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Multiple origins of Cajal-Retzius cells at the borders of the developing pallium

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Cajal-Retzius cells are critical in cortical lamination, but very little is known about their origin and development. The homeodomain transcription factor Dbx1 is expressed in restricted progenitor domains of the developing pallium: the ventral pallium (VP) and the septum. Using genetic tracing and ablation experiments in mice, we show that two subpopulations of Reelin$^+$ Cajal-Retzius cells are generated from Dbx1-expressing progenitors. VP- and septum-derived Reelin$^+$ neurons differ in their onset of appearance, migration routes, destination and expression of molecular markers. Together with reported data supporting the generation of Reelin$^+$ cells in the cortical hem, our results show that Cajal-Retzius cells are generated at least at three focal sites at the borders of the developing pallium and are redistributed by tangential migration. Our data also strongly suggest that distinct Cajal-Retzius subtypes exist and that their presence in different territories of the developing cortex might contribute to region-specific properties.

The cerebral cortex has a laminar organization in which earlier- and later-born neurons accumulate according to an inside-out gradient. Until recently, it was thought that neuronal classes were produced by the local pallium ventricular zone and reached their final laminar destination by means of radial glia–mediated migration[1,2]. This model has now been complemented by evidence supporting the existence of tangential migration from the subpallium to the cerebral cortex[3].

Retzius (1893) and Cajal (1899) described cells with a complex morphology located in the marginal zone of humans at the time of cortical lamination, and now named Cajal-Retzius cells. Similar cells with simpler morphology have been described in the marginal zone of rodents[4]. The number of Cajal-Retzius cells seems to decrease after the completion of cortical lamination[1,5]. The most well documented function of Cajal-Retzius cells is to control the formation of cortical layers by means of the expression of the extracellular glycoprotein Reelin[3,6,7]. Additional functions for Cajal-Retzius cells have been proposed as in the regulation of the radial glia phenotype[8] and in the development of hippocampal connections[9].

Until now, the term 'Cajal-Retzius cells' has been used to identify a heterogeneous population of morphologically and molecularly distinct cell types in the marginal zone/layer I of different species and at different times during embryogenesis and postnatal life[4,5,10,11]. However, the lineage relationship between these cell types is unresolved. The consensus emerging from recent reports is that Cajal-Retzius cells are glutamatergic and express pallial markers[11]. Their pallial origin has been demonstrated by genetic tracing using an *Emx1-Cre* mouse line[12], as *Emx-1* is expressed exclusively in the pallium. They seem to be born between embryonic day (E) 10.5 and E12.5 in mice and are thus among the first neurons to be generated in the developing cortex. In addition, it is now agreed that Reelin in the marginal zone is a marker of Cajal-Retzius cells in the embryonic cortex of several species.

Despite many years of study, the origins and molecular properties of Cajal-Retzius cells are still unresolved. Cajal-Retzius cells were thought to be produced by the local pallium ventricular zone[1] and thus throughout the neocortical neuroepithelium[11,13], but focal pallial and subpallial sources for Cajal-Retzius cells have also been proposed: the retrobulbar area[14], the olfactory primordium[5], the cortical hem[13,15] and the medial ganglionic eminence[16]. Evidence supporting a cortical hem source has come from studies of the IG17 transgenic mouse using *in utero* electroporation[17]. However, several sources of Reelin$^+$ cells in the developing marginal zone remain hypothetical, and the roles of putative subpopulations are largely unknown. The investigation of these questions has been limited so far because of the lack of molecular markers available to identify subclasses of Cajal-Retzius cells and to trace them from their site of origin to the time of neural network formation in the postnatal cortex.

Cell fate allocation and cell diversity are determined at very early stages in progenitor cells at precise coordinates along the dorsoventral and anteroposterior axis[18–20]. The Dbx1 homeodomain transcription factor is expressed in progenitors at the boundary between the dorsal and ventral plates[21–23] of the caudal neural tube, from

