Sim1-expressing cells illuminate the origin and course of migration of the nucleus of the lateral olfactory tract in the mouse amygdala

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Received: 15 October 2020 / Accepted: 16 December 2020 / Published online: 25 January 2021
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Abstract
We focus this report on the nucleus of the lateral olfactory tract (NLOT), a superficial amygdalar nucleus receiving olfactory input. Mixed with its Tbr1-expressing layer 2 pyramidal cell population (NLOT2), there are Sim1-expressing cells whose embryonic origin and mode of arrival remain unclear. We examined this population with Sim1-ISH and a Sim1-tauLacZ mouse line. An alar hypothalamic origin is apparent at the paraventricular area, which expresses Sim1 precociously. This progenitor area shows at E10.5 a Sim1-expressing dorsal prolongation that crosses the telencephalic stalk and follows the terminal sulcus, reaching the caudomedial end of the pallial amygdala. We conceive this Sim1-expressing hypothalamo-amygdalar corridor (HyA) as an evaginated part of the hypothalamic paraventricular area, which participates in the production of Sim1-expressing cells. From E13.5 onwards, Sim1-expressing cells migrated via the HyA penetrate the posterior pallial amygdalar radial unit and associate therein to the incipient Tbr1-expressing migration stream which swings medially past the amygdalar anterior basolateral nucleus (E15.5), crosses the pallio-subpallial boundary (E16.5), and forms the NLOT2 within the anterior amygdala by E17.5. We conclude that the Tbr1-expressing NLOT2 cells arise strictly within the posterior pallial amygdalar unit, involving a variety of required gene functions we discuss. Our results are consistent with the experimental data on NLOT2 origin reported by Remedios et al. (Nat Neurosci 10:1141–1150, 2007), but we disagree on their implication in this process of the dorsal pallium, observed to be distant from the amygdala.

Keywords Hypothalamo-amygdalar corridor · Hypothalamus · Pallial amygdala · Subpallial amygdala · Paraventricular nucleus · Pallium models

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| 3v           | Third ventricle |
| a            | Anterior radial unit |
| AA           | Anterior amygdala |
| Aba          | Anterobasal nucleus |
| ac           | Anterior commissure |
| ACo          | Anterior cortical nucleus |
| AHi          | Amygdalo-hippocampal area |
| ant          | Anterior radial unit |
| Arcs         | Arcuate nucleus |
| b            | Basal radial unit |
| bas          | Basal radial unit |
| BLA          | Anterior basolateral nucleus |
| BLAcap       | Cap portion over BMA of the anterior basolateral nucleus |
| BLP          | Posterior basolateral nucleus |
| BMA          | Anterior basomedial nucleus |
| bp           | Basal plate |
| BST          | Bed nucleus of the stria terminalis |
| BSTM         | Bed nucleus of the stria terminalis, medial part |
| c            | Caudal |
| CeA          | Central amygdalar nucleus |
| CGE          | Caudal ganglionic eminence |
| ch           | Chorioidal tela |
| chf          | Chorioidal fissure |
| CPa          | Caudal paraventricular nucleus complex |
| CSPa         | Caudal subparaventricular area |
| Cx           | Cerebral cortex |
| d            | Dorsal |
| Dg           | Diagonal band |
| DPa          | Dorsal paraventricular nucleus complex |
| Ent          | Entorhinal cortex |

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Introduction

The mammalian amygdalar pallial complex consists of a heterogeneous group of nuclei located in the telencephalic temporal pole, rostrally to the caudoventral hippocampus. They are implicated in reward evaluation of stimuli and emotional learning (Burdach 1819–1822; Johnston 1923; Loo 1930; Weiskrantz 1956; Price et al. 1987, 2004; Alheid et al. 1995; Gloor 1997; Swanson and Petrovich 1998; Amaral et al. 2003; Sah et al. 2003; Phelps and Ledoux 2005; Ledoux 2007; Whalen and Phelps 2009; Rolls 2014, 2015; Olucha-Bordonau et al. 2015; Medina et al. 2017). The nucleus of the lateral olfactory tract (NLOT), the bed nucleus of the accessory olfactory tract (BAOT) and the posteromedial cortical nucleus receive olfactory bulb input (Scalia and Winans 1975; Price et al. 1987, 2004; Martinez-Marcos and Halpern 2006; Pro-Sistiaga et al. 2007; Igarashi et al. 2012).

We examine in this work the developing Sim1- and Tbr1-expressing populations of NLOT layer 2, whose postulated neocortical origin (Remedios et al. 2007) seems inconsistent with our results and standard rodent brain atlas data (Puuelles et al. 2019a; see “Discussion”). The NLOT is an isolated tri-laminar ovoid cell mass, which lies embedded (after its migration) within the subpallial anterior amygdala, just rostrally to the anterior cortical nucleus or ACo (Krettek and Price 1978; De Olmos et al. 1985, 2004; Martinez-Garcia et al. 2012; Igarashi et al. 2012). Nissl staining subdivides the NLOT nucleus in three cell layers. Layer 1 (NLOT1) is a subpial molecular zone with scattered neurons, which receives mitral cell input from the main olfactory bulb (Igarashi et al. 2012). Layer 2 (NLOT2) is a thick and dense corticoid aggregate of pyramidal neurons of medium size, some of them possibly representing inhibitory interneurons of subpallial origin (i.e., they express Dlx5 and Lhx6; Marin and Rubenstein 2001; Garcia-Lopez et al. 2008). Recently Garcia-Calero
et al. (2020) described in addition a cell-sparse NLOT shell formation of genoarchitecturally distinct neurons (Azin2-, Er81, and Cyp26-positive) contributing likewise to layer 3. This shell is continuous with a tail of similar neurons leading backwards along the former migration trail of the NLOT2 up to the medial horn of the basolateral nucleus (BLA). We named the new entity the ‘amygdalo-olfactory stream’ (Garcia-Calero et al. 2020).

The adult NLOT is located as a whole superficially within the subpallial anterior amygdala (AA) domain. Its heterogeneous neuronal composition suggests different neuroepithelial origins of the cells that populate its three layers (Garcia-Lopez et al. 2008; Medina et al. 2017). NLOT2 was hypothesized to represent dorsopallial (neocortical) cells migrated via a characteristic caudal amygdalar migratory stream (CAS), forming ‘a link between neocortex and the amygdala’ (Remedios et al. 2007; Deussing and Wurst 2007; Murillo et al. 2015; Ruiz-Reig et al. 2017). Puelles et al. (2019a) recently expressed doubt about this interpretation of NLOT2, due to the distant position of the molecularly defined dorsal pallium (neocortex) from the amygdala, with large interposed portions of mesocortex and allocortex. The majority of NLOT2 cells express the pallial mantle marker Tbr1, consistently with a pallial origin, whereas other cells express Sim1, a marker otherwise mainly present in the hypothalamus (Fan et al. 1996; Michaud et al. 1998; Balthasar et al. 2005). The transient NLOT2 migratory stream (NLOT2ms), or CAS, appears labelled by the markers Neurod1, Neurod2, Tbr1, Math2 (Neurod6), SCIP and Zic2 (Remedios et al. 2007; Murillo et al. 2015; see also Dach1 signal at the NLOT2ms in the Allen Developing Mouse Brain Atlas). This stream appears in sagittal sections of E14.5–E17.5 mouse embryos, and the definitive nucleus forms within the subpallial anterior amygdala (AA) between E17.5 and E18.5 (Remedios et al. 2007).

Mutant mice lacking Emx1/Emx2, Lhx2, Pax6 or Zic2 functions do not develop the NLOT2 (Remedios et al. 2004; Tole et al. 2005; Murillo et al. 2015). There is so far no rationale indicating how these diverse determinants interact to produce the definitive NLOT structure. We will present a tentative synthesis on how these diverse genes control NLOT2 development, including also the presently studied Sim1 case, since we observed that loss of Sim1 signal likewise impedes the final formation of the NLOT.

The transcription factor Sim1 is expressed in several separate regions of the central nervous system apart the NLOT2, e.g., in the basomedial amygdala, as well as inalar and basal hypothalamic regions (Fan et al. 1996; Wang and Lufkin 2000; Balthasar et al. 2005; Puelles et al. 2012). This gene is necessary for normal development and terminal differentiation of diverse cell types within the paraventricular and supraoptic nuclei in the alar hypothalamic region (Fan et al. 1996; Michaud et al. 1998). Sim1-positive neurons derived from the paraventricular area (Pa) reportedly regulate food intake and energy expenditure, and mice heterozygous for Sim1 are obese (Michaud et al. 2001; Holder et al. 2004; Tolson et al. 2010). The melanocortin-4-receptor pathway (MC4R) apparently mediates the function of Sim1-expressing cells within hypothalamic Pa and amygdalar NLOT (Balthasar et al. 2005). MC4R expression appears only after E18.5 at the post-migratory NLOT2, in parallel with some mesocortical areas and the subiculum (Allen Developing Mouse Brain Atlas). In addition, the Rett syndrome, a pathology belonging to autism spectrum disorders, relates to loss of MeCP2 signal in Sim1-expressing cells at the medial amygdala and the NLOT (Fyffe et al. 2008).

The most probable origin of NLOT2 Sim1-expressing cells is the hypothalamic paraventricular area or Pa (Fan et al. 1996; Puelles et al. 2012). It is already known that Otp-expressing cells of Pa origin migrate into the medial amygdala (Wang and Lufkin 2000; Garcia-Moreno et al. 2010; Morales-Delgado et al. 2011). However, disruption of the Otp gene in mice does not affect the expression of Sim1 in the normally migrated NLOT2 (Wang and Lufkin 2000).

In the present report, we first examined the presumptive extratelencephalic Pa source of Sim1-positive cells, and their migration path across the hemispheric stalk, until they reach the caudomedial pallial amygdala. To this end, we investigated in detail the developmental progression of the Sim1 in situ expression pattern and Sim1-tauLacZ-labeled progeny in correlation with other regional markers. We identified the pathway followed by the Sim1 cells as the hypothalamo-amygdalar corridor (HyA). Next, we explored how such cells, once arrived at the amygdala, incorporate into the NLOT2 migration stream to reach the NLOT2 target.

In the second phase of this study, we related the amygdalar population of Sim1-expressing cells to the rostrally migrating stream of Tbr1-expressing pallial cells which constitute what we call the NLOT2 migratory stream (NLOT2ms), also known as caudal amygdalar stream, or CAS (first described by Remedios et al. 2007). We found that this pallial stream starts at the posterior pallial amygdalar unit we recently defined (Garcia-Calero et al. 2020), which is the anlage of the amygdalo-hippocampal area and the postero medial cortical nucleus (AHi/PMCo complex). This locus is precisely where the migrating hypothalamic Sim1 cells arrive via the HyA. The NLOT2ms thus carries from its origin pallial Tbr1-expressing and hypothalamic Sim1-expressing cells (or Sim1-tauLacZ labeled ones). This mixed cellular stream advances first within pallial amygdala next to the pallio-subpallial limit, until it reaches the medial aspect of the BLA and BMA nuclei. The stream then crosses the pallio-subpallial border roughly at E15.5–E16.5, and the migrating mass approaches radially its superficial target site within the subpallial anterior amygdala area (AA), rounding up thereafter as the NLOT2. This mechanism thus
translocates Sim1- and Tbr1-positive cells from the posterior pallial amygdalar unit (AH/PMCo primordium) into the superficial NLOT2.

While our results on the NLOT2 migration itself corroborate the relevant data of Remedios et al. (2007), we disagree regarding their characterization of the amygdalar locus of origin as a dorsopallial extension, as well as on their implicit parallel conclusion that neocortex reaches caudally the posterior amygdala (being ‘linked to it’, as is affirmed in their title). We consider in the Discussion the doubtful assumptions held for years in this field of research that possibly caused the cited disagreements. We conclude that hypothalamic Sim1 cells incorporate into the NLOT2 pallial migratory stream at its true amygdalar origin, i.e., the posterior pallial amygdalar radial unit, after arriving there at E12.5–E13.5 via the hypothalamo-amygdalar corridor (HyA). We also observed that homozygous Sim1 loss-of-function (obtained from a Sim1-tauLacZ mouse line) does not alter the initial phase of migration of Sim1-expressing cells into the NLOT2s, but these cells stop their advance.
Expression of \textit{Sim1}, \textit{Lhx6}, \textit{Otp}, or \textit{Tbr1} in the developing mouse secondary prosencephalon at E11.5/12.5 (a–m); the section plane is indicated at the side jointly with the gestation day; spatial orientation is indicated at the bottom left-hand corners of panels (a, d, l); d–l illustrations from Website: ©2013 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas. http://developingmouse.brain-map.org.

\textbf{Results}

\textit{Sim1} expression during amygdalar development at early embryonic stages

We first analyzed the hypothalamo-telencephalic expression pattern of \textit{Sim1} in mouse embryos at early developmental stages (E11.5–E13.5), using horizontal sections (relative to the prosomeric forebrain axis), in addition to conventional coronal and sagittal section planes (Figs. 1, 2, 3, 4; see also Fig. 1a in Puelles et al. 2016a; Fig. 1d–i were downloaded from the Allen Developing Mouse Brain Atlas). We compared \textit{Sim1} gene expression with gene/protein markers present in the hemispheric stalk region, such as \textit{Otp}/\textit{Otp} (Pa), \textit{Lhx6} (subpallial diagonal area), \textit{Dlx5} (subpallium as a whole) and \textit{Tbr1}/\textit{Tbr1} (prethalamic eminence). We subsequently compared immunocytochemically \textit{Sim1} signal with the expression of \textit{Otp} and \textit{Tbr1} proteins, and with the radial glia marker RC2, applying the recently proposed \textit{amygdalar radial section plane} (Garcia-Calero et al. 2020). This plane is oblique to the conventional coronal plane, and forms a varying angle of 30°–45° with reference to a line tangent to the entorhinal cortex at the back of the hemisphere; this plane agrees with the spatial disposition of radial glial processes crossing the amygdalar pallial region (Fig. 4h–m).

At E11.5 and E12.5, early \textit{Sim1} transcripts appear in the ventricular and mantle layers of the hypothalamic paraventricular area (Pa; Fig. 1l, m). This pattern extends dorsalward in front of the diencephalic prethalamic eminence (PThE) into a curved spike that enters the telencephalic vesicle through the floor of the interventricular foramen (Pa; PThE; if; ts; Figs. 1a–c, f, g, 4a–d; see also E12.5 whole-mount in Fig. 1n). This labeled spike extends caudalward (topologically dorsalward) along the terminal sulcus (Figs. 1a–c, 4a–d), reaching the posterior amygdalar area at E12.5 (data also at E13.5; Figs. 2a–c, 4a–e). There is partial overlap of the \textit{Sim1} and \textit{Otp} signals at the stalk and terminal sulcus mantle (Figs. 1f–i, 2a–e, 4j, m).

We named the telencephalic spike-like extension of the paraventricular mantle (first described, but left unnamed in Fan et al. 1996) the hypothalamo-amygdalar corridor, interpreting that it carries hypothalamic cells reported to migrate into the amygdala (HyA; Figs. 1a–c, f–i, 2a–e; present data and Wang and Lufkin 2000; Garcia-Moreno et al. 2010; Morales-Delgado et al. 2011; see Fig. 3g–i). The peduncular hypothalamic paraventricular area or Pa (PPa) is continuous rostrally with terminal Pa (TPa) within the terminal hypothalamus (THy); expression of both \textit{Sim1} and \textit{Otp} reaches the acrotterminal optic stalk area (os; PPa; TPa; Fig. 1f–i; see also Morales-Delgado et al. 2011; Puelles and Rubenstein 2015).

At E11.5 the HyA distinctly limits rostro-laterally at the telencephalic stalk with the \textit{Lhx6}-expressing primordium of the medial and caudal ganglionic eminences (MGE, CGE), which include the prospective pallidal and diagonal subpallial areas (Pal; Dg; Garcia-Lopez et al. 2008; Bupesh et al. 2011; Puelles et al. 2016a; Pal; Dg; Fig. 1d, e). Caudomedially, HyA limits with the \textit{Tbr1}-expressing, partially evaginated ‘telencephalic’ portion of the rostral diencephalic prethalamic eminence (PThEt; Fig. 1j, k). The subpallial diagonal (Dg) area will form the...
### Sim1

| WT E13.5 horizontal |
|---------------------|
| a | PM/PRM |
|   | Hi     |
|   | HyA    |
|   | CGE    |
|   | P      |
|   | MGE    |
|   | LGE    |
| b | PM/PRM |
|   | Hi     |
|   | HyA    |
|   | CGE    |
|   | P      |
|   | MGE    |
|   | LGE    |
| c | PM/PRM |
|   | Hi     |
|   | HyA    |
|   | CGE    |
|   | P      |
|   | MGE    |
|   | LGE    |

### Otp

| WT E12.5 horizontal |
|---------------------|
| d | PM/PRM |
|   | Hi     |
|   | ot     |
|   | 3v     |
|   | HyA    |
|   | PThE   |
|   | HyA    |
|   | CGE    |
|   | MGE    |
|   | LGE    |
| e | Hi     |
|   | ot     |
|   | ped    |
|   | 3v     |
|   | HyA    |
|   | PThE   |
|   | HyA    |
|   | CGE    |
|   | MGE    |
|   | LGE    |
| f | Hi     |
|   | 3v     |
|   | PThE   |
|   | PThE   |
|   | HyA    |
|   | CGE    |
|   | MGE    |
|   | LGE    |

### Tbr1

|   | Hi     |
|   | 3v     |
|   | post   |
|   | ant    |
|   | HyA    |

### Dlx5

| h | Hi     |
|   | 3v     |
|   | PThE   |
|   | HyA    |
|   | CGE    |
|   | P      |
|   | MGE    |
|   | LGE    |
| i | Hi     |
|   | 3v     |
|   | PThE   |
|   | HyA    |
|   | CGE    |
|   | P      |
|   | MGE    |
|   | LGE    |
| j | Hi     |
|   | 3v     |
|   | PThE   |
|   | HyA    |
|   | CGE    |
|   | P      |
|   | MGE    |
|   | LGE    |
Fig. 2 Horizontal telencephalic sections at E13.5 (a–c) and E12.5 (d–j), showing expression of Sim1, Otp, Tbr1 and Dlx5, as indicated above the panels. The spatial orientation appears at the bottom right-hand corner of panel a. The images show dorsoventrally ordered section levels through the transition between the hypothalamicus (3v) (plus the dienecphal prethalamic emience; PThE) and the telencephalon, whose ganglionic eminences (LGE, CGE, MGE) are visible in relation to pallial areas (P) and the lateral ventricle (lv). Panels illustrating Sim1-expressing cells dorsally along the hypothalamo-amygdalar corridor at the terminal sulcus (HyA; a), reaching the pallial amygdala behind the CGE and in front of the hippocampus (CGE; Hi; b), and starting to mix laterallywards with pallial amygdalar cells of the posterior amygdalar radial unit, the future AHi (post; c). The separate basal domain of Sim1 expression at the perimammillary/periretromamillary hypothalamic area is visible in the three panels (PM/PRM; a–c). A less populated part of the HyA also appears at its entrance into the interventricular foramen and terminal sulcus, next to the 3v (e, d). Panels immunoreacted for Otp. Dorsally, at the level where the HyA lies just under the floor of the terminal sulcus (HyA; d), there are few Otp cells, whereas their number increases in a more ventral section (HyA; e); the HyA in this case leads into the MeA (MeA; e, f). Panels immunoreacted for Tbr1. Dorsally (f), pallial signal (P) extends around the caudal part of the lateral ventricle (lv) past the hippocampal primordium (Hi) into the evaginated ’telencephalic’ part of the prethalamic emience (PThEt) and the PThE proper; no signal of Tbr1 in the underlying central prethalamus, or in the subpallium (CGE); this section level passes above the pallial amygdala. More ventrally (g), we distinguish anterior, basal and posterior radial parts of the pallial amygdala, the latter next to the HyA (ant, bas, post; HyA). Otherwise hippocampal pallium (Hi), as well as PThEt and PThE are likewise immunoreactive for Tbr1. These direct transitions between Hi and PThEt occur below the end of the choroidal fissure. h–j These panels illustrate Dlx5 subpallial and prethalamic in situ hybridization signal (which excludes the PThE/PThEt areas, as well as the telencephalic pallium (Hi, P)). The reaction delineates the LGE, CGE and MGE ganglionic eminences. The HyA (compare with a–c) appears very weakly stained, caudomedially to the CGE and MGE. For abbreviations, see list. Scale bar represents 300 μm.

