completely define the VPall. We have noted above that anterior olfactory parts of the VPall (OB and AOA) show practically no Dbx1 lineage labeling. We also observed that there are relatively less labeled cells in the dorsal part of the prepiriform and piriform cortex than in the underlying ventral parts (but there is otherwise a sharp molecular boundary of the whole olfactory cortex with the overlying LPall, occupied by claustro-insular structures; Puelles, 2014). To these phenomena we may now add the lack of label in the cited posterior pallial amygdalar nuclei. Jointly, these results suggest that there possibly exists dorsally a rostrocaudal VPall subregion that is not labeled in our material because its progenitor cells do not express *Dbx1*. As we point out below, there is separate evidence that *Dbx1* function is not needed for the histogenesis of VPall derivatives, though it may be relevant for the emergence of the radial and tangential migratory properties of VPall derivatives. In this sense it is relevant to note that the unlabeled posterior parts of the L, BL and BM amygdalar nuclei occupy radial positions next to the ventricular lining, in contrast to the labeled anterior portions, which are more separated from the ventricle.

In any case, the picture provided by present results of genetic lineage analysis is partially different from our previous proposal on the origin of the pallial amygdala, which was based on the combinatorial expression of several genes (Medina et al., 2004). The present approach is more powerful, since it allows permanent and specific labeling of VPall derivatives (at least those related to Dbx1-lineage-generating progenitors; Bielle et al., 2005; Teissier et al., 2010). The pallial amygdala is now also better understood in the context of the recent redefinition of the LPall as a claustro-insular composite histogenetic pallial area, which seems not to contribute to the pallial amygdala at all, either radially or tangentially (Puelles, 2014). This conclusion differs from our previous notion in which we ascribed BL and PLCo to the LPall on the basis of *Emx1* expression (Medina et al., 2004). This observation now needs to be correlated with the different hypotheses that aim to explain singularities at the posterior pallial amygdala (above).

The entorhinal cortex was initially suggested to be a caudal part of the VPall, based on its relative position and shared expression of *Lhx9* (Abellán et al., 2009). More recently, using several transcription factor markers to define the medial pallium (including *Lef1*, *Lmo4* and *Lhx9*), Abellán et al. (2014) claimed that the two classic divisions of the entorhinal cortex have different embryonic origins: the medial part (MEC) derives from the medial pallium, while the lateral part (LEC) derives from a molecularly distinct, novel 'dorsolateral caudal' pallial domain. This interpretation agrees with recent results on pallial protodomains based on transcriptionally regulated enhancers (Pattabiraman et al., 2014), which show that the MEC lies under the same enhancer regulation as the hippocampal formation (see Fig. 2 of that article), while the LEC is excluded from enhancer activity driving reporter expression in the hippocampal formation and MEC, or in the neocortex (see supplementary Figs. 1 and 2 K of that article). Our present data show that MEC is nearly free of Dbx1-lineage VPall derivatives, consistently with its exclusion from the VPall by Abellán et al. (2014). Our data do show that the LEC contains a significant subpopulation of Dbx1-derived cells, but their number is limited, and we suspect that this subpopulation may have immigrated tangentially in from the caudal VPall, representing at two months a remnant of the transient Pierani cells (Teissier et al., 2010; Puelles, 2014). More studies will be needed in order to fully understand the caudal pole of the pallium, including mappings of additional selective transcription factors or other molecules, study of enhancer activity focused on this region, and further analysis of pallio-pallial cell migrations (Puelles, 2011).