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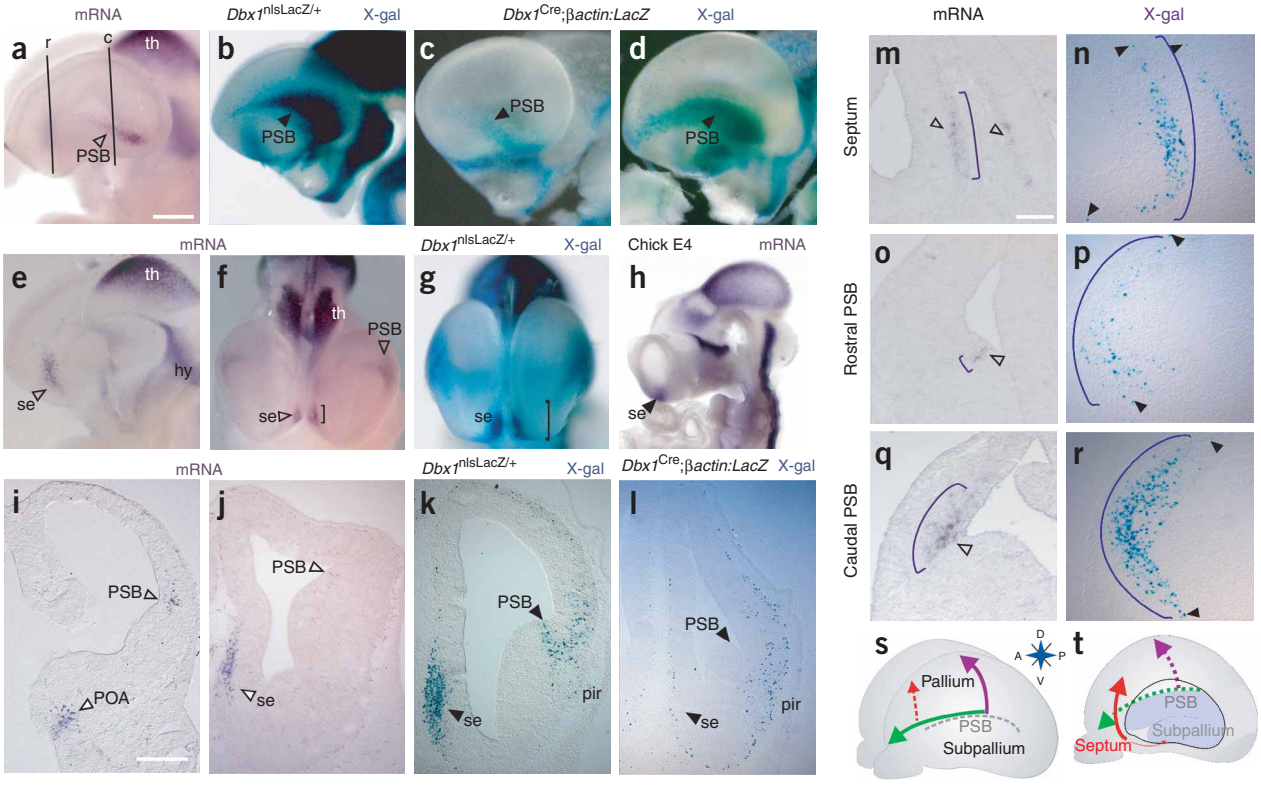

Figure 1 *Dbx1* expression at dorsoventral boundaries in the telencephalon: septum and ventral pallium. (**a**,**e**,**f**) Whole-mount *in situ* hybridization of *Dbx1* mRNA in E11.5 wild-type mouse embryos. (**b**,**c**,**d**,**g**) Whole-mount X-gal staining in E11.5 *Dbx1*$^{nlslacZ/+}$ (**b**,**g**), E11.5 *Dbx1*$^{Cre/+}$;β*actin:lacZ* (**c**) and E12.5 *Dbx1*$^{Cre/+}$;β*actin:lacZ* (**d**) embryos. (**h**) *Dbx1* mRNA is detected only in the septum but not at the PSB in E4 chick embryos. **a**–**d** show lateral views of dissected forebrains; **e**, medial view of a sagittally hemisected brain; **f** and **g**, dorsal views. Black lines in **a** correspond to rostral (r) and caudal (c) sections shown in **m**–**r**. Brackets in **f** and **g** show rostrocaudal extension of mRNA and X-gal detection, respectively. In **a**–**e** and **h**, rostral is at the left; in **f**,**g**, rostral is at the bottom. (**i**,**j**) *In situ* hybridization of *Dbx1* mRNA on cryostat sections of E11.5 caudal (**i**) and E12.5 rostral (**j**) wild-type telencephalons. (**k**,**l**) X-gal staining on cryostat sections of E12.5 *Dbx1*$^{nlslacZ/+}$ (**k**) and *Dbx1*$^{Cre/+}$;β*actin:lacZ* (**l**) telencephalon at comparable rostral levels as **j**. In **i**–**l**, dorsal is at the top. Se: septum; hy: hypothalamus; th: thalamus. (**m**–**r**) Comparison of *Dbx1* mRNA *in situ* hybridization (**m**,**o**,**q**) and X-gal staining (**n**,**p**,**r**) in E11.5 *Dbx1*$^{nlslacZ/+}$ embryos (serial sections). Brackets mark dorsoventral extent of mRNA and X-gal detection. Open arrowheads: sites of *Dbx1* expression; filled arrowheads: β-gal$^+$ cells located at the farthest distance from sites of *Dbx1* expression. Scale bar: 1 mm (**a**–**g**), 50 μm (**m**–**r**), 300 μm (**i**–**l**). (**s**,**t**) Putative migration routes of Dbx1-derived β-gal$^+$ cells.