Sst-expressing principal, supracapsular and medial amygdalar components of the stria terminalis complex ending at the medial amygdala, which are adjacent, but perfectly distinct from our HyA (BST, MA, HyA; see Fig. 3h, i; Morales-Delgado et al. 2011; Puelles et al. 2013, 2016a). Note we previously identified the Dg area as ’anterior entopeduncular area’ (AEP; Bulfone et al. 1993, 1995; Puelles and Rubenstein 1993; Puelles et al. 2000). This name derived from classical literature, but was confusing, since ’entopeduncular’ does not apply properly as a descriptor to a full radial domain (i.e., it refers only to the mantle stratum interstitial to the peduncle). On occasion of preparing the reference atlases and ontology for the Allen Developing Mouse Brain Atlas (during 2008–2011), LP changed the name of this domain to ’diagonal domain or area’ (Dg). This refers explicitly to the radial domain that includes periventricular BST elements, intermediate substantia innominata structures including the basal nucleus of Meynert (SI) and superficial diagonal band (DB) components (Puelles et al. 2013; Thompson et al. 2014). The external anatomic relief of the subpial diagonal band tract serves for topographic identification of the Dg, which limits the HyA throughout its length. Medina and Abellán (2012) refer to the AEP/Dg domain as ‘caudoventral MGE’.

As mentioned above, caudomedially to the evaginated Pa spike, or HyA, lies the evaginated PThE or PThEt (a piece of rostrodorsal prethalamic alar dienecphalon), which already forms at this stage a part of the caudomedial wall of the hemisphere, ending at the choroidal fissure. The ventricular surface of the evaginated portion of PThE bends around the caudal border of the interventricular foramen (just behind the HyA at its floor) into the inner wall of the terminal sulcus (HyA lies along the sulcus proper). Both PThEt and HyA extend all the way into the roof-derived choroidal fissure (see schema in Fig. 15a). The PThE region as a whole represents the dorsalmost histogenetic area of the prethalamic domain (the alar plate of prosomere 3; Puellas et al. 2020), whose Dlx-negative mantle layer (PThE; Fig. 2h–j) is characteristically labelled by Tbr1 and Lhx9 (PThE; Figs. 1j, k; 2f, g; see other markers in Puellas et al. 2012, 2016a, 2020). Tbr1 also labels the mantle layer of the telencephalic pallium, whose hippocampal and amygdalar subdivisions are separated by the final part of the hypothalamo-amygdalar corridor (HyA) from direct contact with the evaginated or ’telencephalic’ PThE (PThEt) at its topologically rostromedial end (PThEt; Hi; Fig. 2f, g).

The selective subpallial Dlx5/Lhx6/Sst markers as well as the pallial/eminential Tbr1 marker accordingly identify differentially at E13.5 and later stages the embryologically diverse structural components present in a cross-section at the cryptic caudal end of the telencephalic stalk and the caudal amygdalar region. Rostromedial to the pallial amygdala there is the Dlx5/Lhx6-expressing (and CB/Sst-positive) subpallial diagonal domain ending at the medial amygdala (BST; MeA; Fig. 3h, i; see also Fig. 15a). Medially to the medial amygdala, there lies at the floor of the terminal sulcus the Sim1/Otp-expressing hypothalamic Pa spike or hypothalamo-amygdalar corridor (HyA; Figs. 3i; 15a). Finally, medially to the HyA there is the evaginated or ’telencephalic’ part of the dienecphalic PThE (PThEt) (Fig. 3i; see also horizontal sections in Figs. 2, 3a–f).

At E12.5/E13.5, Sim1/Otp transcripts and Sim1-tauLacZ labeling still appear along the hypothalamo-amygdalar corridor (HyA; Figs. 2a–e, 3a–i, 4e). The narrow Sim1-expressing area overlaps in part the band of Otp protein expression, but reaches essentially the posterior pallial amygdala coinciding with scattered Tbr1-immunopositive cells (HyA; Figs. 2g, 3a–f). The Otp-positive cells apparently end instead at the subpallial medial amygdala (compare Fig. 3a–c for Sim1 with Fig. 3d for Otp, which displays a more ventral subcapsular section level, where no Sim1 signal is found; see also Wang and Lufkin 2000; Garcia-Moreno et al. 2010; other Otp cells accompany the HyA to its dorsal end at the
The HyA corridor always contacts rostrally and laterally the Dlx5 positive and Tbr1-negative subpallial diagonal domain (along the medial portion of the medial and caudal ganglionic eminences), but is itself a region devoid of Dlx5 transcripts (HyA; CGE; MGE; Figs. 2h–j, 3a, b). It represents the evaginated part of the hypothalamic Pa area along the floor of the terminal sulcus of the lateral ventricle.

The lateral wall of this sulcus is built by the medial and caudal ganglionic eminences, bulges into the ventricle, and is continuous caudally with the medial amygdala. The main part of the ganglionic eminences reaches the brain surface at the olfactory tuberculum (striatum and pallidum), while the diagonal domain ends along the superficially prominent diagonal band, and the preoptic area has its own pial surface. The lateral ventricle wall limiting medially the HyA corresponds to the evaginated part of the PThE, whose surface...
lies at the pial hemispheric sulcus (PThE; chf; Hi; HyA; Figs. 2a–g, 3; see also Fig. 15a). The neuroepithelial HyA corridor lying in between MGE/CGE and PThEt actually represents a radially complete histogenetic area that reaches the brain surface, but its hypothetic intermediate and superficial mantle elements remain poorly studied (probably lumped with the MeA). We estimate that the brain surface corresponding to the HyA corridor proper, which converges ventrally with the hypothalamic Pa area, probably lies medi-ally adjacent to the telencephalic diagonal band (not shown; see Fig. 15a).

We compared Otp immuno-fluorescent signal at the hypothalamic Pa region and related HyA corridor with Tbr1 signal (PThE, and amygdalar or hippocampal pallial areas) and RC2-immunoreactive glial fibres in sections taken in the amygdalar radial section plane (Fig. 4h–m). The Tbr1-positive mantle of the evaginated PThE reaches the roof plate-derived choroidal tela at the prethalamic taenia of the choroidal fissure, and also contacts directly the caudal end of the hippocampal area (PThE; ch; Hi; Fig. 4h), as well as the amygdalo-hippocampal area, beyond the end of the choroidal fissure (AH; Fig. 4k; see also Fig. 15a). The Otp-labeled HyA corridor entering the telencephalic MeA area is separated at this level from the PThE by the compressed walls of the terminal sulcus; there are scarce Tbr1-positive cells (HyA; PThE; Fig. 4h, j, k, m). Otp cells are found later restricted to the medial amygdala, which may include unrecognized remnants of the HyA (Wang and Lufkin 2000; Garcia-Moreno et al. 2010). By co-immunolabeling with the RC2 marker, both HyA corridor-related and PThE-related glial fibres show their distinct radial trajectories; the HyA radial glial cells end superficially at the brain surface outside the peduncular subpallium (ped; HyA; PThE; Fig. 4i–m).

**Telencephalic Sim1 expression at intermediate developmental stages**

To explore further development of the Sim1-expressing cells of the HyA corridor, we mapped this gene in horizontal and coronal sections at stages E14.5 and E16.5 (Figs. 5, 6).

At E14.5, Sim1 signal is still present at the supracapsular periventricular stratum of the HyA corridor. We visualize it in dorsal horizontal sections and intermediate coronal sections along the bottom of the terminal sulcus and next to the subpallial MGE and CGE bulges. The HyA lies orthogonally supracapsular relative to the internal capsule or peduncle, which courses through its underlying intermediate stratum before entering the subpallium (HyA; MGE; CGE; ped; Figs. 5a, f, 6a, b, f, h, i). In lower horizontal sections and rostral coronal sections, the topologically ventral end of the Sim1-positive HyA bendsrostro-medially into the similarly labeled hypothalamic Pa (HyA; Pa; Figs. 5b–d, 6a–c, e). It also reaches caudally the posterior pallial amygdala, past the MeA, as a thin radially stretched domain reaching the pial surface (HyA; Figs. 5b, c, g, 6b, j, k). More rostrally, its para-amygdalar position still appears intercalated between the subpallial CGE (or MeA) territory, lateral to it, and the evaginated telencephalic PThE portion (PThEt), medi ally. The latter also separates the HyA corridor from caudal hippocampal structures (note the choroidal fissure ends slightly above this level; ch; HyA; CGE/MeA; post; Hi; PThEt; Figs. 5a–c, f, 6a, b).

Starting at E13.5, a substantial mass of periventricular Sim1-positive cells, interpreted as hypothalamic HyA-vehiculated Sim1 cells, incorporate into the incipient amygdalar NLOT2 migration stream. The latter appears periventricularly at the posterior amygdalar pallial domain, laterally to the caudal end of the CGE/MeA (post; Figs. 3b, c, 5g). At E14.5, this population extends rostrally through the pallial amygdala as a distinct aggregated cell stream.
At stage E16.5, the Sim1-expressing periventricular HyA corridor appears stretched into a relatively thin supracapsular band. This is presumably due to rostrocaudal morphogenetic growth and increased torsion of the hemisphere. Nevertheless, the HyA retains its previous topologic position along the bottom of the terminal sulcus. It still courses next to subpallial primordia of the diagonal domain and the bed nucleus of stria terminalis that similarly arch back into the amygdalar region (HyA; Dg; BSTM; Fig. 6l–n). These subpallial structures differentially present calbindin immunoreaction and Lhx6/Sst ISH reaction (not shown; Allen Developing Mouse Brain Atlas; García-López et al. 2008; Medina and Abellán 2012; Puelles et al. 2016a). Other Sim1-expressing cells, either sorting out ventralwards out of the dense NLOT2ms, or coming subcapsularly from its dispersed trail elements, form a tenuous medial shell around the amygdalar BMA nucleus, a component of the anterior pallial radial unit. We called this divergent population the para-anterior cell group (PaA; Fig. 6n, o; see also PaA in Figs. 13b–j, 14b). Postnatally, the PaA cells still lie caudally to the NLOT2 and medially to the BMA (Allen Developing Mouse Brain Atlas). At E16.5, most Sim1-labeled cells of the NLOT2ms have separated from the posterior amygdalar unit ventricle, and some pioneering ones may be reaching already the incipient NLOT2 nucleus, after crossing the pallio-subpallial boundary (NLOT2ms; NLOT2; Fig. 6n, o, compare Figs. 11k–m, 13g).

**Description of the Tbr1-expressing NLOT2 migratory stream in the context of Sim1 expression in the telencephalic vesicle**

Since Tbr1 signal is a general pallial marker in the telencephalic mantle it labels the pallial amygdalar NLOT2 migratory stream as well as the definitive NLOT2 itself (layers 2 and 3; Fig. 9g; Remedios et al. 2007). We studied this marker by immunoreaction in horizontal sections (comparable to our Sim1 material at stage E14.5; Fig. 7a–h), as well as in the amygdalar radial section plane (Fig. 7i–l; García-Calero et al. 2020). We hybridized in situ some sections of the horizontal series with the Lhx9 probe, which selectively labels the BMA and ACo nuclei of the anterior amygdalar radial unit, as well as the neighboring ventral subdivision of medial amygdala and the anterior amygdala (García-Calero et al. 2020; García-Calero and Puelles 2021). We also examined the distribution of the subpallial marker Dlx5, whose signal is absent from the NLOT2 and its migration stream (Fig. 8a–h; García-Calero et al. 2020).

This partly counterstained Tbr1 material shows a sharp contrast between the Tbr1-positive pallial amygdalar region and the Tbr1-negative medial amygdala and neighboring amygdalar subpallium (note the CeA and AA domains are distinctly Dlx5 positive areas, whereas MeA shows a weaker
Fig. 5 Horizontal sections through the telencephalon in two embryos at E14.5 (a–e) and E13.5 (f, g), showing $Sim1$ transcripts at the HyA and the beginning of the NLOT2ms. a–e Dorsally, the HyA advances over the peduncle (ped) under the terminal sulcus. As it reaches the posterior pallial amygdala (a; see also f at E13.5), the HyA appears as a labeled thin radial domain intercalated between the MeA (laterally) and the PThEt (medially), both unlabeled (HyA; MeA; PThEt; b, c). Note the close position of the hippocampus and the fimbria/hem (Hi; fi; b, c). At E14.5, the $Sim1$-positive NLOT2ms appears pedunculated, that is, connected by a thinner stalk to the posterior amyg-
dalar ventricular zone, and displaying a rostrally protruding thicker rounded mass (post; NLOT2ms; b, c). Passing into the ventralmost (caudalmost) sections, the NLOT2ms shows a more retarded appearance, and is accompanied by labeled cells apparently passing later-
wards along the posterior amygdalar subventricular zone (post; NLOT2ms; d, e). Compare with the similar aspect found at E13.5 (post; NLOT2ms; MeA; g). The connection of HyA with the Pa area beyond the interventricular foramen appears clearly in c–e. For abbrevi-
ations, see list. Scale bars represent 350 µm (a–e) and 150 µm (f, g).
Dlx5 signal, possibly due to its diagonal nature; Puelles et al. 2016a; Fig. 8a–d). The pallial domain includes at dorso- 
asal section levels the lateral and basal radial units (P; SP; lat/bas; Fig. 7a–c, e; see also Fig. 7i–l in the radial plane), representing the primordia of the prospective lateral (L) and basolateral (BLA/BLP/BLI) nuclei (Garcia-Calero et al. 2020). Underlying horizontal sections also intersect the Lhx9-expressing anterior pallial radial unit (ant; BMA/ACo; Fig. 7f–h), which generally shows a low level of Tbr1 signal (Tole et al. 2005), in contrast to the posterior radial unit, whose periventricular AHi formation is strongly Tbr1-positive (post; AHi; Fig. 7d, g; see also Fig. 7k, l in the radial plane).

The evaginated PThE (PThEt) and its marginal migratory stream are observed as a superficial patch of Tbr1-positive cells limiting medially the medial amygdala, i.e., apparently covering superficially the MeA, though they are separated in fact by the compacted end of the terminal sulcus (PThEt; MeA; Fig. 7b, c, f, g, i, j; black arrow in Fig. 7d, k). The PThEt clearly reaches the caudal hippocampal formation beyond the caudal end of the choiroidial fissure and its fimbrial attachment (PThEt; ch; fi; Hi; Fig. 7b–d, f, g, i–l). In contrast, MeA is distinctly a Tbr1-negative domain (MeD; MeA; Fig. 7b–d, f–h, j–l).

As regards its apparent origin, the Tbr1-positive and Dlx5-negative NLOT2 migratory stream (NLOT2ms) is clearly continuous at E14.5 with the posterior pallial amygdalar domain where Sim1-expressing cells accumulate after E13.5. Once the Tbr1-positive NLOT2ms approaches the pallio-subpallial boundary, it appears intercalated between the Lhx9-positive and weakly Tbr1-expressing anterior radial unit (BMA nucleus) and the non-pallial MeA (NLOT2ms; ant; BMA; MeA; Figs. 7b, c, f, g, i–k, 8a–g). Rostrally, the Dlx5-negative head of the migrating stream approaches the Dlx5-expressing anterior amygdalar area (AA), which was also previously invaded tangentially by Lhx9/Lhx2-positive cells of the anterior radial unit (AA; compare Lhx9 in Fig. 7h and Dlx5 in Fig. 8a–d; Garcia-Calero et al. 2020; Garcia-Calero and Puelles 2021). The dorsal aspect of the NLOT2ms at E16.5 appears covered intimately by Six3-positive neurons apparently related to the CeA nucleus (Fig. 9k).

There may exist both lateral BLA-related and postero- 
medial AHi-related roots of the NLOTms (Fig. 7c), though the postero
ersal root arising at the AHi clearly is the main one, and is the one that incorporates the Sim1-expressing population (see schema in Fig. 15b).