Poor expression of *Dbx1* in progenitors at the theoretic rostral parts of the ventral pallium –the olfactory bulb and the anterior olfactory areas- caused an absence of data for that region with the present approach. A similar cause may explain the general lower level of labeling found in the dorsal part of the piriform cortex. The posterior parts of the pallial L, BL and BM amygdalar nuclei were largely unlabeled. As sketched above, this possibly implies there exists a ventrodorsally decreasing gradient of *Dbx1* expression at the VPall ventricular zone, apart of the well-known caudorostral decreasing gradient (Bielle et al., 2005; Teissier et al., 2010). *Dbx1* expression thus maybe is selectively characteristic of a large part of the VPall, but does not properly define this territory in its entirety. The *pallial extended amygdala* bridging the amygdalo-hypothalamic pallial corridor at the back of the telencephalic stalk (e.g., a domain illustrated by Fan et al. (1996) and Kim et al., 2001 using *Sim1* and *Sfrp2* expression, respectively; see also our Fig. 1) may be considered a continuation of the VPall, which extends beyond purely amygdalar territory and through a thin hemispheric stalk corridor under the terminal sulcus into the pallio-hypothalamic boundary zone. This pallial extended amygdala corridor is contiguous to the broader *subpallial extended amygdala* domain, which is dominated by the *Calb1*-expressing BST complex (Medina and Abellan, 2012; Puelles et al., 2015). On the other hand, our control preparations using *Tbr1* and *Gad67* as pallial and subpallial markers, respectively, indicate that the medial amygdaloid nuclear region receives Dbx1-derived cells of mixed origins, partly migrated tangentially from the subpallial diagonal area (also known as ventrocaudal medial ganglionic eminence; Bupesh et al., 2011a), and partly migrated tangentially from the subpallial septocommissural preoptic area (Carney et al., 2010; Bupesh et al., 2011a), or from the hypothalamic supraopto-paraventricular area (Wang and Lufkin, 2000; García-Moreno et al., 2010; Morales-Delgado et al., 2011; Bupesh et al., 2011a,b). In addition, we cannot discard a contribution to the medial amygdala from the prethalamic eminence, which also produces Dbx1-lineage cells (present results) and is an additional source of cells invading the medial extended amygdala (Bupesh et al., 2011b; Puelles, 2011; Abellán et al., 2013).

4.3. The ventral pallium in comparative context

Earlier genoarchitectonic studies in the mouse, chick and other tetrapods first recognized the existence of a molecularly distinct ventral pallial subdivision (e.g., Smith-Fernández et al., 1998; Puelles et al., 1999, 2000; Brox et al., 2003, 2004), which represents a fragment of the ampler earlier concept of classic "lateral" pallium (Striedter, 1997). The new concept was based on the disposition of radial glial fibers in the area, the combinatory expression patterns

of a handful of developmental regulatory genes, and some ancillary morphological evidence, such as the existence of distinct structural boundaries and differential connections in mammals and other vertebrates (Bruce and Neary, 1995; Smith-Fernández et al., 1998; Puelles et al., 1999, 2000, 2004, 2007; Medina et al., 2004). The idea of the ventral pallium had historic antecedents in the work of Holmgren (1925) and Källén (1953, 1962). Apart of mouse and chicken results, a comparable ventropallial subdivision was observed with homologous gene markers in other vertebrates, including the turtle, frog, teleost fishes, shark and lamprey, and it appears now to be recognized as a common feature in vertebrates (Smith-Fernández et al., 1998; Pombal and Puelles, 1999, 2001; Puelles et al., 1999, 2000; Bachy et al., 2002; Wullimann and Rink, 2002; Brox et al., 2003, 2004; Medina et al., 2004; Ferreiro-Galve et al., 2008; Rodríguez-Moldes, 2009; Abellán et al., 2009, 2014). In the ventral pallium of vertebrates, general pallial markers such as *Pax6* appear expressed selectively in ventricular cells, and *Tbr1* is expressed in postmitotic mantle cells, whereas *Emx1*-otherwise a widespread pallial mantle marker- is expressed only subpially (at the olfactory cortex primordium) and in parts of the amygdalar complex (mostly in its posterior pole), at least at early stages (Puelles et al., 2000; Gorski et al., 2002; Cocas et al., 2009). Eventually, the mature ventral pallium may contain some postmitotic cells expressing *Emx1* or *Dlx2/5* in the mantle, which apparently migrate in tangentially from other pallial subdivisions, or the subpallium, respectively (Smith-Fernández et al., 1998; Puelles et al., 1999, 2000; Gorski et al., 2002; Bachy et al., 2002; Wulliman and Rink, 2002; Brox et al., 2003, 2004; Puelles, 2014); Griveau et al. (2010) reported that Dbx1 progeny does not coincide with Emx1 progeny. The minor subpopulations with subpallial origin include different types of GABAergic interneurons, some of which include calcium binding proteins (Dávila et al., 2005; Legaz et al., 2005a,b) or different neuropeptides (Real et al., 2009).