which postmitotic cells migrate tangentially to their final destination. In the spinal cord, the spatially restricted expression of Dbx1 in progenitors is critical in establishing the distinction between Evx1/2 (V0) and En1 (V1) interneuron cell fates and helps to coordinate diverse phenotypic features[24,25]. In the telencephalon, Dbx1 is expressed in restricted progenitor domains at the borders of the developing pallium: the VP at the pallial-subpallial boundary (PSB), the septum and the preoptic area/anterior entopeduncular area (POA/AEP)[26,27].

In this study, we trace the fate of cells derived from *Dbx1* progenitors in the telencephalon from embryonic to postnatal stages using a genetic approach in mice. By combining genetic tracing and DiI labeling, we show that Dbx1-derived cells migrate from the septum to the medial and piriform cortex and from the PSB to the dorsolateral and piriform cortex. Cells derived from *Dbx1*$^+$ progenitors express Reelin and are Cajal-Retzius cells in the postnatal cortex. These Dbx1-derived Cajal-Retzius cells seem to have distinct origins, onsets of appearance and final destinations, and they differ in expression of calretinin. Our genetic approach proves the existence of two previously unknown sites of origin for Cajal-Retzius cells and suggests that distinct subpopulations of Cajal-Retzius cells are present in different territories of the developing cortex.

RESULTS

Dbx1 is expressed at border regions of the telencephalon

The expression of the *Dbx1* gene is restricted to a narrow domain at the dorsoventral boundary in the spinal cord and telencephalon[21–23,26,27]. We mapped the onset and location of *Dbx1* expression in the telencephalon of mouse embryos using *in situ* hybridization (**Fig. 1**). Expression began around E10.5 in the septum and POA/AEP. At E11.5 it reached its maximum in the septum, in the POA/AEP and near the PSB in the VP with a caudal^{high}rostral^{low} gradient (**Fig. 1a**,**e**,**f**,**i**,**j**)[26]. *Dbx1* expression was restricted to progenitor cells in the ventricular zone. *Dbx1* mRNA was detected on cells located close to the pial surface of the septum (**Fig. 1j**,**m**), whereas it is detected throughout the ventral pallium neuroepithelium (**Fig. 1i**,**o**,**q**). *Dbx1* transcripts and protein were never observed in the ventricular zone of the lateral and medial ganglionic eminences (LGE and MGE, respectively) or in the neuroepithelium of the dorsal and medial pallium (**Fig. 1i**,**j**)[26,27]. *Dbx1* expression progressively declined after E12.5 (ref. 27) but was still detectable during late embryogenesis (data not shown).

Genetic manipulation of the Dbx1 locus

As *Dbx1* expression was transitory and exclusively detected in the ventricular zone, we used a mouse genetic approach to label cells

derived from Dbx1-expressing progenitors. In the *Dbx1*^{nlslacZ} line the insertion of the *lacZ* gene into the *Dbx1* locus allows for short-term lineage tracing[24]. To permanently label Dbx1-derived cells, we generated a *Dbx1*^{Cre} knock-in mouse line, which we subsequently crossed to reporter strains. Animals obtained by crossing *Dbx1*^{Cre} and *βactin:loxP-stop-loxP-nlslacZ*[28] or *Tau*$^{loxP\text{-}stop\text{-}loxP\text{-}MARCKSeGFP\text{-}IRES\text{-}nlslacZ}$ reporter mice will be called *Dbx1*$^{Cre/+}$;β*actin:lacZ* and *Dbx1*$^{Cre/+}$;*Tau*^{GFP}, respectively. The use of these animals allowed us to label Dbx1-derived cells at various times after their generation (mitotic and early postmitotic in *Dbx1*$^{nlslacZ/+}$ versus early- and late-postmitotic in *Dbx1*$^{Cre/+}$;β*actin:lacZ* embryos). Representative images of cryostat sections from E12.5 *Dbx1*$^{nlslacZ/+}$ and *Dbx1*$^{Cre/+}$;β*actin:lacZ* embryos (**Fig. 1k**,**l**) show the subsequent labeling of *Dbx1*-expressing progenitors at the ventral pallium ventricular zone using a *Dbx1* mRNA *in situ* probe (**Fig. 1j**) and Dbx1-derived β-gal^{high} (early postmitotic) and β-gal^{low} (postmitotic) cells spanning from the ventricular zone to the mantle zone in *Dbx1*$^{nlslacZ/+}$ embryos at more ventral and dorsal positions (**Fig. 1k**). Finally, permanently labeled Dbx1-derived postmitotic cells and their arborizations were detected in the mantle zone of *Dbx1*$^{Cre/+}$;β*actin:lacZ* and *Dbx1*$^{Cre/+}$;*Tau*^{GFP} embryos (**Fig. 1l** and data not shown).