The postero
ersal NLOT2ms root links the periventricular stratum of the AHi area, ascribed to the posterior pallial radial unit, to the main NLOT2ms (NLOT2ms; Fig. 7c, f, g, j–l; Garcia-Calero et al. 2020). Our material clearly shows that the NLOT2ms coming out of this root first advances inside the pallial amygdala, next to the boundary of the posterior pallial amygdalar region with the MeA (NLOT2ms; post; AHi; MeA; Figs. 7c, k, l, 8f, g).

At E14.5, the advancing rounded tip of the NLOT2ms has already progressed up to the BLA and BMA pallial amygdalar nuclear primordia (NLOT2ms; lat/bas; ant; BMA; Figs. 7b, c, f, g, i, j, 8c–e), where the ancillary lateral root of the migration may be added (Fig. 7c, f, g, k; see below). The stream crosses immediately thereafter obliquely the pallio-subpallial boundary, as indicated by global comparison of pallial Tbr1 immunoreaction with Dlx5 subpallial signal (Figs. 7, 8). Note the rostral NLOT2ms and the incipi- 
tent NLOT2 primordium within AA are separated laterally from the standard pallium by a band of Tbr1-negative and Dlx5/Six3-positive subpallial cells (Figs. 7f, g, 8b, c, 9k). This band disappears more caudally, where the stream is restricted to amygdalar pallium (Figs. 7b, c, f, g, k, 8d–g).

Marked partial continuity of the pallial BLA nucleus and the NLOT2ms cell mass suggests that a secondary lateral pallial root of the NLOT2ms possibly arises at the baso- 
lateral radial unit (Fig. 7c, f, g, k). The interaction would occur at the locus where the BLA later displays a subpopulation that departs from the standard radial disposition of baso- 
lateral radial unit derivatives, and advances tangentially in lateromedial direction, forming a cap over the BMA nucleus (Garcia-Calero et al. 2020). This aberrant cap population ends forming a medially prominent ‘horn’ of the BLA nucleus, which protrudes into the MeA/AA, and apparently follows partially the transient NLOT2ms rostrwards as it passes by, possibly contributing cells to it (Garcia-Calero et al. 2020). It is however impossible to assess descriptively whether basolateral Tbr1-positive cells indeed pass from the BLA cap and horn into the NLOT2ms, or just stop at the horn. The fact that various BLA markers (including AChE and TH activity; Garcia-Calero et al. 2020) do not appear in the NLOT bears against the hypothesis. Perhaps the BLA horn cells are only partially attracted into the passing NLOT2ms, without further consequences. Remarkably, though, the same BLA horn locus appears related in the adult to a parallel (and molecularly distinct) amygdalo-olfac- 
tory migratory cell stream population (AOS), which extends from the horn all the way into the NLOT, accumulating there into layer 3 and a peripheral shell (Fig. 9i, j; Garcia-Calero et al. 2020).

The Sim1-expressing cells penetrate the NLOT2ms close to the caudal tip of the hypothalamo-amygdalar corridor, apparently passing around the caudal end of the MeA (Figs. 5b, c, g, 6b, j, k, 7i, 8g; see schema in Fig. 15b). Sagittal sections found at the Allen Developing Mouse Brain Atlas, illustrating both Sim1 and Tbr1 markers in E15.5 embryos, corroborate present results (Fig. 9a–e); note AHi expression of Sim1 decreases substantially at E15.5 (Fig. 9c).
This dorsoventrally sectioned series shows dorsoventrally the HyA stretching along the terminal sulcus (HyA; a), its arrival at the posterior pallial amygdala, medially to the caudal end of MeA (HyA; MeA; post; b), and the lateral emergence of the NLOT2ms, with a rounded and cell-dense advance head (NLOT2ms; b–d). e–k This anteroposterior coronally sectioned series shows rostrally the rounded advance head of the NLOT2ms, as well as the connection between HyA and the Pa/SON area (NLOT2ms; HyA; Pa; e; see also PThEt and terminal sulcus—tis—separating it from ganglionic eminences). Sections that are more caudal follow the NLOT2ms backwards into its less dense stalk at the posterior amygdala, laterally to MeA, and continue showing the advance of HyA along the terminal sulcus (NLOT2ms; post; MeA; f–i; g is a magnified detail of f). The last two sections illustrate how the NLOT2ms stalk at the posterior amygdala connects with the HyA under the end of the MeA (HLOT2ms; HyA; post; j, k). Note also the rostral ventralward dissociation from the NLOT2ms of some labeled cells, which correspond to the paraanterior group (PaA; e–g). l–o At E16.5 the relative proportions of different telencephalic cell masses have changed, but a dorsoventrally horizontal section series still shows the HyA stretching along the terminal sulcus next to the diagonal domain (HyA; Dg; l–n) and reaching the posterior amygdala medially to MeA (HyA; post; MeA; m–o). The NLOT2ms emerges laterally to MeA and produces the PaA cell group that forms a medial shell for the BMA, as well as the advancing stream ending in its rounded head (NLOT2ms; PaA; BMA; n, o). For abbreviations, see list. Scale bars represent 200 µm (a–d), 200 µm (e, f, b–k), 150 µm (g), 400 µm (l–o).

On the other hand, the Tbr1-positive pallial neurons which principally build the NLOT2 mainly seem to originate at the same posterior amygdalar locus (AHi) invaded by the Sim1 cells. There is a first phase of pallial intra-amygdalar migration, which runs orthogonal to local radial glia (Remedios et al. 2007; present results). After a decision point next to BLA and BMA nuclei, the migratory stream changes directions, perforates the boundary between pallial amygdala and subpallial amygdala, and proceeds in a descending radial course to form the NLOT primordium within AA (schemata in Fig. 15b). The adult NLOT shows Sim1, Tbr1, and Mc4r transcripts at its layer 2 (Fig. 9f–h), whereas the markers Cyp26 and Er81 label in addition some layer 3 components, which relate to the AOS shell and trilayers (Fig. 9i, j; Garcia-Calero et al. 2020).

Other relevant gene patterns: Zic2, NeuroD1, NeuroD2, NeuroD6, Lhx2, Emx1, Six3.

The transcription factor Zic2 is of interest, since its expression characterizes the initial phase of the NLOT2ms within pallial amygdala, but not the subsequent subpallial phase, or the definitive NLOT nucleus; moreover, lack of function of this gene leads to loss of the NLOT nucleus (Murillo et al. 2015). Our analysis of this pattern uses the Allen Developing Mouse Brain Atlas repository. At E13.5, amygdalar Zic2 expression is weak at the pallial AHi ventricular zone and strong at its incipient mantle (AHi; Fig. 10a). The latter’s medial end lies close, but separate, from a marginal stream of Zic2-expressing cells which spreads out of the PThEt mantle, arching superficially to the cerebral peduncle (black arrow; PThEt; ped; Fig. 10b; see Alonso et al. 2020a, b). There is also Zic2 expression at the terminal portion of the hypothalamic Pa area (TPa; Fig. 10c). At E15.5 Zic2 appears expressed in a caudo-rostral gradient along the NLOT2ms, which reaches the locus of confluence of the two NLOT2ms roots next to BLA. The Zic2-labelled NLOT2ms distinctly originates from the strongly positive AHi mantle. Identification of the latter is certified further by distinct labeling of its characteristic rostrolateral radial subdivision (see Garcia-Calero et al. 2020), which typically ends superficially at the lateral aspect of the PLCo, rather than within the PMCo, like other parts of AHi (NLOT2ms; AHi; PMCoRL; Fig. 10d, e). No connection was visible between the separate labelled mantle layers of AHi and PThEt (not shown). At E18.5, weak Zic2 signal remains at the AHi mantle and its related rostrolateral subdivision, but has practically disappeared from the remnant of the NLOT2ms, and the NLOT2 proper is completely negative (AHi; PMCoRL; NLOT2ms; NLOT; Fig. 10f, g).

We studied Neurod1 transcripts in sagittal and coronal sections (Figs. 10h–j, 11; data from the Allen Developing Mouse Brain Atlas). Signal was already present at E11.5 along an incipient mantle continuum, which communicates the PThEt and the rostrally adjacent hypothalamic Pa area with the pallial amygdala mantle (the latter along the HyA corridor; Fig. 10h). This triple relationship appears clearly in coronal sections at E12.5 (AHi; PThEt; HyA; Fig. 11a). The amygdalar pallial mantle shows at E13.5 a dense Neurod1 labelling of the Zic2-negative later-born AHi periventricular stratum, possibly suggesting later postmitotic expression than Zic2 cells (AHi; Fig. 10i; compare with Fig. 10a). The pattern in coronal sections is reminiscent of the Sim1 pattern at this stage, with posterior amygdalar unit elements labeled lateral to unlabeled MeA and labeled HyA and PThEt (HyA; AHi; PThEt; Fig. 11b–e). We believe that its expanded rostral portion is the beginning of the NLOTms (NLOT2ms; AHi; Fig. 11b–e). A topographically corresponding labeled cell patch stretches even more rostralward in a similar position at E14.5, partly detached now from the AHi periventricular stratum (NLOT2ms; AHi; Fig. 11f–h). The PThEt mantle is massively labelled with Neurod1 at E13.5 and shows a marked tangentially migrated stream of Neurod1-positive cells passing outside the cerebral peduncle (PThEt; ped; Fig. 11b–d); this stream is the same which was revealed by Zic2 signal, seen now more favorably. Notably, there is also labeling of the deep layers of the allocortical and neocortical pallium (Figs. 10i, 11b–f).

At E15.5 the arc-shaped Neurod1-positive NLOT2ms is recognized in sagittal section (Fig. 10j) as described by
Remedios et al. (2007). Interestingly, at E15.5 the migrating stream still partly connects caudally with the periventricular AHi, where it is separated by a distinct negative gap from other labeling seen medial to the MeA, which may be ascribed to the HyA remnant and the marginal PThEt migration mentioned above (NLOT2ms; AHi; MeA; HyA; PThEt; Fig. 11g, h, j). At E16.5 the target NLOT2 locus starts to be reached by the Neurod1 signal. The rostral rounded and larger end of the NLOT2ms is now the strongest labelled part (Fig. 11k); a tenuously labeled tail of the migration stream is still visible, corresponding to the PaA population described above (not shown). The NLOT2ms has lost its caudal connection with the AHi source at this stage (not shown). There remains signal at the deep cortical stratum (unmarked in Fig. 11k). At E17.5 the Neurod1-labelled cells start to aggregate and compact into the prospective NLOT2, leaving
prospective layer 3 less populated; most positive cells adopt a peripheral shell-like position (NLOT; Fig. 11). At E18.5 most labelled NLOT cell are concentrated at the cited shell-like peripheral configuration, appearing as rostral and caudal shells in sagittal sections and as medial and lateral shells in coronal sections (NLOT; Fig. 11m).

Neurod2 shows at E15.5 a rather diffuse and graded expression pattern in the amygdalar BLP and AHí nuclear primordia, as well as in the hippocampal/entorhinal mantle, with strongest signal at the AHí and the related NLOTms, homogeneously labelled throughout (NLOTms; BLP; AHí; Fig. 12a, b). At E18.5, there is distinct labeling at the NLOT primordium, as well as at remnants of the migration stream, and at the now discontinuous AHí mantle and cortical pallium mantle (NLOTms; AHí; Fig. 12c). The NLOT layers are distinct at P4; a few positive cells disperse superficially to NLOT2, possibly within NLOT1; others remain associated to NLOT3 (not shown).

Neurod6 (Math2) transcripts appear at the deep periventricular amygdalar pallium at E13.5 (but are absent at the PThE, an interesting differential characteristic with Neurod1/2). However, the amygdalaburial labelling is mainly present laterally (prospective BLP, BLA) and diminishes towards the caudomedial AHí (not shown). At E15.5, Neurod6 transcripts have practically disappeared at the AHí mantle, but persist mainly in the mantle derivatives of the basolateral radial unit of the amygdala (not shown; see below at E18.5), as well as at the NLOT2ms, which appears labelled in a graded manner, with signal increasing rostralward (NLOTms; AHí; Fig. 12d). At E18.5 Neurod6 expression clearly delineates at lateral sagittal section levels the whole basolateral amygdalar radial unit (the periventricular BLP and associated BLA and BLI intermediate masses, which approach the unlabeled superficial CxAC; Fig. 12e; Garcia-Calero et al. 2020). In more medial sections the BLA can be followed into its smaller medially deviated portion, which forms a cap on top of the BMA (BLAcap; BMA; Fig. 12f), and finally ends via the protruding BLA medial horn (see Garcia-Calero et al. 2020)) into the labeled NLOT2ms (Fig. 12g). Since labelled BLA-related formations form at E18.5 a bridge with the remnants of the labeled NLOT2ms, whereas little labelling was found at the AHí with this marker, this pattern represents circumstantial evidence supporting a hypothetic posterolateral BLA-related lateral root of the NLOTms, which we postulated above tentatively. At E18.5, the NLOT2 adopts a mature shape and continues to express Neurod6 (Fig. 12h). Interestingly, another gene, Lmo3, shows a very similar pattern as Neurod6, appearing expressed from E13.5 onwards mostly at the basolateral radial amygdalar unit (prospective L, BLA, BLI, BLP), as well as at the NLOT2ms, and finally at the NLOT2 (not shown; Allen Developing Mouse Brain Atlas).

Expression of Lhx2 is widespread in the cortex, with reduced signal at the lateral and ventral pallium (Fig. 12i). At E13.5, ventricular zone expression at the hippocampal allocortex (but there is no signal at the cortical hem) is continuous with a small part of the pallial amygdala, identifiable topographically as the AHí primordium, under the caudal end of the lateral ventricle; there appears weaker signal at the anterior radial unit as well (Fig. 12j–k). A strongly labeled AHí ventricular zone and deep mantle, continuous caudally with the hippocampal cortical hem, was also present at E15.5 (Fig. 12l–n). The AHí Lhx2 signal was still identified at E18.5 (not shown; Allen Developing Mouse Brain Atlas). The marginal migration stream of the PThEt also expresses Lhx2 (not shown). At P4, the superficial derivative of this area, the PMCo nucleus, appears positive for Lhx2, jointly with the basolateral amygdalar derivatives (L, BLa, BLP) and the retroentorhinal nucleus (not shown; Allen Developing Mouse Brain Atlas).

Emx1 appears expressed at E13.5 and E15.5 exactly at the same ventricular amygdalar place as Lhx2 (Allen Developing Mouse Brain Atlas), which corresponds to a periventricular lamina of positive mantle cells at the posterior radial unit; there is also ventricular labeling of the hippocampal/entorhinal allocortex and the neocortex (AHí; Fig. 12o, p).

Six3-expressing neurons are present abundantly at the central amygdalar nucleus (CeA), as well as in radially...
related more superficial cells extending from CeA into the caudal part of the olfactory tuberculum (Allen Developing Mouse Brain Atlas). At E16.5, a section cutting horizontally the subpallial phase of the NTOL2ms shows dense $\text{Six}_3$-positive cells contouring intimately laterally and medially the $\text{Six}_3$-negative NTOL2ms (Fig. 9k). Similar cells also cover dorsally the NTOL2ms (not shown). A sagittal section at P2 shows the relationship of the $\text{Six}_3$-positive CeA population with regard to the final NLOT2 formation (Fig. 9l).

**Sim1 loss of function**

As was reported for the hypothalamic Pa derivatives (Michaud et al. 1998), Sim1 loss of function does not alter early differentiation patterns and migrations. Sim1-expressing mantle cells emerge in normal quantity, and migrate into characteristic positions. It is only after E15.5, when they should take the next step in differentiation towards more specific neuronal typologies (e.g., transforming under control of Sim1 and Brn2 into specific peptidergic phenotypes; see Michaud et al. 1998), that they fail to do so and start to die.
Apparently, the earlier born superficial elements, such as the supraoptic nucleus, are more death-resistant than the later born deep ones (Michaud et al. 1998). Our Sim1-tauLacZ material partly shows the same pattern; up to E16.5, the embryos show apparently normal Pa/HyA and amygdalar Sim1-expressing mantle derivatives, but the whole amygdalar NLOT2ms apparently does not advance beyond the amygdalar pallium into the subpallial AA (Figs. 1n, 4a–g).

We studied E16.5 and E18.5 Sim1 tau-LacZ homozygotes. Comparison of mutant and wild-type E16.5 cross-sections in Fig. 13a–j shows in the mutant a somewhat irregular HyA remnant, perhaps more populated than would be expected at this stage, likely reflecting a halted migration, with very limited AHi signal (Fig. 13a, b). There is also an irregularly shaped Pa mantle extending into the supraoptic nucleus locus (post; Pa; SON; Fig. 13b–f). The expected NLOT2ms appears reduced in volume and length, and looks as if a diminished NLOT2ms has diverted entirely into the position of the PaA cell population described above, associated to the medial edge of the BMA nucleus (Fig. 13c–f). This aberrant pattern contrasts to that of the Sim1 tau-LacZ heterozygote, where both the NLOT2 and the PaA are distinct, as well as a normally dimensioned HyA remnant (NLOT2; PaA; post; HyA; Fig. 13g–j). Consistently, homozygote E16.5 wholemounts differ from heterozygous controls by showing at the brain base only one positively labeled patch (instead of the normal two) (Fig. 14a, b). We interpret that this single patch represents the PaA population described above within the amygdalar pallium, associated to the BMA nucleus, so that the subpallial NLOT2 patch is missing. These results suggest, first, that some Sim1-expressing mutant cells fated for the NLOT2 do not complete their migration into the posterior amygdala (AHi), remaining perhaps at the PaA (Fig. 13a, b). Those that do reach the amygdala and advance along the NLOT2ms either die after E15.5 (particularly those destined to the NLOT2), or fail to penetrate the AA (i.e., do not exit the pallial amygdala) and aggregate instead at the PaA. Coronal sections of E18.5 homozygote and wild-type specimens taken at NLOT level fully confirm this interpretation; the post-migratory wild-type NLOT2 is easily recognized in adjacent sections stained for Nissl, or reacted for Sim1 and Brn2 transcripts, whereas no sign of this structure was found in the E18.5 mutant (Fig. 14c–h).