Another transcription factor typically expressed in VPall derivatives in different vertebrates is *Lhx9*, which is especially present at its caudal amygdalar levels (Moreno and González, 2006; Abellán et al., 2009, 2013, 2014; Medina and Abellán, 2009). Postmitotic cells differentiating at the rostral and caudal poles of the VPall (the areas largely devoid of detectable Dbx1 lineage derivatives) express *Lhx9* in different vertebrates, including mouse, chicken, lizard, and frog (Bulchand et al., 2003; Remedios et al., 2004; Moreno et al., 2004; García-López et al., 2008; Abellán et al., 2009; 2013). At rostral levels, this expression characterizes the principal cells of the olfactory bulb (Abellán et al., 2008), whereas at caudal levels the expression is primarily observed at the ventropallial part of the amygdala and the entorhinal cortex (Remedios et al., 2004; Moreno et al., 2004; García-López et al., 2008; Abellán et al., 2009). In the entorhinal cortex of the mouse, *Lhx9* expression is mainly found in its medial part (MEC), which is now considered a part of the medial pallium, characterized by high expression of *Lhx9*, *Lmo4* and *Lef1* (as explained above and discussed in Abellán et al., 2014). Our present results on Dbx1-derivatives agree substantially with these previous reports on the ventral pallial origin of the olfactory cortical areas and a large part of the pallial amygdala (except its caudal pole), and with the current notion of the entorhinal cortex as possibly not belonging to the VPall. In the mouse, the VPall cells are presently shown to derive largely from *Dbx1*-positive progenitors located in a caudorostrally decreasing gradient along the restricted ventricular zone of the ventral pallium (present results; Yun et al., 2001; Medina et al., 2004; Bielle et al., 2005; Teissier et al., 2010, 2012; see also Hirata et al., 2002, 2009). The *Dbx1*-expressing progenitor cells are distinctly most numerous at amygdalar levels.

Curiously, in contrast to the mouse, the ventral pallium of the chicken does not express *Dbx1* (Bielle et al., 2005; Nomura et al., 2009). It is unknown whether this peculiarity is shared by non-mammalian vertebrates in general. Thus, although all gnathostome vertebrates seem to possess a ventral pallial subdivision in the telencephalon that is poor in cells expressing *Emx1* and produces specific olfactory, endopiriform and amygdalar structures, only in some of them (such as the mouse, and probably other mammals) the progenitors of a significant part of this subdivision express *Dbx1*. This raises the issue of the functional role of *Dbx1* in ventral pallial development, which is strictly unresolved as yet. Attempts to identify a clear ventral pallial phenotype in *Dbx1*-/- mice, using gene expression markers of the ventral pallium and adjacent regions, apparently were inconclusive (Borello and Rubenstein, unpublished). This suggests, consistently with the knowledge of its absence in the chick, where a large VPall is present, that *Dbx1* function may not be needed *per se* for the production of the VPall as a pallial area distinct from the lateral pallium. This function may correspond to a set of as yet undisclosed genetic determinants, possibly shared by all vertebrates (e.g., see Pattabiraman et al., 2014).

According to findings in the spinal cord (Pierani et al., 2001), it may be conjectured that *Dbx1* may have a role in promoting radial and tangential migration, a capacity that clearly characterizes the mammalian VPall postmitotic neurons, as opposed to non-mammalian counterparts, which stratify in an outside-in sequence and do not migrate tangentially (Tsai et al., 1981a,b; Goffinet et al., 1986; Bielle et al., 2005; Hirata et al., 2009; Teissier et al., 2010, 2012; Suzuki et al., 2012; Puelles et al., 2016). There is however little information about the existence of tangential pallio-pallial migrations in non-mammals (Métin et al., 2007; Striedter et al., 2008; Puelles, 2011; Puelles et al., 2016). Nomura et al. (2009) performed elegant transgenic experiments in quail suggesting that *Dbx1* function reorganizes local telencephalic radial glia cells via signals released by reelin-secreting cells, creating conditions favourable for radial migration which would be normally absent without this signalling.