Dbx1-derived cells are highly motile

We compared *Dbx1* expression and β-gal activity at E11.5 in *Dbx1*$^{nlslacZ/+}$ and *Dbx1*$^{Cre/+}$;β*actin:lacZ* telencephalons using whole-mount *in toto* preparations. *Dbx1*$^{nlslacZ/+}$ embryos showed broader 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) activity with respect to the detection of the *Dbx1* mRNA along the rostrocaudal axis at the PSB and the septum, and along the dorsoventral axis at the PSB (compare **Fig. 1a**,**b** and **Fig. 1f**,**g**). Notably, *Dbx1*$^{Cre/+}$;β*actin:lacZ* telencephalons at E12.5 showed even more X-gal cells along the rostral PSB, suggesting that Dbx1-derived cells migrate rostrally along the PSB. In addition, scattered X-gal cells were observed in the dorsofrontal half of the developing cortex (**Fig. 1d**).

To better map the position of Dbx1-derived β-gal$^+$ cells with respect to the sites of *Dbx1* mRNA production, we analyzed serial sections of E11.5 *Dbx1*$^{nlslacZ/+}$ embryos for *Dbx1* transcripts by *in situ* hybridization and X-gal staining. At E11.5 in the telencephalon, β-gal$^+$ cells were located at a distance from *Dbx1*$^+$ cells, dorsally and ventrally to the rostral and caudal PSB ventricular zone and to the septum (compare **Fig. 1m** to **Fig. 1n**, **Fig. 1o** to **Fig. 1p** and **Fig. 1q** to **Fig. 1r**). β-gal$^+$ cells were observed in the forming preplate of the medial, dorsolateral and ventral pallium (**Supplementary Fig. 1**). β-gal$^+$ cells were located mostly dorsal to the PSB at rostral levels (**Fig. 1o**,**p**) and preferentially ventral to the PSB at more caudal levels (**Fig. 1q**,**r**). Between E11.5 and E12.5 Dbx1-derived β-gal$^+$ cells seemed to be streaming ventrally and dorsally (**Fig. 1k**), and many were located in the preplate/marginal zone of the piriform territory and of the medial and dorsal pallium. β-gal$^+$ nuclei in the marginal zone were oriented both parallel and perpendicular to the pial surface, and the number of nuclei and cell bodies oriented perpendicular to the surface increased with time (**Supplementary Fig. 1**). We believe that round nuclei perpendicular to the pial surface correspond to cells that have stopped migrating and have reached their final position. Thus, Dbx1 progenitors in the septum and VP generate mostly preplate/marginal zone cells between E10.5 and E12.5, and some Dbx1-derived cells seem to have already reached their final destination at E12.5.

In order to test the capacity of Dbx1-derived cells to migrate, explants containing Dbx1$^+$ progenitors were grown in a collagen matrix. Beginning at 1 d *in vitro* (DIV), β-gal$^+$ cells were observed within and at a distance from rostral PSB ($n = 6$) and caudal PSB ($n = 6$) explants of *Dbx1*$^{nlslacZ/+}$ E12.5 telencephalons (**Supplementary Fig. 1**). Similar results were obtained using septum and caudal PSB explants of E11.5 *Dbx1*^{Cre};β*actin:lacZ* embryos after 3 DIV (data not shown). Compared with interneuronal migration from MGE explants at E12.5, both speed and distance of Dbx1-derived β-gal$^+$ cells in PSB and septum explants was very similar (data not shown). Taken together, these results suggest that Dbx1-derived cells are highly motile and migrate from their sites of origin to populate different cortical regions.

Dbx1$^{Cre/+}$;*Tau*^{GFP} cells migrate both dorsally and ventrally

To study the routes of migration of Dbx1-derived β-gal$^+$ cells, DiI labeling experiments were performed on coronal slices of E12.0–E12.5 *Dbx1*$^{Cre/+}$;*Tau*^{GFP} telencephalons cultured *in vitro*. One DIV after the insertion of a crystal in the rostral septum ($n = 4$; **Fig. 2a**,**d–f**), DiI$^+$ cells were observed dorsally and ventrally in the marginal zone of the medial and ventral wall (**Fig. 2b**), respectively, and had traveled a long distance up to the dorsal cortex. Several DiI$^+$ cells were colabeled with GFP (**Fig. 2d–f**, white arrow) and had a morphology consistent with migrating cells. However, when DiI crystals were inserted at progressively more caudal levels of the medioventral wall, the extent of dorsal migration decreased, whereas that of ventral migration remained unchanged ($n = 14$, **Fig. 2c** and data not shown). DiI$^+$/GFP$^+$ cells were detected in a ventral stream up to the ventrolateral wall at the level of the piriform territory.