**Discussion**

The first goal of this study was to examine the origin of the Sim1-expressing young neurons that later populate the migrated NLOT2 nucleus. The second goal was to determine how the Sim1 population relates to the stream of migrating pallial NTOL2 cells that occurs in the mouse roughly between E14.5 and E17.5 (Remedios et al. 2007). Our results unexpectedly illuminated the parallel issue of the ambiguous statements made by the cited authors on pallial origin and migration course of the NLOT layer2 cell population. We found that the Tbr1-positive neurons of the NLOT2ms (or CAS) originate from the posterior amygdalar radial unit (Garcia-Calero et al. 2020). Remedios et al. (2007) previously deduced that this component arises from a caudal extension of the dorsal pallium. Their origin and our origin seem topographically identical (present data), confirming an amygdalar and non-cortical source of the phenomenon.

Our recent analysis of the cortical and amygdalar pallium fields (Puelles et al. 2019a; Garcia-Calero et al. 2020; Garcia-Calero and Puelles 2020, 2021) leads us to doubt that it is possible to extrapolate cortical pallium sectors into the histogenetically separate amygdalar pallial field. This now obsolete analytic approach was actually initiated by our group (Puelles et al. 2000, and Medina et al. 2004), and its use is still common (e.g., Desfils et al. 2018; Ruiz-Reig et al. 2018), but should be discontinued. Along the Discussion, we will argue that all the data considered by Remedios et al (2007) are coherently reinterpretable according to a simpler amygdalar origin hypothesis, without involving wider cortical relationships other than the hippocampal/entorhinal close neighbors. This option is advantageous at least in offering higher consistency with the known anatomy of developing and adult rodent brains, by recognizing that the primordium of the neocortex is always distant from the amygdalar field, due to their absolute separation by interposed mesocortical and allocortical pallial domains (Puelles et al. 2019a; Garcia-Cabezas et al. 2019; Pattabiraman et al. 2014; Bayer and Altman 1991; Swanson 1987).

With regard to Sim1 cells, we described a Sim1-expressing hypothalamo-amygdalar corridor (HyA). Fan et al. (1996) first mapped it at E10.5 and E12.5, but left it unnamed (see their Figs. 1n, 15a). In agreement with tentative schemata of Puelles and Rubenstein (2003, 2015) on this point, we think that the HyA is a dorsal prolongation of the hypothalamic paraventricular area (Pa) that results co-evaginated into the early telencephalic vesicle jointly with the neighboring rostrodorsal ‘telencephalic’ part of the diencephalic prethalamic eminence (PTHeEt; Puelles 2019). This implies a novel concept which may be of interest in comparative neuroanatomy, namely the existence of an alar hypothalamic subdomain that stretches into the telencephalic roof without losing its original molecular character (Fig. 15a). This hypothesis explains the course of HyA through the floor of the interventricular foramen and of the terminal sulcus (Fig. 15a). The HyA always lies next to neighboring subpallial formations (e.g., main BST nuclei, supracapsular BST and MeA) and finally reaches the posterior pallial amygdala at the end of the choroidial fissure (Fig. 15a).
The Pa/HyA progenitor domain represents the apparent origin of all forebrain alar Sim1-expressing cells (there are separate basal ones; Fig. 1n). This implies that the derivatives of this area must include the population that reaches the amygdala and eventually enters the NLOT2. The HyA can be understood as the migratory pathway for the arrival of paraventricular Sim1 and Otp cells to pallial or subpallial parts of the amygdala, as was already demonstrated for Otp cells (Wang and Lufkin 2000; Garcia-Moreno et al. 2010; Morales-Delgado et al. 2011; Morales et al. 2021; present results; see also Bardet et al. 2008 for alternative perspective). Considering that various names applied previously to this pathway were conceptually inappropriate (see below), we renamed it ‘hypothalamo-amygalaric corridor’ (HyA), emphasizing its hypothalamic origin and molecular profile, as well as its amygdalar ending.

The topographically caudal end of the HyA corridor (which topologically is actually its dorsal end, where the hypothalamus reaches the choroidal roof plate; Fan et al. 1996) allows the access of Sim1 cells to the extreme caudomedial part of the pallial amygdala (namely its posterior radial unit, or prospective AHi/PMCo complex). The invasion occurs specifically at its rostromedial subdivision (AHiRM; Garcia-Calero et al. 2020), which characteristically protrudes into the medial brain surface with the underlying PMCo nucleus, behind the subpallial MeA (p; MeA; Fig. 15a; De Olmos 2004).

Analysis of the posterior amygdalar radial unit in horizontal sections at E13.5 and E14.5 revealed that the migrated Sim1-expressing cells first shift laterally within AHi, passing behind the MeA, and adopting a new position within the rostromedial AHi subdivision found lateral to the MeA (Fig. 15b). Here they join the Tbr1-positive local elements that start to migrate into the arc-shaped NLOT2 migration stream. The latter, aptly (though somewhat ambiguously) named the ‘caudal amygdaloid stream’ (CAS), arrives at its target between E16.5 and E17.5 (Remedios et al. 2007). Our NLOT2ms results corroborate absolutely these earlier data, adding some points of interest.

We examined in more detail the course of the arc-shaped Sim1-expressing NLOT2ms/CAS using Tbr1 immunoreaction and Dlx5 ISH, which respectively mark the pallium versus subpallium domains. We thus were able to divide the migration into successive pallial and subpallial phases. The first phase traverses several parts of the pallial amygdala, always next to the pallio-subpallial boundary, proceeding orthogonally to the local radial glia between E14.5 and E15.5 (as already noted by Remedios et al. 2007, and corroborated by us; Fig. 15b). Once the stream reaches at about E15.5 the medial side of the BLA and BMA nuclei (basolateral and anterior radial amygdalar units; b, a; in Fig. 15b; Garcia-Calero et al. 2020), the NLOT2ms/CAS proceeds into its second subpallial phase. To this end it crosses the pallio-subpallial border into the subpallial anterior amygdala (AA), and advances therein in a radial course into its target locus (Fig. 15b, d), surrounded by dispersed Dlx5- , Six3-, Pax6-, calbindin, and Lhx9/Lhx2- expressing cells. Here the migration ends, and the NLOT nucleus forms. Remedios et al. (2007) observed these two phases as regards the changing relationship with radial glial processes (tangential to radial), but they apparently did not notice that the change also coincides with the pallial versus subpallial character of the tissue surrounding the migrating cells. We think this result has relevance towards understanding the roles of diverse genes known to control this migration (see below). The decision point where the cells change into the second phase lies next to the anterior radial unit of the palial amygdala (prospective BMA/ACo nuclei), and in the medial vicinity of the BLA nucleus (intermediate mass of the basolateral unit; Garcia-Calero et al. 2020).

The convergence of the hypothalamic Sim1-expressing HyA pathway with the posterior amygdalar source of migrating Tbr1-positive pallial cells (Fig. 15b) clearly identifies the origin of the NLOT2ms/CAS migratory process as the posterior unit of the pallial amygdala. The latter contacts caudally the hippocampus and entorhinal cortex, both of them allocortical (De Olmos 2004; Franklin and Paxinos 2013; Puelles et al. 2019a; Garcia-Calero et al. 2020). In contrast, Remedios et al. (2007), referring to our amygdalar AHi/PMCo complex, reported that the migration originates from a caudal part of the dorsal pallium, thus pretending to establish a ‘link between the amygdala and neocortex’. This conclusion is clearly contradictory with the easily observable caudal direct boundary of the AHi posterior unit of the pallial amygdala with the hippocampus (as appears reflected in its conventional name, ‘amygdalo-hippocampal transition area’). Several other commentaries, reviews or reports...
accepted the Remedios et al. (2007) interpretation of the CAS migration as a cortical dorsal pallium phenomenon without raising doubts or objections (Deussing and Wurst 2007; Subramanian et al. 2009; Murillo et al. 2015; Ruiz-Reig et al. 2017; Chou and Tole 2019). In a recent review of cortical models featuring classically defined concentric ring-shaped domains (Puelles et al. 2019a) we did express doubts about this hypothesis of Remedios et al. (2007), since all these cortex models showed the neocortex to be totally separated from the amygdala by the massive outer allocortical ring and the thinner mesocortical ring.

We studied carefully the Remedios et al. (2007) paper, exploring various ways to understand what seemed a remarkable error of interpretation coming from first-rate researchers. We reached the conclusion that the paper includes various sorts of doubtful assumptions and interpretive errors that

Fig. 10 Sagittal sections illustrating amygdalar \textit{Zic2} transcripts at E13.5, E15.5 and E18.5 (a–g), jointly with \textit{Neurod1} transcripts at E11.5, E13.5 and E15.5 (h–j). The spatial orientation appears at the bottom left-hand corner of a. At E13.5, \textit{Zic2} signal appears at the intermediate AHi mantle (a), as well as at the PThEt and its marginal migration stream passing medially caudal to the peduncle (PThEt; ped; black arrow; b, c), as well as the terminal part of the paraventricular area (TPa; e). At E15.5, the AHi \textit{Zic2} signal continues labeling the AHi, and, particularly the full radial extent of its rostral subdivision (AHi; PMCoRL; d), as well as the NLOT2ms (e; note neighboring ERh and Hi cortex). At E18.5, the \textit{Zic2} labeling is less marked, but persists at some of the places seen before (AHi, PMCoRL, NLOT2ms, but not at the NLOT2; f, g). h–j \textit{Neurod1} transcripts appear at the paraventricular hypothalamic area (Pa) and HyA at E11.5 (Pa; HyA; h). At E13.5, \textit{Neurod1} signal appears at the AHi mantle, deep to the level labeled with \textit{Zic2} (AHi; compare a with i; see also the relative positions of dorsal and medial pallium, DP, MP). At E15.5, \textit{Neurod1} signal clearly identifies the NLOT2ms and AHi periventricular stratum, the latter connected under the end of the lateral ventricle (lv) with ERh and Hi cortex derived from medial pallium (NLOT2ms; AHi; ERh; Hi; j). For abbreviations, see list. Scale bar represents 400 µm
Fig. 11 Anteroposteriorly ordered coronal sections illustrating Neurod1 amygdalar expression at E12.5, E13.5, E14.5, E15.5, E16.5, E17.5 and E18.5 (note coronal sections cut obliquely the NLOT2ms, as well as the whole pallial amygdala). The spatial orientation appears at the bottom left-hand corner of a. At E12.5, Neurod1 signal appears at the Pa, HyA, AH1i and PThEt (a). At E13.5, the signal within the PThEt mantle expands medioventrally into its marginal migration stream at the back of the peduncle. Medially to HyA (PThEt; ped; HyA; b–e). The labeled NLOT2ms appears cut obliquely in a more lateral position, as a positive patch stretching back to the AH1 domain (NLOT2ms; AH1; b–e). A similar anteroposterior sequence through NLOT2ms appears at E14.5 (NLOT2ms; AH1; f–h). At E15.5, the Neurod1-labeled NLOT2ms appears still connected caudally to the AH1 (NLOT2ms; AH1; i, j); note also the labeled medial superficial marginal stream of the PThEt at (j). At more advanced stages, the definitive NLOT nucleus starts to conform, with Neurod1 signal increasingly restricted to its layer 2 (NLOT2; k–m). For abbreviations, see list. Scale bar represents 400 µm.
were actually widely shared (including by us) in the field of pallial studies back in the first years of 2000. We will divide our resulting interpretation into three levels of analysis.

First, we will examine whether there is discrepancy between Remedios et al. (2007) and present results about the observed embryonic location and adult identity of the origin of the NTOL2ms/CAS phenomenon. The possible use of ambiguous terms might have approximated at least implicitly our respective interpretations. It seems clear that we both see the origin of the NTOL2ms at exactly the same place of the embryonic telencephalon, but for various reasons we classify this locus as posterior pallial amygdala, whereas Remedios et al. (2007) classified it as dorsal pallium. We cannot be both right.

In a second, more semantic level of analysis, we consider whether there existed in 2007, and still exist today, solid grounds to classify the particular telencephalic locus where the CAS originates as ‘dorsal pallium’. This question possibly needs a ‘yes and no’ answer, because such a notion was indeed possible, and even conventional, during a number of years, but this has changed now, turning it into a risky idea. We also must address the scientific meaning of ascribing an embryonic brain pallial locus to a given pallial cortical sector, such as the ‘dorsal pallium’. The molecularly distinct pallial sectors with which Remedios et al. (2007) dealt were partly corroborated postulates within a conceptual pallium model that is now 20 years old (Puelles et al. 2000; Medina et al. 2004; Tole et al. 2005, and has recently evolved for good reasons to different postulates. The relevant assumptions have indeed changed significantly in recent years (Puelles 2014, 2017; Puelles et al. 2016b, c, 2017, 2019a).

Finally, we will discuss the notion of Remedios et al. (2007) that the dorsal pallium, the primordium of neocortex, actually extends caudalwards between the caudal ends of hem and antehem to establish as its caudalmost subregion the locus of CAS origin. This idea seems inconsistent with available developmental and adult rodent brain neuroanatomic knowledge. Tradition in this field does not detect any direct contiguity whatsoever between neocortex and pallial amygdala (Puelles et al. 2019a; Garcia-Cabezas et al. 2019; Pattabiraman et al. 2014; Bayer and Altman 1991; Swanson 1987).

Proceeding now to our first topic, we inquire whether we really disagree about the embryonic location of the source of NTOL2ms or CAS. As stated above, interpretation of our results within our recent radial amygdala model (Garcia-Calero et al. 2020) allows little doubt that this origin lies in the posterior pallial amygdalar unit (AHi/PMCo complex). On the other hand, Remedios et al. (2007) vaguely described this locus as the ‘caudal telencephalic neuroepithelium’ or ‘the caudal extreme of the telencephalon’ (for instance, in the legend to their Fig. 2, while locating their electroporation experiment). They probably referred to the local end of the lateral ventricle. According to us, the neuroepithelium at the front of the end of the ventricle is in large part pallial amygdalar (note subpallial amygdala also steps in somewhere), whereas the neuroepithelium that lines caudally the end of the ventricle is hippocampal and entorhinal (check the plates in Garcia-Calero et al. 2020). Remedios et al. (2007) seem to have lacked these anatomic references. The description of their important electroporation experiment did not include any suggestion that the experimental site lies rostral to the lateral ventricle, as is clearly visible (see their Fig. 2). This anatomic locus is systematically ascribed to the amygdala in all developing rodent brain atlases (e.g., Altman and Bayer 1995; Alvarez-Bolado and Swanson 1996; Jacobowitz and Abbott 1997; Foster 1998; Paxinos et al. 2007; Ashwell and Paxinos 2008).

After proceeding to an analysis of ventral, lateral, medial and dorsal pallium markers which was concluded to exclude the first three options, Remedios et al. (2007) jumped to the conclusion that the caudal locus of CAS origin had to be an integral part of the remaining option, the dorsal pallium, adducing some molecular evidence (considered below) and the existence of a gap between the hem and antihem organizer territories at the periphery of the pallium, which might allow the dorsal pallium to extend into the area of CAS origin. The ambiguous term ‘caudal amygdaloid stream’ (CAS) they used does not refer to an amygdalar origin of the NLOT2, but to a caudal dorsopallial (neocortical) origin of this migrated,
finally ‘amygdalar’ structure. The authors apparently did not conceive any primary amygdalar non-cortical pallial neuroepithelial region at the ‘caudal telencephalic neuroepithelium’, though their own radial glia preparations clearly suggested it (their Figs. 7a–c, 8h). They apparently believed, probably inspired by our earlier study of ventral and lateral pallium parts of the amygdala (Medina et al. 2004), or by other contemporaneous sources, that all
parts of the pallial amygdala originate in one of the four postulated cortical pallial sectors, and secondarily migrate to distinct final amygdaloid sites. To illustrate this notion, Remedios et al. (2007) drew dashed lateral and ventral pallium arrows in their Figs. 1 and 8, complemented by their dorsopallial CAS, jointly depicting the hypothetic paths of amygdalopetal migrating cells originated in various parts of the cortex. Similar arrows were reproduced by Deussing and Wurst (2007; their Fig. 1), reflecting editorial approval of this notion. The whole Discussion of Remedios et al. (2007) developed thereafter out of the initial ‘dorsal pallium’ diagnosis. We did not find any place where Remedios et al. (2007) considered even hypothetically that the CAS locus of origin might be intrinsically pallial amygdalar (this option might have been qualified to represent an analog of cortical dorsal pallium in terms of its molecular profile). We understand that Murillo et al. (2015) and Ruiz-Reig et al. (2017) later simply accepted the scientific authority of the respected research group and Nature Neuroscience.

We initially also thought in much the same way, since we also shared the assumption of varied cortical origins of distinct amygdalar components, all the way from Puelles et al. (2000) to Puelles et al. (2016b). Subsequently, we started to doubt such interpretations, as we gradually assimilated various contradictory data accruing from the work of Gorski et al. (2002), Puelles (2014) and Puelles et al. (2016c), as we will comment below. We have since realized that this conventional viewpoint on the mode of pallial amygdala formation is unfortunately erroneous. This happens, partly, but importantly, because all studies performed in this period confided on coronal sections oblique to radial amygdalar structure. In such sections, we never see the true amygdalar ventricular zone, because the latter appears in other coronal sections caudal to the amygdala, precisely at the ‘caudal telencephalic neuroepithelium’ of Remedios et al. (2007).

That is the simplest reason why these authors missed the true amygdalar identity of the CAS origin.

We thus conclude that Remedios et al. (2007) did not discover or deduce that the CAS/NLOT2 origin is at the posterior part of the pallial amygdala because they ignored (as did everybody else at the time) that the latter, equivalent to their ‘caudal neuroepithelium’, is a local pallial amygdalar progenitor domain, that does not derive from the cortical dorsal pallium, or from any other cortical subregion. The cause of this error was the widely shared assumption that cortical pallial sectors produce claustral and amygdalar nuclear populations that migrate long distances into extracortical adult positions, such as the amygdala. This false assumption, tied methodologically to the use of coronal sections, misled Remedios et al. (2007) into the simplistic and, as it turns out, false conclusion involving the actually distant dorsal pallium as the origin of the pallial cells of the CAS/NLOT2.