It can therefore be conjectured that co-option of *Dbx1* expression in the VPall of ancestral mammals (possibly preceded by transpositional changes in the cis-regulatory region of the *Dbx1* gene) was a significant factor leading to emergence of radial migration in early mammals, at least within the VPall, possibly with widespread consequences on the evolution of the cerebral cortex. Compare the massive dorsal ventricular ridge of sauropsids – essentially a large *periventricular* mass of cells extending to the brain surface (Puelles et al., 2016) -versus the superficially migrated cortical, endopiriform and amygdaloid derivatives in mammals – essentially keeping some caudal periventricular remnants. (Holmgren, 1925; Striedter, 1997; Aboitiz, 1999; Puelles et al., 2000; Puelles, 2001, 2011). The expression of *Dbx1* is known to be regulated by retinoic acid signals in caudal and rostral parts of the neural tube, including the telencephalon (Pierani et al., 1999, reviews by Campbell, 2003; Lupo et al., 2006), but we lack relevant data on the comparative distribution of this developmental regulatory mechanism.

In contrast to the situation in most vertebrates, the radial dimension of the ventral pallium is strikingly changed in its apparent topography in rodents and other mammals, probably accompanying the histogenetic change from outside-in to inside-out layering and a differential regulation of proliferation and neurogenesis (Misson et al., 1991; Nieuwenhuys et al., 1998). This morphogenetic effect is also evident in our results with *Dbx1*-derived cells. In topographic terms, the ventropallial radial glia of the mouse forms a vertical curtain extending from a thin longitudinal matrix area next to the pallio-subpallial boundary and the lateral angle of the lateral ventricle down to subpial glial endfeet at the surface of the olfactory cortex (Misson et al., 1991; Valverde and Santacana, 1994; Hirata et al., 2002). In contrast, the disposition along the ventriculo-pial axis of the massive

non-mammalian VPall is variously oblique topographically, due to the large intraventricular protrusion (the dorsal ventricular ridge), which causes radial glia fibers to course generally caudo-rostrally, with various degrees of mediolateral and latero-medial obliquity (Bachy et al., 2002; Wullimann and Rink, 2002; Brox et al., 2003, 2004; Puelles et al., 2000, 2007; Sandoval et al., in preparation). Nevertheless, the topology of the mammalian VPall relative to its immediate neighbors within the telencephalic wall, the striatum and the LPall, remains invariant across the described evolutionary change in morphogenesis (Puelles et al., 2016). As is abundantly remarked in the literature, the ventralwards change in position of the mammalian olfactory cortex (correlative with ventralward deformation of ventropallial radial glia fibers) occurs in parallel with the disproportionate enlargement of both the neocortex and the striatum.

The mammalian ventropallial migratory stream proceeds along a particularly dense ventropallial packet of radial glia fibers (Medina et al., 2004; Hirata et al., 2009; present results). This cellular current was previously frequently misinterpreted as a "lateral migratory stream", supposed to bring dorsopallial cells (diverted tangentially from a normal radial route into the cortex) to a lateral "reservoir", where they awaited ulterior targeting into the 'ventrolateral' cortex (Bayer and Altman, 1991; see critical comments on this notion in Puelles, 2014). Our present data corroborate the recent appreciation that the so-called *reservoir* largely corresponds to the bed nucleus of the external capsule (BEC), which represents a permanent remnant of the ventropallial migratory stream, that is, contains radially migrated VPall cells that do not reach the ventral endopiriform nucleus or the olfactory cortex.

An additional consideration is that recruitment of *Dbx1* to the VPall molecular identity tool-kit may well relate causally to the evolutionary emergence of tangential pallio-pallial cell migrations from the VPall into the DPall (Bielle et al., 2005; Teissier et al., 2010, 2012; Puelles, 2011), insofar as there is evidence that such VPall migrations do not exist, at least in chicken embryos (Hirata et al., 2009; Suzuki et al., 2012). This is a phenomenon that may be of crucial importance for the evolution of the mammalian neocortex (Borello and Pierani, 2010; Puelles, 2011; Arai and Pierani, 2014). While Bielle et al. (2005) demonstrated an early contribution of tangentially migrating VPall cells (produced at E11.5) to the population of mouse neocortical Cajal-Retzius cells, Teissier et al. (2010, 2012), working in the same laboratory, identified a second and sizeable set of tangentially migrating VPall cells (produced with a peak at E12.5), which colonize transiently as a subpopulation all layers of the whole neocortex, and later largely die postnatally. However, these Pierani cells (Puelles, 2014) migrate through the cortical subventricular zone, and, doing so, they exert an important mitogenic effect on the dorsopallial progenitor cells, causing them to increase the production of pyramidal neurons by 25% (Teissier et al., 2010, 2012). No such phenomenon is known to occur in the sauropsidian pallium (Hirata et al., 2009; Suzuki et al., 2012, Suzuki and Hirata, 2014).