When crystals were inserted at the PSB ($n = 11$; **Fig. 2g–r**), DiI$^+$ cells were observed ventrally (**Fig. 2h**,**i**), as expected from previous reports[29–31]. We were surprised to find that DiI$^+$ cells were also detected dorsally in the intermediate zone and marginal zone of the dorsal pallium (**Fig. 2g**,**h**,**j–r**). The migration in the intermediate zone was more prominent in caudal sections. Colabeling of DiI and GFP was observed in cells in the marginal zone of the ventral pallium (**Fig. 2i**) and the dorsal pallium (**Fig. 2j**,**o**,**p**). DiI$^+$/GFP$^+$ cells were observed reaching as far dorsal as the prospective isocortex and as far ventral as the prospective piriform cortex. The extent of dorsal and ventral migration was quite similar at all caudorostral levels at this stage. DiI$^+$/GFP$^-$ cells were also present in the migrating stream (about 30–50% of the total DiI$^+$ cells were GFP$^-$). These results reflect either an incomplete expression of the IRES in Dbx1-derived cells of *Dbx1*$^{Cre/+}$ animals or that cells derived from progenitors other than Dbx1-expressing cells, most probably from the ganglionic eminences, can also migrate through the DiI-labeled area toward the cortex. Many GFP$^+$ cells are present at this stage in the basal telencephalon in caudal sections and are likely to have been derived from Dbx1 progenitors in the POA.

Thus, Dbx1-derived cells generated in the septum and the ventral pallium ventricular zone migrate in culture dorsally and ventrally along superficial routes of migration and reach the piriform cortex/isocortex and the medial wall/piriform cortex, respectively, within 24 h.

Early-born Dbx1-derived cells express Reelin

To begin investigating the identity of the cells derived from septum and PSB Dbx1$^+$ progenitors, we first analyzed the time and position of their first appearance in the telencephalon. The first β-gal$^+$ cells to be detected in *Dbx1*$^{nlslacZ/+}$ embryos were located in the area of the septum at E10.5 (**Fig. 3a** and data not shown). At this stage, very few β-gal$^+$ cells were present in proximity to the caudal PSB, whereas cells were detected between E11.0 and E11.5 at the rostral PSB (**Fig. 3a**,**b**,**i**,**n** and **Fig. 1m–r**). Dbx1-derived β-gal$^+$ cells were among the first postmitotic cells to be generated, as suggested by their superficial position and by the lack of BrdU colabeling upon injection of BrdU at E10.75 (**Fig. 3a**,**b**).