The implications of such biased thinking, given the real distance of the amygdalar target from the molecularly delimited dorsal pallium (Puelles et al. 2019a), are reduced to the absurd in our Fig. 15c, d (red dots and lines). The data produced by Remedios et al. (2007), and notably their excellent electroporation experiment (whose position we represent as a black dot within the posterior amygdala; Fig. 15c, d), do not support long-distance migration of the CAS. Such translocation would be needed if these cells really came from the dorsal pallium (red dot). The site labeled by electroporation clearly corresponds to a posterior amygdalar pallial site, not to any distant dorsal pallium cortical sector. We will come back to this conclusion under the third point of discussion.

As a second topic of clarification, we examine now how solid were the grounds used by Remedios et al. (2007) to classify as ‘dorsal pallium’ the caudal telencephalic locus where the CAS/NLOT2ms originates, and whether their argument remains valid today. We think that they reasonably excluded the ventral and lateral pallial sectors, but not so the medial pallium, as we will see below. We also address the meaning and interest of ascribing an embryonic pallial locus to a given pallial sector postulated within a model (Puelles et al. 2000).

In the molecular era of pallium developmental studies, started roughly between 1998 and 2000, ascriptions of pallial elements to the postulated four sectors of the Puelles et al. (2000) pallium model proceeded by demonstrating a particular molecular marker profile shared by the corresponding territory. This was the case even when the pallial portion of interest was not cortical in structure (there are pallial parts of the septum and of the amygdala which essentially lack cortical structure; see also more general comments on comparative studies of non-corticoid pallium regions of non-mammals in Puelles et al. 2017 and Puelles et al. 2017).
Remedios et al. (2007) employed the standard approach in their Results and Discussion, but did not consider first whether their area of interest—the ‘caudal telencephalic neuroepithelium’—was cortical or nuclear (i.e., amygdalar) in nature. This possibly distracted their attention from the theoretical possibility of a primary amygdalar classification of the CAS origin. Eventually, after thinking they had excluded the molecular profiles of the ventral, lateral and medial cortical sectors, they concluded that the data available pointed clearly enough to the single remaining option, the ‘dorsal pallium’. We think in retrospect that the markers they considered specific of the dorsal pallium, mainly Emx1 and Lhx2 (as well as Neurod1/2 and Tbr1) were not really ‘dorsal pallium’ specific. These signals also extend importantly at appropriate early stages within the mediopallial primordia of hippocampal and entorhinal cortical areas, even though the hippocampal hem is indeed negative for Lhx2, as was pointed out by these authors (see our Figs. 9d, e, l, 10, 11a–j, 12). These mediopallial or allocortical areas are close neighbours of the amygdala (see Hi, ERh, MP; Fig. 12a, b, d, j–p), much closer than the dorsal pallium or neocortex (DP). This close relationship was also evident in some of the illustrations of Remedios et al. (2007), such as their Figs. 1b, c, 2a, 3b, i–m, 4t, 8a, as well as in various other reports of the same group illustrating Lhx2 expression. The lapse in recognizing a lack of specificity of these markers is difficult to understand. Probably other less relevant comparative considerations, like the sharing of a reelin/Dab1/Cdk5 neuronal migration control mechanism at both the CAS and the dorsal pallium, or their new data on a gap between the hem and antihem organizers, added salience to the ‘dorsal pallium’ hypothesis. The interesting comparison made in their Fig. 4w between Wnt2b (a hem marker) and sFrp2 (an antihem marker) against the pallial Emx1 signal should have been accompanied by a similarly oriented image comparing the same landmarks with Lhx2. Note the Lhx2 pattern shown in their Fig. 4t is quite different from that of sFrp2 in Fig. 4r. To identify the hem versus antihem is not the same as identifying the medial pallium versus the dorsal pallium, because these specializations lie topologically outside the medial and ventral pallium allocortical regions, and do not contact the dorsal pallium at all (Fig. 15c). We thus believe Remedios et al. (2007) did not attend to this diagnostic task optimally, according to available evidence. In our opinion, the authors should have concluded that the area of interest could as likely be either medial pallium or dorsal pallium. Between these two possibilities, the most parsimonious option was to ascribe the caudal area of interest to cortical medial pallium, rather than to dorsal pallium, due to the observable vicinity of mediopallial cortical areas to the area of interest. More indirectly, the then known joint requirement of hem-related Emx1/Emx2 function for NLOT development also bespoke of a mediopallial relationship (Tole et al. 2005; Shinozaki et al. 2004; Suda et al. 2010). This viewpoint was indeed reached 7 years later by Abellán et al. (2014), who emphasized a number of shared LIM-homeobox genes between the
medial pallium and the AHi/PMCo amygdalar complex, that is, the amygdalar source of the CAS.

We next consider critically the rationale of ascribing any amygdalar pallial parts to one of the four pallial cortical sectors defined by Puelles et al. (2000). We presently hold that, in any case, the seemingly reasonable pallial structure assumptions that Remedios et al. (2007) employed back in 2007 are now outdated (followed also, implicitly, by Deussing and Wurst 2007; Subramanian et al. 2009; Muriello et al. 2015; Puelles et al. 2016c; Ruiz-Reig et al. 2017; Chou and Tole 2019).

Historically, Puelles et al. (2000) and Medina et al. (2004) used a peculiarity in Emx1 expression (its absence in a previously non distinguished portion of cortical pallial mantle lying next to the subpallium) to develop a tetrapartite mouse/chick pallium model that left behind the classic tripartite pallium model (the latter only contemplated lateral, dorsal and medial constituents; see Striedter 1997). Four (ventral, lateral, dorsal and medial) pallium sectors were distinguished at middle levels of the hemisphere. Though it was based on rather sketchy molecular data, this model apparently was valid ab initio for tetrapods (Smith-Fernández et al. 1998; Puelles et al. 2000). Corroborations of the new tetrapartite model (demonstrating presence of the Emx1-negative VPall) accrued thereafter in various amniote species, as well as in man, giving ample credibility to the model. It was initially unclear whether the four pallial sectors extend throughout the whole length of the telencephalic pallium, that is, e.g., whether they reach the caudal amygdalar pole in a parallel arrangement of longitudinal partitions, as had been theorized previously by Kuhlenbeck (1973), Holmgren (1925), and other classic authors. Recently we have learned that the four cortical pallial sectors do not reach the pallial amygdala, due to the emerging alternative concentric ring-shaped arrangement of allocortical and mesocortical areas around a central iso/neocortical island (García-Cabézas et al. 2019; Puelles et al. 2019a; see other references therein, an idea ranging back at least to Swanson 1987). Medina et al. (2004) first addressed the partial demonstration of parallel longitudinal pallial cortical sectors expected to reach the amygdala, using chosen markers thought to define the novel ventral and lateral cortical pallium sectors. The conclusion was that, according to the distribution of such markers, both ventropallial and lateropallial cortical sectors seemed to extend caudalwards into the pallial amygdala, each having specific radially migrated amygdalar nuclear derivatives. This tendentious, rather preconceived conclusion inspired in what one sees in coronal sections lamentably soon became a sort of established fact, or strong assumption, in the field, leading to the non-fundamented stream arrows drawn by Remedios et al. (2007) and Deussing and Wurst (2007).

Indeed, the illustrations of the Medina et al. (2004) report, based on standard coronal sections and artist drawings, arbitrarily suggested that the sources of any claustral or amygdalar nuclear structures lay at the ventropallial or lateropallial cortical ventricular zones appearing in the same sections, next to the pallio-subpallial boundary. This was an error inconsistent with the glial structure already known at the time, because no radial glia processes extend from the cortical ventral pallium into amygdalar pallial territory (the ventropallial glial processes rather plunge straightforwardly into the local olfactory cortex). Nobody recognized this conceptual error at that moment, due to the conventional massive use of coronal sections and the mentioned assumptions. These induced subliminally the belief that the nuclei you see in one coronal section come from the ventricular zone you have in that same section (disregarding other possible origins more rostrally or caudally in the brain).

It so happens that telencephalic coronal sections are oblique by some 45 degrees to amygdalar radial glial structure, because the ventricular zone where amygdalar nuclei actually arise lies behind the amygdalar nuclei, precisely at the ‘caudal telencephalic neuroepithelium’ whose glial structure was examined by Remedios et al. (2007), whereas the corresponding pial surface lies ventrally to the amygdala (García-Calero et al. 2020; García-Calero and Puelles 2020). Many authors accepted at face value the fore mentioned arbitrary conclusion about cortical origins of amygdalar cell masses held to migrate via ‘ventropallial and lateropallial migration streams’ into secondary amygdalar positions. The list of reports incurring in this inherited error includes Remedios et al. (2004, 2007), Tole et al. (2005), Subramanian et al. (2009), Martínez-García et al. (2007, 2012) and Olucha-Bordonau et al. (2015), and there surely are other cases, including Puelles et al. (2016c).

A lateropallial migration stream that ends superficially in a dorsal part of the olfactory cortex, as proposed by Puelles et al. (2000) and Medina et al. (2004), was never demonstrated in terms of radial glia or experimental analysis. A later re-examination of this conundrum led LP to redefine the concept of lateral pallium as a radially organized ‘claustral-insular’ complex, now believed to represent the true lateral pallium, whereas the whole olfactory cortex resulted ascribed to the updated ventral pallium (Puelles 2014). Remarkably, the new lateropallial cortico-nuclear unit also appears with corresponding topology and selective gene markers in chick and reptiles, where nobody had previously expected to find claustrum and insula homologs (Puelles et al. 2016b, 2017, Puelles 2017; these surprising results were recently corroborated with a transcriptomic approach in reptiles by Tosches et al. 2018 and Norimoto et al. 2020). However, as concluded in the cited reports, as well as in Puelles et al. (2019a), this updated lateral pallium has no molecularly identifiable derivative in the mouse amygdala.

Contrarily, the postulated ventropallial migration stream is easily observable with radial glia stains (Puelles 2014;
Schemata summarizing our findings according to the updated prosomeric model (Puelles and Rubenstein 2015). a This schema visualizes our present conception of the right half of the forebrain, after eliminating the upper alar plate and part of the roof plate of telencephalon and diencephalon by a horizontal section. The hypothalamo-telencephalic structural unit (secondary prosopencephalon) appears separated from the diencephalon by a thick transverse black line. A thinner topologically transversal black line separates the hypothalamo-telencephalic neuromeres h1 and h2 units one from another. Similar lines separate the three diencephalic neuromeres (p3, p2, p1) and their major alar derivatives, prethalamus (with the prethalamic eminence bulging into the interventricular foramen), thalamus and pretectum (PTh/PTHE, Th, PT; a part of the PTHe is evaginated into the medial wall of the hemisphere (its ‘telencephalic’ part, marked here with a ‘+’). The medial hemispheric wall surface is identified in dark grey. At the midline there is caudally the basal plate part, marked here with a ‘t’). The medioventral roof plate present at the HyA tip is the dorsalmost hypothalamic alar derivative, because it touches the similarly deformed choroidal roof plate present at the choroidal fissure in the medial hemispheric wall; chf). The roughly longitudinal (but rather oblique) hypothalamo-telencephalic boundary is marked as a thick blue line separating the HyA from the subpallium (in pale grey) and the amygdalar pallium (in pale yellow). The major septo-amygdalar subpallial domains, preoptic area, diagonal area, pallial area and striatum are identified (PoA, Dg, Pal; St). We simplified here the schema, because in fact the caudal subpallium would partly cover much of the pallial amygdala. We represent a state in which the torsion of the hemisphere is not completed; just imagine the central Pal and St extending backwards above the MxP and pallial amygdala, pushing the latter below the plane of the schema. All subpallial parts converge upon the septum (which is not a ventral, but a dorsal topologic entity, against what is repeated in the literature, since it encompasses the median commissural roof (e.g., the anterior commissure, ac, plus other telencephalic commissures more caudally, and adjacent extreme alar telencephalic subregions). The caudalmost subpallial regions are amygdalar, and include the anterior amygdala (AA) and the medial amygdala (MeA). The pallial amygdala (in pale yellow) appears divided into its five radial macrounits, anterior, lateral, basal, posterior and retrotipiform (a, I, b, p, rep; based on Garcia-Calero et al. 2020). Note the HyA corridor leads to the rostromedial part of the posterior unit (p), just behind the end of the subpallial MeA. The schema identifies as well-general cortex (Cx) and hippocampal allocortex (Hi; note this receives the telencephalic insertion of the roof choroidal fissure (chf) at its alar border, the cortical hem (not identified). b This second schema is essentially the same as in a, without most letterings, and is used to make clear the course followed by Sim1-expressing cells (arrows). These originate within the hypothalamic CPas and the HyA (see “Discussion”). They reach the posterior amygdalar pallium unit (p) behind the MeA, and incorporate (orange arrow) into the caudorostral NLOT2 or CAS pallial Tbr1-expressing migration stream (black arrow, with main posterior, p, origin and possible secondary, smaller origin at the basal unit, b; see schema a). The mixed arc-shaped migration stream courses first through amygdalar pallium, but finally crosses the limit of the subpallium and ends superficially at the anterior amygdala (AA). The conclusion is that the primary origin of the Tbr1-positive CAS coincides with the locus where the hypothalamic Sim1 cells arrive at the posterior pallial amygdala, and both populations compose the CAS or NLOT2 stream. c This figure shows a flattened topological schema distinguishing the amygdalar pallium (pale yellow) from the cortical pallium, which is divided into a central neo-cortex island (NCx, white) surrounded by a thin mesocortical inner ring (MCx; light grey) and a partly broader allocortical outer ring with olfactory, entorhinal/schizocortical and hippocampal subregions (OACx, SchCx, HiACx; green). The violet colored line alongside the OACx symbolizes the antihem (AH), whereas the blue colored line alongside the HiACx represents the cortical hem (CH). This cortical map reproduces notions reported in Puelles et al. (2019a), partly inherited from previous literature. As shown in a, normally the cortex covers topographically the amygdalar pallium, causing the erroneous impression that the pallial amygdala is a cortical derivative. In c, we represent its true topological subjacent position, obtained if the respectiveventricular zones (seen with the same color-code in d) are separated and flattened out. The posterior pallial amygdala (p) falls close to the SChx and the caudal ends of the OACx and HiACx (and of the AH, CH). The position of the final NLOT nucleus within subpallium (AA) appears in c at the bottom of the schema. According to us (compare b), the mixed CAS migration starts from the black dot within (p), and then proceeds along the red arrow into the AA. According to Remedios et al. (2007), the CAS migration would start at the NCx (red dot) and follow a longer route to reach the AA (but they also wrongly think that the NCx extends initially into the posterior amygdala; however, note the intercalated mesocortical and allocortical expanses of cortex). d This schema is complementary to c, and represents a sagittal section through it passing from the rostral olfactory bulb across the whole cortex, and in particular the SchCx, into the pallial amygdala (thin line in e). The cortical pallium is represented with the same color code as in c (NCx, white; MCx, light grey; OACx/SchCx, green). In this schema, the structure is unflattened, and shows the standard sagittal section configuration (lateral ventricle cavity in black). Note the rostral relationship of the pallial amygdala (yellow) with the subpallium that partly covers it, as mentioned above (Subpall.; white; compare a, b) and its caudal relation with allocortical cortex domains, closest to the posterior amygdalar radial unit (p) where the CAS migration originates (p; black dot; red arrow into NLOT/AA). As in c, we also represent with an alternative red dot origin of the red arrow the longer course predicted from an hypothetic NCx origin of the CAS (unless it is proven that the NCx ends caudally at the posterior pallial amygdala, disrupting the continuity of the meso- and allocortical rings).
the Gorski et al. (2002) result in a lizard, and proposed a possible distinct ‘ventrocaudal pallial sector’, restricted to the amygdalar domain, as the origin of amygdalar Emx1 cells (such solution was already mentioned as a possibility in mouse in Puelles et al. 2016c; however, this hypothesis has not yet been correlated with the recent radial model of the mouse amygdala which we presently use; Garcia-Calero et al. 2020). At the time of the Gorski et al. (2002) paper, we did not understand what this sharp contradiction might mean, but we were motivated to accommodate these and other discrepant data in a better model. Unfortunately, we also remained dominated by coronal section-driven assumptions for years.

Our last coronal section-based effort to define the ventropallial contribution to the amygdala used analysis of the theoretically conclusive Dbx1-derived progeny, since Dbx1 expression was held to be strictly restricted to cortical ventral pallium progenitors (Medina et al. 2004; Puelles et al. 2016c). What we did not consider significant was that Dbx1 also appears expressed primarily, and, actually, more importantly, at the ‘caudal telencephalic neuroepithelium’ (Bielle et al. 2005; Teissier et al. 2010), probably because we thought we already knew where the amygdalar nuclei came from. Our standard coronal analysis surprisingly produced various results that seemed contradictory, inconclusive, or difficult to explain. This included parts of olfactory cortex (and the whole olfactory bulb) which lacked Dbx1 derivatives largely, or altogether, or only halves of some amygdalar nuclei appearing to be positive. It was also unclear that the observed Dbx1-LacZ labeled amygdalar elements had actually migrated through the distinctly labelled ventropallial migration stream seen reaching olfactory cortex just outside of the pallio-subpallial boundary and of the pallial amygdala. Importantly, the amygdalar periventricular masses at the back of the amygdala oddly belonged likewise to Dbx1 progeny (partly seen, for instance, in Figs. 3c–e and 4a–e in Puelles et al. 2016c). These data were unexplainable by the standard model we were using. This led to offering two alternative interpretations (so far both unverified, and maybe both wrong), proposed respectively by Medina and Puelles, since our previous updated model (Puelles 2014). The concentric cortical map already showed that the pallial amygdala is a different neuroepithelial field that is adjacent, but separate, from the cortex. The two adjacent fields nevertheless do clearly share general pallial molecular characteristics, and thus are both pallial (Puelles et al. 2000).