4.4. Ventropallial versus lateropallial parts of the pallium

Apart of the initially detected paucity of *Emx1* signal in the VPall (Smith-Fernández et al., 1998; Puelles et al., 1999, 2000; Griveau et al., 2010), the list of markers characterizing the VPall differentially relative to the LPall accrued subsequently: e.g., *Sfrp2*, *Wnt7b*, several members of the *epidermal growth factor* (*EGF*) family and *Fgf7* (Kim et al., 2001; Assimacopoulos et al., 2003), as well as *Dbx1*, *Ngn2*, *Sema5A* and *Cadh8* (Medina et al., 2004) and *Lhx9* (García-López et al., 2008; Abellán et al., 2009; 2013). However, the signals of these markers do not overlap precisely at the ventricular zone, and, excepting *Lhx9*, the derivatives of the progenitors expressing them have not been traced in the mantle zone yet.

The genoarchitectonic distinction of VPall and LPall pallial domains (these were previously considered to build an unitary "lateral pallium"; Striedter, 1997) served to highlight a possible division of both the piriform cortex and the non-amygdalar deep hypopallial nuclei (pallial nuclei that develop deep to olfactory cortex) into dorsal and ventral parts ascribed respectively to LPall and VPall (Puelles et al., 1999, 2000; Medina et al., 2004). Dorsolateral and ventromedial portions of the claustrum and dorsal and ventral endopiriform nuclei were thus postulated, held to form strictly deep (radially) to the dorsal and ventral parts of the piriform cortex. The relevant partition of hypopallial nuclei is hardly visible cytoarchitecturally in most mammals, but seemed consistent with anatomical observations obtained in the brain of insectivores (Sanides, 1969; Dinopoulos et al., 1992; Narkiewicz and Mamos, 1990); these "basal" mammals, and possibly also monotremes and marsupials (Puelles, 2014), show less morphogenetic deformation of the VPall/LPall area than is found, e.g., in rodents, carnivores and primates. The sauropsidian dorsal ventricular ridge (postulated as the field homolog of the mammalian hypopallium plus piriform cortex complex; Holmgren, 1925; Striedter, 1997) shows in birds a clearcut dorsoventral division in two main parts, known modernly as the mesopallium (=LPall) and nidopallium (=VPall) (Reiner et al., 2004; Puelles et al., 2007, 2016).

Our present results in mouse, analyzed in the light of the recent mapping of claustral derivatives marked selectively by *Nr4a2* expression (Puelles, 2014), confirm this author's conclusion that a change in the cited VPall/LPall configuration needs to be contemplated, affecting the postulated ascriptions of individual grisea to either the VPall or LPall pallial sectors. In that study, it was observed that radial glia processes traversing the principal (dorsolateral) part of the claustrum do not end subpially in the olfactory cortex, as was assumed previously, but in the insula. This unexpected result, jointly with *Nr4a2* gene expression data, led to the conclusion that the LPall is really formed by the claustrum and the insular cortex superficial to it. This left the whole piriform cortex as an apparent derivative of the VPall, as had been suggested by Hirata et al. (2009); indeed, this cortex receives the thick packet of radial glial fibers that bypasses medially the main claustrum, coinciding with the ventropallial migratory stream (Misson et al., 1991). Moreover, the same analysis mapping *Nr4a2* revealed that the dorsal endopiriform nucleus (EPd) expresses this marker (in contrast to EPv, which shows no trace of it, and belongs to ventropallial Dbx1 progeny; Teissier et al., 2010; present data). The EPd cells can be observed to migrate tangentially at early stages from the claustral primordium (in the LPall) into the adjacent VPall, where this population becomes established deep to the dorsal part of the piriform cortex (Puelles, 2014). There is a caudal extension of the dorsal endopiriform nucleus, which is found at amygdalar levels, and shows the same labelling and migratory origin. None of these populations (known to express *Emx1* and derive from *Emx1*-progeny; Gorski et al., 2002) were found among the VPall progeny in the present study. This result corroborates the thesis that they are lateropallial (claustral) in *origin*, though ventropallial in postmigratory *topography* (Puelles, 2014). *Viceversa*, the BEC/ventral endopiriform nucleus complex belongs to VPall Dbx1-lineage and was identified deep to the ventral part of the piriform cortex (BEC; EPv). The BEC corresponds to what we previously called 'ventromedial claustrum' (Puelles et al., 1999, 2000; Medina et al., 2004), but does not express *Nr4a2* (nor other claustral markers, such as *Cadh8* -Medina et al., 2004- or latexin -Arimatsu et al., 2003), and shows instead distinct *Dbx1*^{nlslacZ} signal, a combination consistent with a ventropallial origin. The previous concept of the BEC as 'ventromedial claustrum' already implied