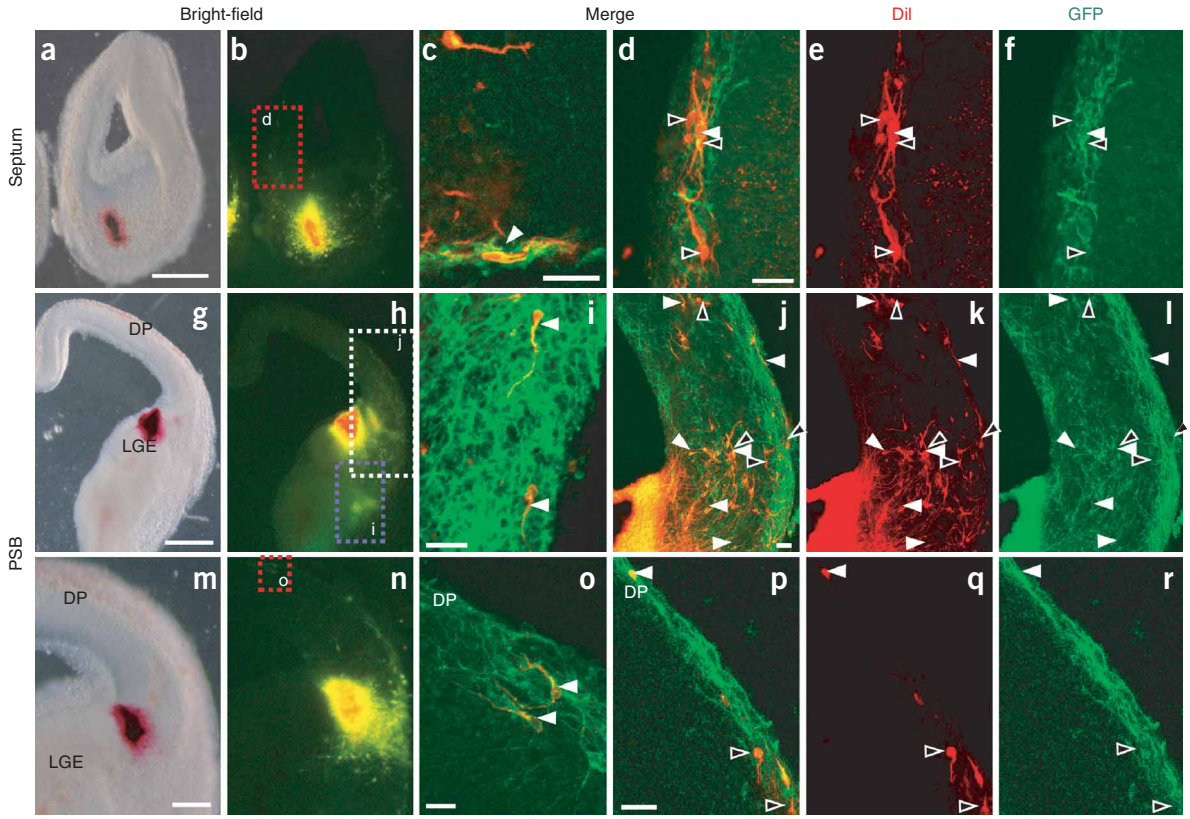

Figure 2 *Dbx1*^{Cre};*Tau*^{GFP} cells migrate dorsally and ventrally from the PSB and the septum *in vitro*. DiI$^+$/GFP$^+$ cells reach as far as the dorsal cortex in 24 h. (**a**,**g**,**m**) Bright-field images of DiI crystals inserted in the rostral septum (**a**) and PSB (**g**,**m**). (**b**,**h**,**n**) Dark-field images using a rhodamine filter of **a**,**g**,**m**, respectively. (**c**,**d**,**i**,**j**,**o**,**p**) Confocal images of double-labeled DiI and GFP cells. Areas enlarged in **d**,**i**,**j**,**o** are indicated by boxes in **b**,**h**,**n**. (**d**–**f**,**j**–**l**) **d**–**f** show medial wall dorsal to the septum; **c** shows basal wall of the telencephalon lateral to the septum; **h**,**j**–**l**,**m**–**r** show dorsal cortex and **i** ventral cortex after DiI insertion at the caudal PSB. (**e**,**k**,**q**) DiI single-label images. (**f**,**l**,**r**) Single-label GFP images. **c**,**d**,**i**,**j**,**o**,**p** show merged images (DiI and GFP). White arrowheads indicate DiI$^+$/GFP$^+$ double-labeled cells and black arrowheads DiI-only labeled cells. Scale bars = 400 μm (**a**,**b**,**g**,**h**), 100 μm (**m**,**n**), 40 μm (**c**,**d**,**i**,**j**), 20 μm (**o**,**p**).

As Reelin seems to be a specific marker for the early-born Cajal-Retzius neurons in the marginal zone[4], we tested whether Dbx1-derived cells express Reelin. The majority of Reelin$^+$ cells at E10.5 are concentrated in the septum and rostral pallium and few in the caudomedial hem (**Fig. 3d**). Very few, if any, scattered Reelin$^+$ cells are detected in the forming preplate in other telencephalic regions at this stage. Most (98%, $n = 153$) postmitotic preplate Dbx1-derived cells in *Dbx1*$^{nlslacZ/+}$ embryos expressed Reelin at E10.5–E11.0 (**Fig. 3a–c**). These results strongly suggest that Reelin$^+$ neurons derived from Dbx1 progenitors in the septum were born at E10.5 and are consistent with the reported generation of a vast proportion of Reelin$^+$ layer I neurons at this stage[11]. In addition, in *Dbx1*$^{nlslacZ/+}$ embryos at E11.5 and E12.5, β-gal$^+$/Reelin$^+$ cells were detected at progressively longer distances from the Dbx1 progenitor zones (septum and PSB) up to the medial pallium, dorsal pallium and piriform cortex (**Fig. 3e–s**). A large proportion of β-gal^{low} (differentiating Dbx1-derived) cells in the marginal zone of *Dbx1*$^{nlslacZ/+}$ embryos between E10.5 and E12.5 expressed Reelin (rostrally 82%, $n = 116$; caudally 67%, $n = 30$ at E11.5). Rostrally, Dbx1-derived Reelin$^+$ cells were scattered in the marginal zone around the whole telencephalic vesicles including the dorsal cortex (data not shown). Furthermore, at E11.0–E11.5 in the rostral half of the telencephalon, 72% of Reelin$^+$ cells ($n = 57$) in the piriform region, dorsolateral and medial pallium preplate and 50–60% of Reelin$^+$ cells ($n = 84$) in the superficial layer of the basolateral telencephalon express β-gal and thus derive from Dbx1 progenitors.