This first step allowed us conceptually to investigate next what happens inside the isolated amygdalar pallial field. We eventually produced a radial model of the pallial amygdala (García-Calero et al. 2020), which postulates that all amygdalar nuclei originate in different subareas within the ‘caudal telencephalic neuroepithelium’ locus identified by Remedios et al. (2007). The model derives all amygdalar nuclei from no less than 9 molecularly different amygdalar radial units, subsumed under 5 macrounits (Fig. 15a), each of which develops molecularly distinct periventricular, intermediate and superficial strata. This new, much more complex amygdalar model now explains easily the previously contradictory data of Gorski et al. (2002), Puelles (2014) and Puelles et al. (2016c). García-Calero and Puelles (2021) reexamined and reinterpreted the Dbx1 data of Puelles et al. (2016c) within the novel radial amygdalar model. They reached the conclusion, consistent with both radial glia pattern and Bielle et al. (2005), that amygdalar Dbx1 derivatives are intrinsic
to the amygdalar field, and cannot be assimilated to cortical ventral pallium Dlx1 derivatives. This is precisely the conclusion that also satisfactorily explains the Emx1-LacZ data in Gorski et al. (2002).

In the wake of these advances, the effort made by Remedios et al. (2007) to trace the amygdalar CAS migration to the ‘dorsal pallium’ cortical sector seems outdated and devoid of explanatory meaning, when reexamined under the light of the radial model (and the abandonment of coronal sections). There is simply no interest in extracting amygdalar pallial nuclei from parts of the cortical pallium, because no part of pallial amygdala (including the CAS/NLOT2ms) comes out of any cortical domain. Moreover, the concentric ring cortical model provides wider significance and novel lines of analysis for the hem and antihem functions, admitting other possible organizer sites as well. Note our Fig. 15c illustrates that the dorsal pallium does not even come close to the hem and antihem organizers, because the inner mesocortical and outer allocortical cortical rings are interposed (i.e., these organizers lie topologically outside the entire concentrically organized cortex; interestingly, the gap illustrated by Remedios et al. 2007 that separates ‘longitudinally’ the hem from the antihem relates to the new amygdalo-allocortical or amygdalo-entorhinal border proposed by us). This new topologic concept of the cortical field and its organizers recovers the classic notions of allocortex and mesocortex (the two concentric peripheral cortical rings) as the natural evolutionary environment of the central neocortex/isocortex island (Puelles 2001, 2011; Puelles et al. 2019a).

It was thus a commonly held, but not wholly supported, or solidly evidence-based, assumption of many workers in the pallial developmental field at the time of the Remedios et al. (2007) study that cortical pallial sectors might or must have derivatives in the amygdala. It is important to realize that all the referred amygdalar molecular mappings aiming to establish cortical origins of amygdalar parts stood systematically on coronal sections, which are oblique to amygdalar radial glia.

The new amygdala model agrees with the facts that the respective cortical and amygdalar structures are different (layers versus nuclei). Note that amygdalar neurogenetic stratification is outside in, whereas the cortical one is inside out (Garcia-Calero and Puelles 2020), not to speak of differential connections and functions. Consequently, pallium models postulating a number of parallel longitudinal pallial sectors extending throughout the whole telencephalon are presumably on the wane. We should study the cortical pallium and the amygdalar pallium as causally separate complex fields, each with quite diverse molecular profiles, and probably with correspondingly diverse causal mechanisms, irrespective of the known partially shared gene patterns.

As regards our third discussion point sketched above, we refer to atlases of developing rodent brains (e.g., Altman and Bayer 1995; Alvarez-Bolado and Swanson 1996; Jacobowitz and Abbott 1997; Foster 1998; Paxinos et al. 2007; Ashwell and Paxinos 2008), which unanimously identify the posterior pallial region (AHI) where the NLOT2ms originates as an integral part of the amygdala. This clearly includes the caudal pallial area where Remedios et al. (2007) electroporated at E11.5 a GFP-fluorescent label, which later nicely concentrated in the CAS migration stream (their Fig. 2). The AHI name (‘amygdalo-hippocampal transition area’) given conventionally in atlases to this posterior amygdalar area actually alludes to its easily visible extensive caudal neighborhood with the hippocampus (and entorhinal cortex), rather than with the distant neocortex. This fact obviously already stood ab initio against the conclusions of Remedios et al. (2007). These authors explicitly identified the ‘dorsal pallium’ as the primordium of the neocortex, and even announced a ‘link between the amygdala and neocortex’ in their title. However, no part of the amygdala is histologically neocortical or eulaminate (i.e., six-layered; Garcia-Cabezas et al. 2019), or even contacts neocortical structures (recent reviews in Barbas 2015; Garcia-Cabezas et al. 2019; Puelles et al. 2019a; Garcia-Calero et al. 2020; see also Paxinos and Franklin 2013, and other rodent brain atlases; see also our Fig. 15c, d). This means that there is no solid anatomic or developmental evidence for the neocortex-amygdala link, irrespective that the posterior amygdala seems to share given molecular markers with both dorsal and medial pallium (as was argued above).

Neuroanatomists know for a long time that the neocortex lies far away from the amygdala. If neocortex really produced the CAS cells, these would have to migrate across the interposed mesocortex and allocortex rings that surround the neocortex, possibly having to pass across the entorhinal schizocortex, in order to reach the amygdala beyond the caudal end of the lateral ventricle (see red dot and red arrow schematics in our Fig. 15c, d). Actually, Remedios et al. (2007) did not really think, or wanted to imply, that their postulated ‘neocortical origin’ was distant, since they did not explore that possibility. After all, they knew from their crucial electroporation experiment that the migration originates entirely in front of the caudal end of the lateral ventricle (compare Fig. 15c, d with their Fig. 2).

These authors accordingly just played with the hypothesis that the relatively unexplored caudal part of the dorsal pallium might extend caudalwards through the caudal gap they had discovered between hem and antihem, passing beyond the caudal end of the ventricle into a similarly unexplored ‘caudal telencephalic neuroepithelium’. This implies an aberrant model of cortical development which we need to criticise, because patterning studies are brought into significant confusion by arbitrary modifications of the ‘area
map’ that needs to be explained (see comments in Puellès et al. 2019a).

In holding this hypothesis, Remedios et al. (2007) implied (probably unwittingly) that the limits of neocortex are wrong in all precedent publications, atlases and specialized book chapters on the cortex since Brodmann (1909) and von Economo (1927). These sources do not show that the neocortex extends backwards to contact the posterior pallial amygdala (García-Cabezas et al. 2019; Puellès et al. 2019a). It is a sign of a certain dismissive attitude to conventional neuroanatomy in the molecular era that Remedios et al. (2007) actually got this conclusion published in Nature Neuroscience! However, these authors did not even explore in their Discussion the conventional interpretation suggesting that any cortex immediately caudal to the amygdala most probably had to be of the hippocampal allocortical sort, that is, mediopallial. This involves either entorhinal cortex (a variant non-eulaminate sort of allocortex, also termed ‘schizocortex’; Puellès et al. 2019a), or hippocampal subicular or Ammon’s horn areas. We represented these diverse amygdalar caudal vicinity relationships in optimal horizontal sections in García-Calero et al. (2020; e.g., our Fig. 5). Various cortex models supporting this notion were recorded since 1987 (e.g., Swanson 1987; Bayer and Altman 1991; Pattabiraman et al. 2014; García-Cabezas et al. 2019). See also Witter (2012; his Figs.5.1 and 5.2) and Martínez-García et al. (2012; their Fig. 6.3).

Alternatively, the conclusion of Remedios et al. (2007) might imply that the postrhinal, entorhinal and hippocampal cortex domains are actually developmentally and molecularly neocortical, against the majority of existing opinions in the field, and a host of inconsistent molecular data (Thompson et al. 2014; Allen Mouse Brain Atlas, and other analogous repositories). We thus think that Remedios et al. (2007) were not aware of this anatomical difficulty, and did not realize that the hypothetic existence of dorsal pallium passing through the hem-antihem gap to contact the amygdala implied a highly improbable rupture of the allocortical ring present around the whole mesocortex and neocortex, as was already known in 2007 (Swanson 1987; Bayer and Altman 1991; Fig. 15c).

All this implies that it is possible in 2020 to reinterpret coherently the objectively beautiful and rich results of Remedios et al. (2007) as we presently did here, by substituting a primary posterior amygdalar origin of the CAS/NLOT2ms migration, and leaving the dorsal pallium apart in its central place within the cortical ‘area map’. We eliminated doubtful assumptions based on the use of coronal sections, and other conceptual errors of the near past. We refrained from deciding the issue at hand with hardly specific gene expression patterns (Lhx2, Ems1), or from believing that selective cortical gene patterns inform us about the origin of differently fated amygdalar structures, or intrinsic amygdalar migratory phenomena. We will see below that we can insert the duly corroborated gene requirements observed by Remedios et al. (2007), into a different and more complete explanatory rationale that does not involve the dorsal pallium, and does not disrupt on the sly the anatomic traditions preserved by rodent brain atlases and other relevant literature.

The real CAS origin at the amygdalar AHi/PMC0 primordium (posterior amygdalar unit) is consistent, moreover, with our present results showing that this amygdalar unit is also the target of the HyA corridor and its migrated hypothalamic Sim1-positive cells. These are fated to help the CAS reach the NLOT2 locus by their apparently required presence in this cell stream. Divergent evolution of an equivalent NLOT2 homolog in other vertebrate groups thus may be also linked to co-evolution of the Sim1-expressing population and its timely arrival at the pallial amygdala, in order to participate in the NLOT migration stream.

**Precise origin of Sim1 cells targeting the pallial amygdala and Sim1/Otp comparison**

Present results suggest that Sim1-expressing NLOT2 cells originate along the HyA and Pa areas, since both belong strictly to the same progenitor domain, merely deformed in part during hemispheric evagination. There is the caveat that the Pa (and thus perhaps also the HyA) is presently held to be divided into 3 dorsoventral subdomains with some variant properties (dorsal, central and ventral Pa, or DPa, CPA, VPA; see Fig. 15a; Puellès et al. 2012). All of them produce Sim1- and Otp-expressing cells (Michaud et al. 1998; Wang and Lufkin 2000), but only the CPA and VPA subdomains display Brn2 expression, while only DPA co-expresses Foxg1 (Morales et al. 2021). The dorsoventral subdivision of Pa leads to phenotypic and migratory differential properties before or after the terminal differentiation step of the neurons produced in each sector (Schonemann et al. 1995; Michaud et al. 1998; Wang and Lufkin 2000; note none of these authors were aware of the existence of Pa subdomains; see also Diaz et al. 2015). DPA apparently produces mainly TRH and SST cells, while CPA and VPA jointly produce CRH, AVP and OT cells. We had difficulties in confirming this pattern (except for CRH cells, which are clearly related to CPA) on the basis of mappings at the Allen Developing Mouse Brain Atlas, possibly due to short-range tangential migrations that redistribute the cells. Remarkably, only the TRH cell type appears later in the pallial amygdala (at the PMC0 part of the AHi/PMC0 posterior complex), though this happens in a much delayed chronology (first tenuous expression at P4; strong signal at P14). Dispersed SST cells are abundant in various amygdalar nuclei, due to massive tangential migration of SST-expressing interneurons from the subpallial diagonal domain (Puelles et al. 2016a).
Therefore, we cannot identify potential intrinsically developed, HyA-related SST elements. 

Morales et al. (2021) interestingly observed that the DPa subdomain (interpreted by these authors as being telencephalic rather than hypothalamic, due to its coincident FoxG1 marker signal) extends into a rostrrodorsal part of our HyA. These authors show that DPa contributes glutamatergic Otp cells to the medial amygdala, extended medial subpallial amygdala, and part of the BSTM nucleus (curiously, all of them targets practically devoid of Sim1-expressing cells). The CPA subarea extends instead into the caudalventral part of HyA, which presumably leads into the pallial amygdala (as represented in Fig. 15a). This HyA division idea was already advanced by Puelles and Rubenstein (2003, their Fig. 3 schema) and Puelles and Rubenstein (2015, their Fig. 10 schema). In any case, the fact that the wild-type E18.5 NLOT2 expresses both Sim1 and Brn2 (Fig. 14e, g) suggests that its hypothalamicic cells originate at the extended CPA subarea, rather than the DPa, where Brn2 reportedly is not expressed (Michaud et al. 1998). Hence, there are at least two molecularly distinct parts dividing lengthwise the HyA corridor, which extend the FoxG1/Brn2 molecular border existing between DPa and CPAa (Fig. 15a). Our observation of overlapping Sim1 and Brn2 signal in NLOT2 at E18.5 suggests that the latter marker may render the CPA-HyA subdivision ostensible.

Further studies should explore why Otp-expressing cells apparently target preferentially subpallial centers (Morales et al. 2021; Wang and Lufkin 2000; Garcia-Moreno et al. 2010), whereas Sim1-expressing cells target selectively the pallial amygdala, and mainly NLOT2 and PaA therein (present analysis). Wang and Lufkin (2000) misidentified the locus of Sim1 cells within the amygdala (see below).

The NLOT2 migration and the presence of Sim1 cells in the postnatal NLOT2 are unaffected in Otp mutant mice, though the Otp-expressing cells migrated early on into the MeA are substantially reduced in number after E15.5 (Wang and Lufkin 2000; their Figs. 2i–l, 3). In Fig. 7a, b of the same report it can be noticed that the HyA appears Otp-labeled under the terminal sulcus in the Otp-LacZ heterozygote at E15.5, but not in the homozygote, though the MeA is invaded equally. Probably most of the Otp cells migrated into MeA arrive there via the subcapsular part of the HyA (LP and EG-C, unpublished observations). Wang and Lufkin (2000) also showed in their Fig. 6e, f (at P1), Fig. 7q, r (at E15.5), and Fig. 8s, t (at E13.5) images of pairs of comparable sections from wild type and Otp−/− embryos reacted for Sim1 transcripts, which compare perfectly with our present material. The P1 amygdalar cell mass expressing Sim1 (their Fig. 6e, f) clearly is the NLOT2, though it was mislabeled as ‘MeA’ (there is no Sim1 signal at the MeA, nor any Otp signal at the NLOT, and the position and shape shown correspond to NLOT). The authors apparently were unaware of the fact that the amygdalar targets of Sim1 and Otp cells are different. In fact, it is unclear to us whether something similar occurs also in the hypothalamus, where a subtle differential topography of Sim1 versus Otp cells might have passed undetected, unless the mixed pattern accepted conventionally truly reigns there. The amygdalar E15.5 Sim1 image of Wang and Lufkin (2000; their Fig. 7q, r) shows a normally labeled NLOT2ms in both heterozygote and homozygote, while the E13.5 image (their Fig. 8s, t) exactly duplicates our Fig. 5g, illustrating incipient Sim1 penetration of the periventricular posterior pallial amygdala (future AHi). It is of interest as well that heterozygotic Otp-LacZ whole-mounts show no labeled HyA at E11.5, whereas a fully formed HyA appears at E12.5 (Wang and Lufkin 2000; their Fig. 2e, g; in the legend, the authors misidentified the HyA as ‘amygdaloid nuclei’). This result points to an independent, possibly consecutive production of earlier Sim1-fated cells versus slightly later Otp-fated cells, at least at the HyA. Part of the timing and positional subtleties detected later in the patterns of delayed cell death resulting from Sim1 or Otp loss of function (Michaud et al. 1998; Wang and Lufkin 2000; present results) probably obey to this temporal dissociation.

The production of Sim1 neurons at the Pa/HyA indeed begins precociously at E10.5 or earlier (Fan et al. 1996), whereas Acampora et al. (2000) detected Otp transcripts in the hypothalamus already at E9.5. The corresponding ventricular zone is transiently Sim1-positive between E10.5 and E11.5, but the expression becomes subsequently restricted to the mantle layer as of E12.5 (Fan et al. 1996; present results—see Fig. 1m; this coincides with the first emergence of Otp-LacZ positive HyA; Wang and Lufkin 2000; their Fig. 2G). One may thus conjecture that Otp cells possibly start to emerge at the HyA once the Sim1 gene is downregulated at the ventricular zone, leaving only Otp activated there. This change in molecular profile might underlie causally the change in neurogenetic timing between Sim1 and Otp cells at the HyA, and possibly affect as well their differential molecular profile, terminal differentiation, migratory interactions and targets.

**The present concept of the hypothalamo-amygdalar corridor**

We described for clarity the evaginated paraventricular HyA corridor as a supracapsular anatomic entity lining ventricularly and periventricularly the floor of the interventricular foramen and the terminal sulcus, in planar continuity with the hypothalamic Pa area and the posterior pallial amygdala (Fig. 15a). However, we must assume, based upon general knowledge on neuroepithelial histogenesis (Nieuwenhuys and Puelles 2016), that the HyA surely develops its own mantle layer containing narrow periventricular, intermediate and superficial strata derivatives. This radially complete
HyA territory of the neural wall must end at the pial surface of the telencephalic stalk continguously with that of the non-evaginated Pa, intercalated between the prethalamic pial surfaces of the prethalamtic eminence and the subpallial diagonal band (Fig. 5b, c). We do not know anything about the non-periventricular HyA mantle derivatives, which apparently do not express significantly Sim1. They may contain Otp cells, and be lumped with components of the MeA in atlases and publications (Fig. 15a).

Previously we conceived the HyA as a ‘pallial corridor’, that is, as a part of telencephalic pallium that descended behind the MeA and other parts of the ganglionic eminences to contact the alar hypothalamus (implicit in Puelles et al. 2013, 2016a). In a similar vein, we identified the avian counterpart as ‘pallial extended amygdala’, given its parallel disposition relative to the subpallial extended amygdala complex (e.g., Puelles et al. 2007, 2019b). Our present work made us realize finally that the HyA represents permanently an evaginated portion of the hypothalamic Pa area, sharing some of its molecular and neurogenetic properties, and even reaching the chorioidal root plate (see choriod fissure -chf- in Fig. 15a). The presence of this thin hypothalamic band extending into the roof plate separates the telencephalic cortical and amygdalar pallial fields from the diencephalic pretelencephalic eminence (we previously wrongly supposed that the prethalamic eminence contacts both the hippocampus and the pallial amygdala; e.g., Puelles 2013; Puelles et al. 2015; Alonso et al. 2020a).