that it was placed and was originated within the VPall domain. In conclusion, we regard now the BEC and EPv as straightforward VPall derivatives that should be strictly distinguished from claustral populations such as the claustrum proper and the migrated EPd cell population, which represent hypopallial LPall derivatives (Puelles, 2014; note the EPd was called 'ventral claustrum' in older times, a name that results again in a way appropriate, if we want to clearly distinguish this migrated griseum from the ventropallial EPv). The claustral complex, which is found largely rostral to the level of the posterior limb of the anterior commissure seems to derive entirely from the LPall (together with the insula), whereas the VPall found at these levels produces no claustral components, but generates its own separate derivatives, BEC, EPv, and the piriform cortex. The overall scenario thus results simplified.

According to comparative considerations, the reduced BEC population together with the EPv parallels in position (and embryologic origin within the pallial Bauplan) the massive nidopallium of birds. It thus might be searched for reception of some thalamic input as a residual feature in mammals, whereas this trait is massively developed and diversified in sauropsids (Striedter, 1997; Puelles, 2001; Guirado and Dávila, 2002; Reiner et al., 2004; Butler et al., 2011). Another hypothesis that needs to be examined is how far the BEC/EPv plus the VPall-derived part of the pallial amygdala of mammals compare with the avian and reptilian nidopallium plus arcopallium (Bruce and Neary, 1995; Bruce, 2007, 2012; see also Martínez-García et al., 2007; Abellán et al., 2009, 2013; Medina et al., 2011).

As noted above, our data indicate that the ventral pallium progeny is indeed represented by many cells in the ventral part of the piriform cortex, but less abundant cells are present in the dorsal piriform cortex at E18.5, indicating the possible existence of a dorsal subdomain of ventricular VPall that hardly expresses *Dbx1* (see also Hirata et al., 2009). Given the sharp molecular, structural and functional differences between the olfactory and insular cortex areas, it is unlikely that the dorsal piriform cortex originates from the LPall, now that we know it essentially produces the claustro-insular complex (Puelles, 2014). We therefore favor the hypothesis that there is a dorsal part of the VPall progenitor domain that does not express *Dbx1*, causing its derivatives to appear unlabeled in our material. This hypothesis suggests that the whole piriform cortex jointly with the cortico-amygdaloid transition cortex derive from the VPall, as was suggested likewise by Puelles (2014). At coronal levels caudal to the posterior limb of the anterior commissure, a large part of the basal nuclear amygdalar complex (including the anterior parts of the lateral, basomedial and basolateral nuclei), plus the anterior cortical amygdalar area and the pallial extended amygdala, were found by us to be VPall derivatives, due to their massive *Dbx1*^{nlslacZ} and *Dbx1*^{CreLacZ} labelling. The discrepant presence of abundant *Emx1*-expressing cells in the ventral and posterior parts of the basolateral amygdalar nuclei and in the posterior cortical amygdalar areas (Gorski et al., 2002; Medina et al., 2004; Cocas et al., 2009) coincides with the local scarcity of Dbx1-derived cells, leading to the alternative interpretations sketched above, i.e., that they either are not part of the VPall domain, contain large numbers of tangentially migrated non-VPall-derived cells, or originate from the presently postulated part of the caudal VPall. A further causal possibility that seems conceivable is that a distinct progenitor sector that gives rise to the caudal pole of the pallial amygdala expresses early on *Emx1*, discontinuously with LPall, and this marker results secondarily downregulated, after the early BLv/BLp/PLCo/PMCo neurons have been born. Cocas et al. (2009) suggested that these early-born *Emx1*-expressing cells of the posterior basal amygdalar complex are produced in the LPall or DPall, since they co-express *Tbr1* and *Pax6*, and may migrate tangentially into the amygdala; this aspect is also consistent with a *Dbx1*-negative VPall origin. Puelles (2014) did not observe any early claustral LPall cells expressing *Nr4a2* that invade tangentially the pallial amygdala, but he did not examine later-born insular LPall cells.