As β-gal expression is lost in late postmitotic cells in *Dbx1*$^{nlslacZ/+}$ animals, we analyzed *Dbx1*^{Cre} embryos to trace later derivatives of Dbx1 progenitors (**Fig. 3h**). In the marginal zone of E12.5 *Dbx1*$^{Cre/+}$; β*actin:lacZ* embryos, about 50–98% of β-gal$^+$ cells were Reelin$^+$, depending on the cortical or subcortical zones, the highest percentage being detected in the septum (98%) and in the rostral and caudal piriform cortex (80–85%). In addition, different proportions of Reelin$^+$ cells were β-gal$^+$ in distinct regions of the telencephalic vesicles. Rostrally and caudally, 90 to 43%, respectively, of Reelin$^+$ cells colabeled with β-gal in the piriform territory, 40–50% in the lateral cortex, 12–20% in the dorsal-most cortex (medial and lateral), 99% in the intermediate medial wall (dorsal to the septum) and around 50–78% in the basal telencephalon. Moreover, beginning at E12.5, not all β-gal$^+$ cells in the cortex expressed Reelin, and β-gal$^+$/Reelin$^-$ cells were also detected in the caudal intermediate zone. As we did not observe colabeling of GABA and β-gal in PSB explants of E12.5 *Dbx1*$^{nlslacZ/+}$ embryos kept in culture for 48 h (**Fig. 6m**), and no interneurons seem to be generated from the PSB until at least E12.5–E13.5, we believe that β-gal$^+$ cells in the caudal intermediate zone represent populations of interneurons derived from Dbx1 progenitors in the caudal/ventral septum or AEP/POA (data not shown). These data strongly suggest that between E10.5 and E12.5, Reelin$^+$ marginal zone neurons derived

from the septum and the PSB preferentially populate rostral and dorsolateral/piriform cortex, respectively. As Reelin$^+$ cells are homogeneously distributed around the telencephalic vesicles at E12.5, our results also suggest that caudodorsal and caudomedial cortical regions are populated by Reelin$^+$ cells derived from a Dbx1-independent caudal source, which is likely to be the cortical hem[17].

Dbx1-derived Reelin$^+$ cells are layer I Cajal-Retzius cells

As Cajal-Retzius cells are defined by morphological criteria in layer I of the developed cortex, we genetically traced the fate of early Dbx1-derived neurons in the late embryonic and postnatal cortex. β-gal$^+$ cells were observed in the marginal zone/layer I and the cortical plate (CP) of the piriform cortex and the isocortex and in the hippocampus at E17