The HyA schema in Fig. 15a clarifies our presently updated understanding of the hypothalamic-telencephalic, hypothalamo-subpallial, hypothalamo-pallial-amygdalar, and diencephalo-telencephalic boundaries. It suggests the need to continue thinking about relevant topologic notions relative to this cryptic brain area (Puelles 2019; Puelles et al. 2019a).

**The NLOT2 migratory pathway re-examined within the radial amygdala model**

As mentioned above, we proposed recently a radial model of the pallial amygdala (Garcia-Calero et al. 2020). This model postulates the existence of 5 histogenetic pallial-amygdalar radial macrounits. By conservative topological criteria we defined the macrounits as lateral, basal, anterior, posterior, and retroendopiriform radial units (there are some subdivisions, leading actually to nine molecularly distinguishable radial structural complexes; see loc.cit., and Table 1 in Garcia-Calero and Puelles 2020, 2021). Each of these radial units shows a different combinatorial molecular profile (with variously shared markers), and produces characteristic amygdalar nuclei in a stratified outside-in arrangement relative to birthdates (i.e., superficial nuclei are born before intermediate ones, and these before the periventricular nuclei; see discussions in Garcia-Calero et al. 2020, and Garcia-Calero and Puelles 2020, 2021). It is thus meaningless to expect inside-out radial migrations within the pallial amygdala (e.g., Subramanian et al. 2009).

Hypothalamic (paraventricular) Sim1-expressing cells first translocate into the telencephalon along the HyA; note this displacement is topologically strictly restricted to a caudal neighbourhood (it occurs next to the hypothalamo-diencephalic border) and advances dorsally (into the telencephalon), even though the HyA appears in whole-mounts as a caudally oriented arc; this bespeaks of the morphogenetic deformation caused by the development of the caudal telencephalic pole (Fig. 1n; see also Fan et al. 1996). At the end of this corridor, the migrating cells bypass the caudalmost part of the MeA, and invade directly the posterior radial amygdalar unit (between E13.5 and E14.5; Fig. 15b). They enter it through its rostromedial subdivision, intercalated between the MeA and the ventral hippocampus. The Sim1 cells immediately mix there with local Tbr1 cells, and, together, they start to migrate tangentially rostralwards within the amygdala, advancing now laterally to the MeA and alongside the local pallio-subpallial boundary (Fig. 15b). We corroborated the immediate formation of a dense composite Tbr1/Sim1-expressing mass which also shows NeuroD1/2/6 and Zic2 signal, as was initially described by Remedios et al. (2007) and Murillo et al. (2015). See also selective CAS expression of Dach 1 in the Allen Developing Mouse Brain Atlas. This mass forms the CAS or NLOT2ms (NLOT2ms; Fig. 7i).

At E15.5 the mixed NLOT2 migratory stream continues advancing rostralwards through the pallial side of the pallio-subpallial boundary, passing from the posterior unit (AHi) to the medial aspect of the basal and anterior radial units (p, b, a; in Fig. 15a, b). The stream ends where we call the **pallial phase** of its course at a locus just medial to the immature basolateral anterior nucleus (BLA) and slightly above the basomedial anterior nucleus (BMA). BLA and BMA are intermediate strata derived respectively from the basal and anterior radial units (a, b; Fig. 15a, b; Garcia-Calero et al. 2020). It is at this locus where we noted that Zic2 expression is downregulated within the NLOT2ms (Murillo et al. 2015 did not comment on this point, visible in their material). Zic2 expression, apart from defining **selectively** the pallial phase of the NLOT2ms, also happens to be clearly selective for the amygdalar AHi, excluding any cortical expression, apart from defining the rostromedial subdivision of the posterior radial unit or AHi/PMCo complex, which ends superficially separately from the PMCo, next to the PLCo formation (RL.; Fig. 10d, f; details in Garcia-Calero et al. 2020).

Some additional Tbr1-positive cells from the BLA primordium may incorporate at this level into the NLOT2ms, after becoming displaced lateromedially from the radial axis.
of the basolateral subunit. This deviation apparently results in the characteristic non-radial BLA cap over BMA, and the typical medial horn of the BLA nucleus (see Garcia-Calero et al. 2020). Another marker identifying the BLA horn is Lmo3 (Abellán et al. 2009; their Fig. 1E). This formation penetrates the AA in correlation with the molecularly distinct amygdalo-olfactory cell stream (AOS), which thereafter trails the NLOT2ms from this point into the definitive adult NLOT (see these details in Garcia-Calero et al. 2020). We noted here that Neurod6 (Math2) seems to label selectively this possible secondary rostrolateral root of the NLOT2ms (see selectively labeled BLA, BLAcap, BLI, BLP, and NLOT2ms in Fig. 12e–g); a similar combined expression pattern was recently noted in Er81 preparations at E15.5 and E18.5 at the Allen Developing Mouse Brain Atlas. Indeed, Neurod6 signal appears scarcely if at all at the AHi main NLOT2ms origin (Fig. 12d, g), but nevertheless labels the NLOT2ms and the mature NLOT as well (Fig. 12g, h). We thus believe that Neurod6, and possibly also Er81, label selectively a minor basolateral Tbr1-positive source of the NLOT2ms (b in Fig. 15a, b). This maybe resolves in the AOS stream and the few Tbr1 cells aggregating later at the layer 3 of NLOT (Fig. 9g), also forming a tenuous shell around this nucleus. The AOS cells were first detected by their Azin2-LacZ signal, also present at the BLA and AHi (not shown; see Garcia-Calero et al. 2020), and were later found to express selectively Er81 (Etv1) and Cyp26, signals, which appear, jointly with Tbr1, at the postnatal layer 3 of the NLOT (Fig. 9g–i, j).

In contrast to Zic2, transcripts of Neurod1 and Neurod2 (as well as of Dach1, discovered at the Allen Atlas after our first submission) are selectively present at the AHi, NLOT2ms and definitive NLOT, as well as at the entorhinal and hippocampal cortex in the case of Neurod1/2 (Figs. 10i, j, 12a–c). Neurod6 signal is also present at the neighboring cortex (though hardly at E13.5), but does not label significantly the AHi at any stage examined (Fig. 12d, g, h).

The NLOT2ms next passes from the BLA/BMA neighborhood into the anterior amygdalar subpallium (AHi; Fig. 15b), crossing the local pallio-subpallial boundary. This is an obvious decision point where the migratory stratification into the anterior amygdalar radial unit (a into AA; this migration is not marked specifically in Fig. 15a, b; see Garcia-Calero and Puelles 2021). A good number of subpallium-derived cells expressing Pax6 or calbindin (not shown) accompany these migrated pallial cells at the AA. Tole et al. (2005) reported that absence of Pax6 function generates anomalies in the formation of NLOT. Moreover, as the NLOT2ms enters the subpallium, it is surrounded rostrodorsally (perhaps guided) by a fairly dense Six3-expressing population of subpallial cells filling the radial space between the central amygdalar nucleus and the olfactory tuberculum (CeA; OT; Fig. 9k, l).

We conjecture that pallial and subpallial cells populating the AA may produce attractive signals acting on the NLOT2 cells when these reach the BLA/BMA decision point and downregulate Zic2. Such attracting signals surely include reelin, as suggested by Remedios et al. (2007), who demonstrated reelin and Dab1 signals at the AA, and showed experimentally that the progress of the migration depends on the reelin/cdk5 pathway (shared by neocortical radially migrating neurons). It is remarkable that no violation of the pallio-subpallial border occurs until the stream contacts the AA; Fig. 15b). This suggests that the reelin attraction effect only reaches up to the aforementioned decision point.

Finally, the NLOT creates its own encapsulated place (with practically no AA cell mixing) within AA; this locus relates to the subpial passage of the lateral olfactory tract mediallywards. Over E16.5–E18.5, the trailing part of the NLOT2ms gradually disappears as the cells reach the target, and the definitive tri-layered NLOT forms in conjunction with the other layer components. The latter have still uncertain origins. Layer 1 was reported to express selectively Lhx2 (Tole et al. 2005) and Lhx9 (Remedios et al. 2007), a pattern it possibly shares with the neighboring, likewise olfacto-recipient, superficial BAOT nucleus (García-López et al. 2008; their Fig. 14D). Layer 3 expresses partially Tbr1, as well as Azin2-LacZ, Er81(Etv1) and Cyp26 signals, as we have reported (Fig. 9g, i, j). This pattern apparently relates the layer 3 NLOT cells to the BLA medial horn and the distinctive Azin2-LacZ-positive AOS (Garcia-Calero et al. 2020), though we have also observed a good number of probably tangentially migrated subpallial Dlx5/6-LacZ-labelled neurons mixed therein (LP; unpublished observations). As mentioned above, a few Sim1-expressing cells named by us para-anterior cell group (PaA) remain dispersed medially to the BMA (part of the anterior radial pallial amygdalar unit), caudally to the definitive NLOT (PaA; Figs. 6n, o, 13c–f, h–j, 14a, b; see postnatal stages in the Allen Developing Mouse Brain Atlas). We are not sure whether these
cells split off from the NLOT2ms, or migrate independently along a subcapsular route from the hypothalamic paraventricular area, or the HyA. Remarkably, a remnant of the Sim1-expressing HyA located next to the AHi also remains visible, intercalated between the MeA and the PThEt up to P4, but apparently disappears afterwards (not shown; see Allen Developing Mouse Brain Atlas). Curiously, Otp cells are also observed postnatally at this extreme caudal HyA locus, next to the choroidal tela of the choroidal fissure; these Otp cells persist even in the adult brain (LP; EG-C; unpublished observations).

**Synthesis of loss of function mouse phenotypes affecting NLOT development**

Mice mutants for genes such as Tbr1, Emx1/Emx2, Lhx2, Pax6, Zic2 (and also Sim1, according to present results) affect NLOT formation (Remedios et al. 2004, 2007; Tole et al. 2005; Murillo et al. 2015), but their analysis has not been brought yet to a synthetic conception of how the NLOT migrates and forms under their joint influences. We offer a tentative synthetic interpretation that seems consistent with presently available data, and suggests some possibilities apt to be tested experimentally.

The joint mutation of Emx1/Emx2 leads to absence of the NLOT (Tole et al. 2005). This phenotype shows mainly a severe loss of hippocampal portions including the dentate gyrus (e.g., Bishop et al. 2003; Shinozaki et al. 2004; Suda et al. 2010; see also Mangale et al. 2008), apparently due to failure of hem patterning effects. We postulate that at least the rostromedial subdivision of the posterior radial amygdalar unit, which lies just in front of the caudoventral end of the hippocampus and of the attached hem organizer (Fig. 12o, p), possibly lacks in this situation needed hem patterning signals, apart from its intrinsic Emx1 signal (Remedios et al. 2007; present results; Fig. 12o, p). This molecular abnormality at the origin of the CAS migration would lead to a primary failure of NLOT formation, due to abnormal specification of its origin. Such hem influence upon the posterior amygdalar unit may explain as well the existence of several shared hippocampal genes at this locus (Abellán et al. 2014). We do not believe that this implies that the origin of the CAS migration is mesopallial cortical, for the same reasons that we discard the notion that it may be dorsopallial (see above), or perhaps caudo-ventropallial (Ruiz-Reig et al. 2018). These notions implicitly (and arbitrarily) refer to cortical pallial sectors, and we are dealing with the pallial amygdala.

A similar case may apply to mutants devoid of Lhx2 signal (Mangale et al. 2008; Chou and Tole 2019). Since this gene appears strongly expressed in combination with Emx1 at the posterior radial unit and neighboring allocortex (Fig. 12i–n) at the stage in which the NLOT2ms starts to form. Lack of Lhx2 function within the posterior amygdala possibly alters the normal local specification of the origin of this migration, so that as a result the NLOT2ms does not form. We do not believe that Lhx2 lack of function phenomena described within cortical pallium, particularly phenomena taking place in the allocortical primordia (apparently not contemplated by Mangale et al. 2008, Subramanian et al. 2009, or Chou and Tole 2019), affect per se the posterior pallial amygdala, unless the hem functions are affected (see case of Emx1/Emx2). Secondly, if the CAS migration should emerge anyway in this mutant (this point can be examined), lack of Lhx2 function might have a different sort of amygdalar relevance. The Lhx2/Lhx9 cells migrated into AA may normally participate in generating the anterior amygdalar reelin signal needed for the final attraction of the NLOT2 cells into their definitive AA position (Remedios et al. 2007). Such attraction may result somehow compromised by lack of Lhx2 function in these AA cells. Interestingly, the pallial amygdalar nuclei co-expressing Lhx2 and Lhx9 (BMA; ACo) seem to attract on their own the para-anterior cell group (PaA) of Sim1-expressing cells, which forms a persistent medial shell next to the BMA nucleus, thus remaining within the pallial amygdala. This structure apparently resists loss of the Sim1 signal (Figs. 13a–f, 14a, b), but we lack data about its possible absence in Lhx2 mutants.

The Lhx2/Lhx9 expressing amygdalar pallial cells were earlier thought to derive from the cortical ventral pallium (Medina et al. 2004; Tole et al. 2005; Puelles et al. 2016b). However, García-Calero et al. (2020) and García-Calero and Puelles (2021) reconsidered this notion, visualizing the Lhx9/Lhx2 combination as restricted primarily to the embryonic anterior radial amygdalar unit. They also described the secondary tangential migration of superficial BMA/ACo Lhx9/Lhx2 cells into the AA. García-López et al. (2008) visualized other local migrations of similar cells that penetrate the MeA, a point later verified experimentally by Bupesh et al. (2011).

Tbr1 and Zic2 mutants show a disorganization of the migration and the NLOT nucleus is not formed (Remedios et al. 2004, 2007; Murillo et al. 2015). Since the NLOT2ms is a pallial migration, lack of the fundamental pallial Tbr1 marker may cause abortion of the origin of the migration. As regards Zic2, we noted that it only appears expressed by the NLOT2ms cells during their pallial phase of migration, and this gene results downregulated afterwards as the stream moves into the subpallium (shown, but not discussed by Murillo et al. 2015; present results). This peculiarity suggests that the migration needs Zic2 function only during the initial pallial phase (while it proceeds orthogonal to glial processes, according to Remedios et al. 2007), that is, between the posterior radial unit origin and the BLA/BMA decision point. This part of the migration possibly does not obey yet the reelin/cdk5 signaling pathway, a mechanism
which appears to apply mainly to the second subpallial phase (Remedios et al. 2007; Subramanian et al. 2009), and perhaps even participates directly or indirectly in the downregulation of Zic2 at the decision point. The shorter CAS migration observed in absence of reelin/cdk5 signaling (Remedios et al. 2007) possibly occurs thanks to Zic2 in concert with the Neurod1/2 genes (see also in vitro data of Murillo et al. 2015).

We observed that Sim1 mutant homozygotes reproduce in absence of Sim1 function the initial phase of Sim1-tau-LacZ-labelled NLOT2ms migration up to the pallial-subpallial decision point. However, once Sim1 cells start to die at about E15.5-E16.5 (as found by Michaud et al. 1998 in hypothalamic paraventricular Sim1 derivatives), progression of the NLOT2ms (with majority Tbr1 cells, and a Sim1/Brn2-expressing subpopulation) into the subpallial migration phase fails to occur. No cytoarchitectonic or molecular trace of the NLOT nucleus appears subsequently within AA at E18.5 (Fig. 14c–h). This result possibly indicates an intimate migration-facilitating or perhaps trophic relationship needed for the second phase of migration, which unifies the further migration of both Tbr1- and Sim1-expressing cells within the stream. This interaction seems needed at least from the intermediate decision point onwards, once Zic2 activity is repressed. Accordingly, Sim1 function is somehow required to advance the whole migration stream into its second subpallial phase. One interesting possibility is that the Tbr1-Sim1 interaction makes the newly Zic2-negative NLOT2 migrating cells able to respond to the subpallial reelin signals related to Lhx2 and Pax6 signals within AA, thus allowing the second phase to begin. Moreover, Sim1 function possibly is also needed less critically during earlier stages in the migration into and inside the amygdala of the Sim1 cells, since we also observed abnormal accumulations of Sim1 cells along the HyA at E16.5 (Figs. 13, 14a, b).

Our tentative hypothesis of a second basolateral root of the migrating NLOT2 stream, connected to Neurod6 and Etv1 signals, maybe does not hold, since we have only circumstantial evidence so far (Neurod6 or Etv1 knockout might be informative). In any case, we think that the notable adult BLA cap and horn elements, which singularly protrude anti-radially into the amygdalar subpallium in the wake of the advancing NLOT2ms (as suggested by Garcia-Calero et al. 2020) is perhaps understandable alternatively as a sketched but unfinished reaction to the subpallial attracting reelin signals which bring the NLOT2ms into AA. The AOS cells extending from the BLA horn into the NLOT layer 3 and related shell formation (Garcia-Calero et al. 2020) seem to represent another AA-attraction phenomenon starting at or near the decision point.

Our present synthetic hypothesis accordingly suggests that hem Emx1/Emx2 expression (with added Emx1, Lhx2 and Neurod1/2 signals at the posterior radial unit) specifies molecularly the posterior amygdalar territory where the CAS originates (Tole et al. 2005). Failure of this early step, or lack of local Tbr1 function, compromises the whole migration. Subsequently Zic2 jointly with Neurod1/2 and Dach1 acting at the origin of the migration are crucial for the activation and control of the first phase of migration (pallial steps orthogonal to glial structure; Murillo et al. 2015). The head of the NLOT2ms (which strongly expresses Dach1) thereafter downregulates Zic2 expression, and Sim1 function is needed at least at the intermediate decision point for the progression of the stream into its second phase of migration into AA, now parallel to radial glia. This phase crucially requires as well an active reelin/cdk5 signaling pathway, as shown experimentally by Remedios et al. (2007). The latter aspect possibly involves Pax6 function in AA subpallial neurons or in corroborative radial glia cells (Tole et al. 2005; Remedios et al. 2007), and perhaps this depends partly on Lhx2 in the migrated anterior pallial amygdalar cells populating AA (Remedios et al. 2004; Subramanian et al. 2009). Whether subpallial Six3-expressing cells that cover rostrally and perhaps this depends partly on Lhx2 in the migrated anterior pallial amygdalar cells populating AA (Remedios et al. 2004; Subramanian et al. 2009). Whether subpallial Six3-expressing cells that cover rostro-dorsally the subpallial phase of NLOT2ms migration (present results) are also involved in its control requires investigation; an involvement is suggested by their closeness to the migrating stream (present results; Fig. 9k). A further concurring circumstance is that the Lhx2 mutant does not develop a normal lateral olfactory tract under the AA and NLOT (Saha et al. 2007); it is so far unclear whether this defect also affects the NLOT2 final migration into the AA.