Briefly, the present listing of VPall derivatives generally corroborates previous proposals (Puelles et al., 2000; Medina et al., 2004; García-López et al., 2008), but entails some corrections and clarifications:

First, regarding the claustral complex, we previously proposed that the dorsal endopiriform nucleus was a ventropallial derivative (Medina et al., 2004). However, present results revealed few *Dbx1*-derived cells there (EPd, Fig. 6D); moreover, Gorski et al. (2002) identified Emx1-lineage ascription of its cells, and we now know about its claustral migratory origin and characteristic expression of *Nr4a2* and various other claustral markers (Puelles, 2014). We therefore ascribe it now to the LPall, representing a new case of pallio-pallial tangential migration (LPall into VPall; Puelles, 2011, 2014).

Second, regarding the basal nuclear amygdalar complex, we previously proposed that only the lateral and basomedial nuclei, plus ACo and PMCo, derive from VPall, whereas the basolateral nucleus and PLCo were held to derive from the lateral pallium (Medina et al., 2004). However, our present results indicate that in addition to the lateral and basomedial nuclei, the anterior part of the basolateral nucleus also contains large numbers of *Dbx1*-derived cells and therefore also should be ascribed to VPall, jointly with ACo; in contrast, PMCo does not seem to belong to VPall. Moreover, the BLv/BLp and PLCo/PMCo do not express *Nr4a2*, though they partially express *Emx1* (Medina et al., 2004). This apparently excludes a claustral LPall origin (Puelles, 2014). Though we tentatively have considered above several explanatory alternatives for this discrepant territory, the simplest and most conservative option is the postulate that a dorsal and caudal part of the VPall ventricular zone does not express *Dbx1*. This would explain the paucity or absence of Dbx1-derived progeny observed at the OB, AOA, dorsal PirCx and posterior amygdala, and also evades the conclusion that the classic amygdalar nuclei are wrongly defined (this conclusion seems to follow all other interpretations). Patterning features obtaining exclusively at the posterior pallial amygdala (e.g., via cortical hem or eminential signals; see Puelles, 2011) might be responsible for the local expression of *Emx1* in absence of both *Dbx1* and *Nr4a2*. The dorsal pallium is a potential alternative source of amygdalar *Emx1*-expressing cells, as commented by Cocas et al. (2009), but this seems less probable than a local origin, due to the longer distance from that possible source, and the absence so far of any specific evidence of such a long migration. Curiously, it was postulated that the NLOT amygdaloid nucleus is a tangentially migrated derivative of the DPall (Remedios et al., 2007), but this nucleus largely does not express *Emx1*. We accordingly feel that this issue of the origin of the posterior pallial amygdala still needs further analysis according to the various conceivable alternative interpretations, before definitive conclusions can be reached. Puelles (2014) advanced the novel conclusion that the LPall as a whole (which includes the insular/perirhinal cortex and the hypopallial claustral derivatives) does not extend caudally into amygdalar levels (the caudal tail of the EPd relates to the piriform cortex, but not to amygdaloid nuclei), though this issue also needs more analysis. Nevertheless, the notion emerges that the classic concept of an unitary claustro-amygdaloid complex may be devoid of molecular fundament.

Third, regarding the medial amygdala, we previously proposed that this region contains a subpopulation of neurons derived from the ventral pallium, based on the expression of *Lhx9* (García-López et al., 2008). Our present results are still consistent with this idea, but they also reveal, jointly with those of Hirata et al. (2009), the existence of other potential sources of *Dbx1*-derived cells for the

medial amygdala, including the *Dbx1*-positive preoptic septocommissural area of the subpallium and the *Dbx1*-expressing hypothalamic SPV (Figs. 1A-C; see the next section of the Discussion).