**Experimental procedures**

**Animal preparation and tissue analysis**

The day of the vaginal plug was counted as embryonic day E0.5. The brains from sacrificed mouse embryos were dissected out, and fixed overnight in 4% paraformaldehyde in pH 7.4 phosphate-buffered saline (PBS) at 4 °C. The brains were embedded in 4% agarose in PBS, and 100 μm sections were cut in horizontal, sagittal, coronal and oblique planes with a Leica vibratome (VT1000 S), to be processed for in situ hybridization and immunohistochemistry.

The generation and genotyping of mice carrying Sim1-tau-LacZ was described previously in Marion et al. (2005). Briefly, a gene cassette encoding tauLacZ was inserted into the first exon of the Sim1 gene to generate the Sim1tau-LacZ allele. The β-galactosidase activity protocol for detection of the Sim1tau-LacZ allele was also previously described in Marion et al. (2005).
In situ hybridization

We used the restriction enzymes and polymerases suitable for specific riboprobe synthesis in the presence of digoxigenin-11-UTP. The hybridization protocol used was according to Shimamura et al. (1994). Mouse cDNA probes used for in situ hybridization analysis were Dlx5 and Sim1 (J.R. Rubenstein), Brn2 (J.L.Michaud) and Lhx9 (our own lab).

Immunohistochemistry

For immunostaining we followed the protocol published in Garcia-Calero and Scharff (2013). The primary antibodies used in this study were: rabbit anti-Opt (F. Vaccarino), rabbit anti-Tbr1 (1:200; sc-48816, Santa Cruz Biotechnology, Inc), mouse anti-RC2 (1:10; Developmental Studies Hybridoma Bank, Iowa City, IA, USA).

Image capture, manipulation and figure assembly

Digital photomicrographs were acquired using an Aperio CS2 digitalizing device and a confocal microscope (TCS SP8 AOBS; Leica Microsystems GmbH, Mannheim, Germany). The z-stack images were acquired with LCS software. Digital images were processed with Aperio ImageScope (Leica Microsystems GmbH, Mannheim, Germany), ImageJ (NIH, http://rsb.info.nih.gov/ij) and Adobe Photoshop and Adobe Illustrator softwares (Adobe Systems Mountain View, CA, USA).

Acknowledgements

We thank the Allen Institute for Brain Science for public availability of the markers analyzed (Website: ©2013 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas. http://developingmouse.brain-map.org).

Funding

This work was supported by a Spanish Ministry of Economy and Competitiveness Grant BFU2014-57516P (with European Community FEDER support), and a Seneca Foundation (Autonomous Community of Murcia) Excellency Research contract, reference: 19904/MUR/2010 (with European Community FEDER support), and a Seneca Foundation (Autonomous Community of Murcia) Excellency Research contract, reference: 19904/MUR/2010 (with European Community FEDER support), and a Seneca Foundation (Autonomous Community of Murcia) Excellency Research contract, reference: 19904/MUR/2010 (with European Community FEDER support), and a Seneca Foundation (Autonomous Community of Murcia) Excellency Research contract, reference: 19904/MUR/2010 (with European Community FEDER support), and a Seneca Foundation (Autonomous Community of Murcia) Excellency Research contract, reference: 19904/MUR/2010 (with European Community FEDER support).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards Not applicable. See below about animal care.

Human participants This article does not contain any studies involving human participants.

Research involving animals All experimental protocols and handling, use, and care of laboratory animals were conducted in compliance with the current normative standards of the European Union (Directive 2010/63/EU), the Spanish Government (Royal Decree 1201/2005 and 53/2013; Law 32/107), and with the approval of the University of Murcia committee for animal experimental ethics (No. A13170406).

Informed consent All authors consent to participate and publish the data included in this manuscript.

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References

Abellán A, Legaz I, Vernier B, Rétaux S, Medina L (2009) Olfactory and amygdalar structures of the chicken ventral pallium based on the combinatorial expression patterns of LIM and other developmental regulatory genes. J Comput Neurol 516:166–186. https://doi.org/10.1002/cne.22102
Abellán A, Desflis E, Medina L (2014) Combinatorial expression of Lef1, Lhx2, Lhx5, Lhx9, Lmo3, Lmo4 and Prox1 helps to identify comparable subdivisions in the developing hippocampal formation of mouse and chicken. Front Neuroanat 8:59. https://doi.org/10.3389/fnana.2014.00059
Acampora D, Postiglione MP, Avantaggiato V, Di Bonito M, Simone A (2000) The role of Otx and Otp genes in brain development. Int J Dev Biol 44:669–677
Alheid GF, de Olmos J, Beltramino CA (1995) Amygdala and extended amygdala. In: Paxinos G (ed) The rat nervous system. Academic Press, San Diego, pp 495–578
Alonso A, Trujillo CM, Puelles L (2020a) Longitudinal developmental analysis of prethalamic eminence derivatives in the chick by mapping of Tbr1 in situ expression. Brain Struct Funct 225:481–510. https://doi.org/10.1007/s00429-019-02015-3
Alonso A, Carmen María Trujillo CM, Puelles L. (2020b) Experimental analysis of neuronal tangential migrations exiting from the prethalamic eminence in chick embryos. Brain Struct Funct (submitted).
Altmann J, Bayer SA (1995) Atlas of prenatal rat brain development. CRC Press, Boca Raton
Alvarez-Bolado G, Swanson LW (1996) Developmental brain maps: structure of the embryonic rat brain. Elsevier, Amsterdam
Amaral DG, Bauman MD, Schumann CM (2003) The amygdala and autism: implications from nonhuman primate studies. Genes Brain Behav 2:295–302
Ashwell KWS, Paxinos G (2008) Atlas of the developing rat nervous system 3rd edit. Academic Press, Amsterdam
Balthasar N, Dalgaard LT, Lee CE, Yu J, Funahashi H, Williams T, Ferreira M, Tang V, McGovern RA, Kenny CD, Christiansen LM, Edelstein E, Choi B, Boss O, Aschkenasi C, Zhang CY, Mountjoy K, Kishi T, Elmqquist JK, Lowell BB (2005) Divergence of melanocortin pathways in the control of food intake and energy expenditure. Cell 123:493–505
Loo YT (1930) The forebrain of the opossum, Didelphis virginiana. Parts 1–2 J Comput Neur 51:13–64/52:1–148.
Mangale VS, Hiroko KE, Satyaki PRV, Gokulchandran N, Chikibire S, Subramanian L, Shetty AS, Martynoga B, Paul J, Mark V, Mai MV, Li Y, Flanagan LA, Tole S, Monuki ES (2008) Lhx2 selector activity specifies cortical identity and suppresses hippocampal organizer fate. Science 319:304–309
Marin O, Rubenstein JL (2001) A long, remarkable journey: tangential migration in the telencephalon. Nat Rev Neurosci 2:780–790
Marion JF, Yang C, Caqueret A, Boucher F, Michaud JL (2005) Sim1 and Sim2 are required for the correct targeting of mammillary body axons. Development 132:5527–5537. https://doi.org/10.1242/dev.02142
Martínez-García F, Novejarque A, Gutiérrez-Castellanos N, Lanuza N, Nieuwenhuys R, Puelles L (2016) Towards a new neuromorphology. Academic Press, Oxford, pp 313–392
Martínez-García F, Novejarque A, Gutiérrez-Castellanos N, Lanuza E (2012) Piriform cortex and amygdala. In: Watson C, Paxinos G, Puelles L (eds) The mouse nervous system. Academic Press, San Diego, pp 140–172
Martínez-Marcos A, Halpern M (2006) Efferent connections of the main olfactory bulb in the opossum (Monodelphis domestica): a characterization of the olfactory entorhinal cortex in a marsupial. Neurosci Lett 395:51–56. https://doi.org/10.1016/j.neulet.2005.10.052
Medina L, Abellán A (2012) Subpallial structures. In: Watson C, Paxinos G, Puelles L (eds) The mouse nervous system. Academic Press, Amsterdam, pp 173–220
Medina L, Legaz I, González G, de Castro F, Rubenstein JLR, Puelles L (2004) Expression of Dbx1, neurogenin 2, semaphorin 5A, caderhin 8, and emx1 distinguish ventral and lateral pallial histogenetic divisions in the developing claustrum-amygdaled complex. J Comput Neurol 474:504–523
Medina L, Abellán A, Vicario A, Castro-Robles B, Desfils E (2017) The Amygdala. In: Kaas J (ed) Evolutionary neuroscience. Academic Press, Oxford, pp 313–392
Michaud JL, Legaz I, González G, de Castro F, Rubenstein JLR, Puelles L (2004) Expression of Dbx1, neurogenin 2, semaphorin 5A, caderhin 8, and emx1 distinguish ventral and lateral pallial histogenetic divisions in the developing claustrum-amygdaled complex. J Comput Neurol 474:504–523
Michaud JL, Abellán A, Vicario A, Castro-Robles B, Desfils E (2017) The Amygdala. In: Kaas J (ed) Evolution of nervous systems, vol 1, 2nd edn. Elsevier, Oxford, pp 427–478
Michaud JL, Rosenquist T, May NR, Fan CM (1998) Development of neuroendocrine lineages requires the bHLH-PAS transcription factor SIM1. Genes Dev 12:3264–3275
Michaud JL, Boucher F, Melnyk A, Gauthier F, Goshu E, Lévy E, Legaz I, González G, Puelles L, Rubenstein JL (2014) Transcriptional regulation of enhancers active in protodomains of the developing cerebral cortex. Neuron 82:989–1003. https://doi.org/10.1016/j.neuron.2014.04.014
Paxinos G, Franklin KBJ (2008) The mouse brain in stereotaxic coordinates, 4th edn. Academic Press/Elsevier, Amsterdam
Paxinos G, Halliday G, Watson C, Koutcherov Y, Wang H (2007) Atlas of the developing mouse brain at E17.5, P0, and P6. Academic Press/Elsevier, London
Phelps EA, LeDoux JE (2005) Contributions of the amygdala to emotion processing: from animal models to human behavior. Neuron 48:175–187
Price JL, Russchen FT, Amaral DG (1987) The limbic region. II. The amygdaloid complex. In: Björklund A, Hökfelt T, Swanson LW (eds) Handbook of chemical neuroanatomy: integrated systems of the CNS, vol 5, Part 1. Elsevier, Amsterdam, pp 279–388
Pro-Sistiaga P, Mohedano-Moriano A, Ubeda-Batlon I, Del Mar A-J, Marcos P, Artacho-Pérula E, Crespo C, Insausti R, Martínez-Marcos A (2007) Convergence of olfactory and vomeronasal projections in the rat basal telencephalon. J Comput Neurol 504:346–362
Puelles L (2001) Thoughts on the development, structure and evolution of the mammalian and avian telencephalic pallium. Philos Trans R Soc Lond B Biol Sci 356:1583–1598. https://doi.org/10.1098/rstb.2001.0973
Puelles L (2011) Pallio-pallial tangential migrations and growth signaling: new scenario for cortical evolution? Brain Behav Evol 78:108–127. https://doi.org/10.1159/000327905
Puelles L (2013) Plan of the developing vertebrate nervous system relating embryology to the adult nervous system (prosomere model, overview of brain organization). In: Rubenstein JLR, Rakic P (eds) Comprehensive developmental neuroscience: patterning and cell type specification in the developing CNS and PNS. Academic Press, Amsterdam, pp 187–209
Puelles L (2014) Development and evolution of the claustrum. In: Smyrnis J, Ramachandran VS, Edelstein L (eds) Functional neuroanatomy of the claustrum. Academic Press, New York, pp 119–176
Puelles L (2017) Comments on the updated tetrapartite pallium model in the mouse and chick, featuring a homologous claustrum-amygdala complex. J Comput Neurol. https://doi.org/10.1101/2020.07.17.207936
Puelles L (2019) Survey of midbrain, diencephalon, and hypothalamus. In: Rubenstein JLR, Rakic P (eds) The mouse nervous system, 16-020-1993-6
Puelles L (2011) Pallial-pallial tangential migrations and growth signaling: new scenario for cortical evolution? Brain Behav Evol 78:108–127. https://doi.org/10.1159/000327905
Puelles L (2019) Survey of midbrain, diencephalon, and hypothalamus neuroanatomic terms whose prosomeric definition conflicts with columnar tradition. Front Neuroanat 13:20. https://doi.org/10.3389/fnana.2019.00020
Puelles L, Rubenstein JLR (1993) Expression patterns of homeobox genes in the embryonic mouse forebrain suggest a neuromeric organization. Trends Neurosci 16:472–479
Puelles L, Rubenstein JLR (2003) Forebrain gene expression domains and the evolving prosomeric model. Trends Neurosci 26:469–476
Puelles L, Rubenstein JLR (2015) A new scenario of hypothalamic neuroanatomy: rationale of new hypotheses introduced in the updated prosomeric model. Front Neuroanat Front Neuroanat 9:27. https://doi.org/10.3389/fnana.2015.00027
Puelles L, Kuwana E, Puelles E, Kelleher J, Bulfone A, Rubenstein JLR (2000) Pallial and subpallial derivatives in the chick and mouse
Puelles L, Martínez-de-la-Torre M, Pachinós G, Watson C, Martínez S (2019) Gene maps and related histogenetic domains in the forebrain and midbrain. In: Pachinós G (ed) The rat nervous system, 4th edn. Academic Press/Elsevier, San Diego, pp 3–43

Puelles L, Morales-Delgado N, Merchán P, Castro-Robles B, Martínez-de-la-Torre M, Díaz C, Ferrán JL, Martínez-de-la-Torre M (2016a) Selective early expression of the orphan nuclear receptor Nr4a2 identifies the claustrum homolog in the mouse diagonal area. Brain Struct Funct 221:3027–3065. https://doi.org/10.1007/s00429-015-1086-8

Puelles L, Medina L, Borello U, Legaz I, Pierani A, Rubenstein JLR (2016c) Mouse ventral pallium derivatives traced to olfactory cortical and amygdaloid areas with Dbx1-LacZ reporters. J Chem Neuroanat 75:2–19

Puelles L, Ayad A, Alonso A, del Corral R, Ferrán JL, Martínez-de-la-Torre M (2017) The pallium in reptiles and birds in the light of the updated tetrapartate pallium model. In: Kaas J (ed) Evolution of nervous systems, vol 1, 2nd edn. Elsevier, Oxford, pp 519–555

Puelles L, Alonso A, García-Calero E, Martínez-de-la-Torre M (2019) Concentric ring topology of mammalian cortical sectors and relevance for pattering studies. J Comput Neurol 527:1731–1752. https://doi.org/10.1002/cne.24650

Puelles L, Martínez-de-la-Torre M, Martínez S, Watson C, Pachinós G (2019) The chick brain in stereotaxic coordinates: an atlas featuring neureomorphic subdivisions and mammalian homologies, 2nd edn. Academic Press, San Diego

Puelles L, Díaz C, Stühmer T, Ferrán JL, Martínez-de-la Torre M, Rubenstein JLR (2020) LacZ-reporter mapping of Dlx5/6 expression and genoarchitectural analysis of the postnatal mouse prethalamus. J Comput Neurol. https://doi.org/10.1002/cne.24952

Remedios R, Subramanian L, Tole S (2004) LIM genes parcellate the telencephalon, traced by the embryonic expression profiles of the genes Dlx-2, Emx-1, Nkx-2.1, Pax-6 and Tbr-1. J Comput Neurol 424:409–438

Ruiz-Reig N, Studer M (2017) Rostro-caudal and caudo-rostral migrations in the telencephalon: going forward or backward? Front Neurosci 11:692. https://doi.org/10.3389/fnins

Muñoz-Dorado J, del Río A (2020) The pallium and limbic cortex: a general overview. In: Swanson LW, Cowan WM (eds) The rat brain in stereotaxic coordinates, 8th edn. Elsevier, Oxford, pp 179–227

Thompson KL, Ghersi E, Calvo DM, Eichenbaum H, Moscovitch M, Buckner RL (2011) The hippocampus and the process of memory consolidation. Nat Rev Neurosci 12:429–439. https://doi.org/10.1038/nrn3030
of several nuclei of the amygdaloid complex. J Neurosci 25:2753–2760
Tolson KP, Gemelli T, Gautron L, Elmquist JK, Zinn AR, Kullaoui BM (2010) Postnatal Sim1 deficiency causes hyperphagic obesity and reduced Mc4r and oxytocin expression. J Neurosci 30:3803–3812. https://doi.org/10.1523/JNEUROSCI.5444-09.2010
Tosches MA, Yamawaki TM, Naumann RK, Jacobi AA, Tushev G, Laurent G (2018) Evolution of pallium, hippocampus, and cortical cell types revealed by single-cell transcriptomics in reptiles. Science 360:881–888. https://doi.org/10.1126/science.aar4237
von Economo C (1927) Cellular structure of the human cerebral cortex, 2009 transl/ed. LC Thriarhou. Karger, Basel
Wang W, Lufkin T (2000) The murine Otp homeobox gene plays an essential role in the specification of neuronal cell lineages in the developing hypothalamus. Dev Biol 227:432–449
Weiskrantz L (1956) Behavioral changes associated with ablation of the amygdaloid complex in monkeys. J Comput Physiol Psychol 49:381–391
Whalen PJ, Phelps EA (2009) The human amygdala. The Guilford Press, New York
Witter M (2012) Hippocampus. In: Watson C, Paxinos G, Puelles L (eds) The mouse nervous system. Academic Press, Amsterdam, pp 112–139

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