Fourth, we previously proposed that the amygdalo-hippocampal transition area (Medina et al., 2004) and the lateral entorhinal cortex (Abellán et al., 2009) possibly derive from the ventral pallium. However, this seems now hardly supported by recent data, which suggest that the amygdalo-hippocampal transition area together with the medial entorhinal cortex are medial pallium derivatives (based on the combinatorial expression of *Lef1*, *Lhx9* and *Lm4* and their relative positions during development), while the lateral entorhinal cortex (LEC) was postulated to derive from a separate dorsolateral caudal pallial domain (Abellán et al., 2014; see discussion above). Indeed, these areas only include minor subpopulations of Dbx1-derivatives (see Fig. 7C). The patchy distribution of *Dbx1* expression in the periventricular mantle zone of the lateral entorhinal cortex at E18.5 (specially at medial levels; Fig. 5D) suggests a patchy tangential migration from VPall towards this area, which may be reflected later in other peculiarities. For instance, the formation of discrete stellate cell clumps in the superficial layer of this cortical area is characteristic of the mammalian lateral entorhinal cortex; these aggregates contain cells of similar molecular profile and origin, which invite comparison with the avian aggregates or islands with ventropallial origin studied amongst others by Striedter and Keefer (2000) and Heyers et al. (2003).

Fifth, regarding the amygdalar nuclei of the lateral olfactory tract (NLOT) and of the accessory olfactory tract (BAOT), we previously proposed, based on *Lhx9* expression, that the BAOT and the superficial layer of the NLOT (LOT1) derive from the ventral pallium (García-López et al., 2008; see also Tole et al., 2005). In contrast, layer II of the NLOT (LOT2) has been proposed to originate in the dorsal pallium (Remedios et al., 2007); we do not regard their evidence as supporting conclusively this point, since the ventricular origin identified by these authors lies far away from the DPall (at the amygdalo-hippocampal interface). Finally, layer III (LOT3) was proposed to originate in the diagonal area (MGEcv) of the subpallium (García-López et al., 2008; Puelles et al., 2013). We could not identify these nuclei in our material during embryonic development, but in juvenile mice the LOT1 and LOT3 layers contain only few *Dbx1*-derived cells (Fig. 6B), as well as higher numbers of *Dlx5/6*-derived cells (Puelles and Rubenstein, unpublished observations on Dlx5/6-derived progeny), which agrees with their respective putative origins in the ventral pallium (LOT1) or the diagonal area of the subpallium (LOT3). In contrast, LOT2 contains practically no *Dbx1*-derived cells, is *Dlx5/6* negative, and is *Tbr1*- and *Sim1*-positive (see Fan et al., 1996), which agrees with a different – possibly hypothalamic – origin, in contrast with the majority of the cells in the surrounding pallial amygdala.

4.5. The medial extended amygdala: the confluence of cells of different origins

Our results indicate the existence of a thin supracapsular pallial cell corridor of *Dbx1*-derived cells that connects the ventropallial amygdala with the hypothalamic supraopto-paraventricular domain of the alar hypothalamus (SPV) and the prethalamic eminence. This corridor passes under the bottom of the terminal sulcus, medially and parallel to subpallial diagonal area elements belonging to the medial BST complex (Puelles et al., 2015). This little-known entity represents a *pallial* extended amygdala, which partially correlates with at least one of the cell corridors of the recently defined medial extended amygdala (Puelles et al., 2004, Puelles et al., 2007, 2013, 2015; García-López et al., 2008; Abellán and Medina, 2008, 2009; Abellán et al., 2013; Bupesh et al., 2011a,b). Different parts of the medial extended amygdala (including the medial amygdala and BSTM) and the olfactory tuberculum may receive cells tangentially through this pallio-hypothalamic corridor, which derives from Dbx1-expressing progenitors in the hypothalamo-amygdalar stalk continuum, or perhaps also from the prethalamic eminence (Bulfone et al., 1995; Bupesh et al., 2011b; Abellán et al., 2013; see García-Moreno et al., 2010; Morales-Delgado et al., 2011). In addition, our results indicate that the medial extended amygdala appears to receive cells from *Dbx1* expressing progenitors in the preoptic septocommissural area (POC) and diagonal domain (or MGEvc) of the subpallium, in agreement with data on genetic and experimental fate mapping (Hirata et al., 2009; Carney et al., 2010; Bupesh et al., 2011a).

Ethical approval

Animals were kept and handled following the rules implemented by law in the European Community.

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