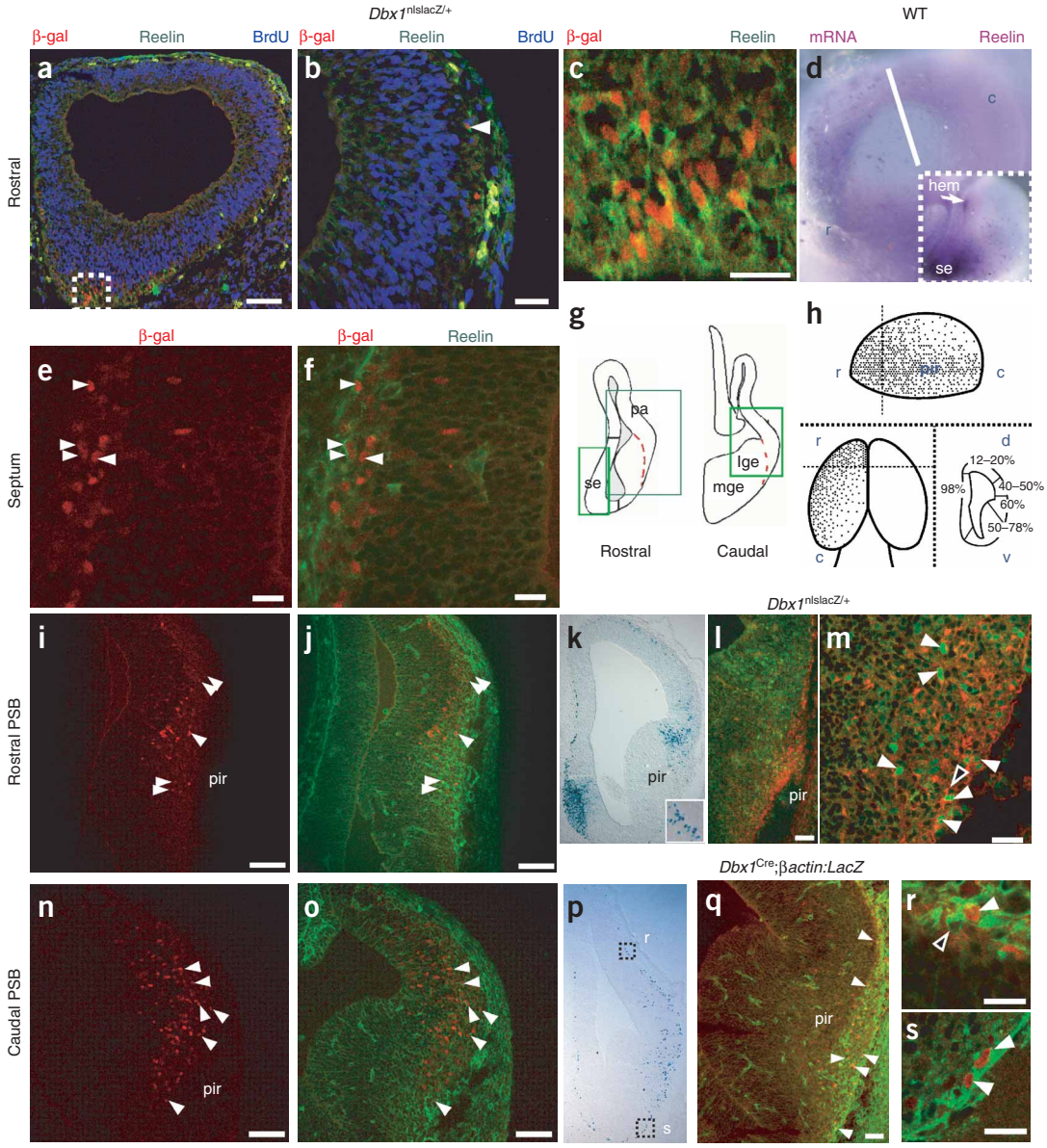

Figure 3 Early-born Dbx1-derived cells in the preplate/marginal zone express Reelin. (**a**,**b**) Immunohistochemistry on a rostral section at septum level of an E10.75 *Dbx1*$^{nlsLacZ/+}$ embryo labeled with BrdU 1 h before collection. **b** A higher-magnification view of **a** in PSB area. (**c**) High-magnification views of boxed area in **a**. (**d**) Whole-mount *in situ* hybridization of *Reelin* mRNA in E10.5 wild-type embryos; lateral view of telencephalic vesicle. Inset: frontal view of the right part of the head. (**e**–**j**,**n**–**o**) β-gal and Reelin staining in E11.5 *Dbx1*$^{nlsLacZ/+}$ telencephalon at the level of the septum (**e**,**f**) rostral PSB (**i**,**j**) and caudal PSB (**n**,**o**). **g** shows the rostrocaudal level and the areas of confocal images acquisition. **h** shows schematic representation of Dbx1-derived β-gal$^+$/Reelin$^+$ cells in different regions of E12.5 *Dbx1*$^{Cre/+}$;*βactin:lacZ* telencephalons (coronal section, lateral and dorsal views). (**k**) X-gal staining of an E12.5 *Dbx1*$^{nlsLacZ/+}$ telencephalon section at an intermediate level along the rostrocaudal axis. Inset: magnification of marginal zone in lateral cortex of an E14.5 *Dbx1*$^{Cre/+}$;*βactin:lacZ* embryo showing β-gal$^+$ nuclei parallel (migrating) and perpendicular (resident) to the pial surface. (**l**,**m**) β-gal (green) and Reelin (red) staining in E12.5 *Dbx1*$^{nlsLacZ/+}$ telencephalon sections at the same level as **k**. **m** shows high-magnification view of the piriform cortex. (**p**) X-gal staining of an E12.5 *Dbx1*$^{Cre/+}$;*βactin:lacZ* section at the same level as **k**. (**q**–**s**) β-gal (red) and Reelin (green) staining in E12.5 *Dbx1*$^{Cre/+}$;*βactin:lacZ* telencephalon sections at the same level as **p**. **r**,**s** are high-magnification images of areas indicated in **p**. White arrowheads: double-labeled β-gal$^+$/Reelin$^+$ cells. Black arrowheads: Reelin$^+$-only cells. Immunohistochemical images were acquired with a confocal microscope. r: rostral; c: caudal; d: dorsal; v: ventral; se: septum; pir: piriform territory; pa: pallium. Scale bars = 100 μm (**a**,**i**,**j**,**l**,**n**,**o**,**q**), 40 μm (**b**), 20 μm (**c**,**e**,**f**,**m**,**r**,**s**).

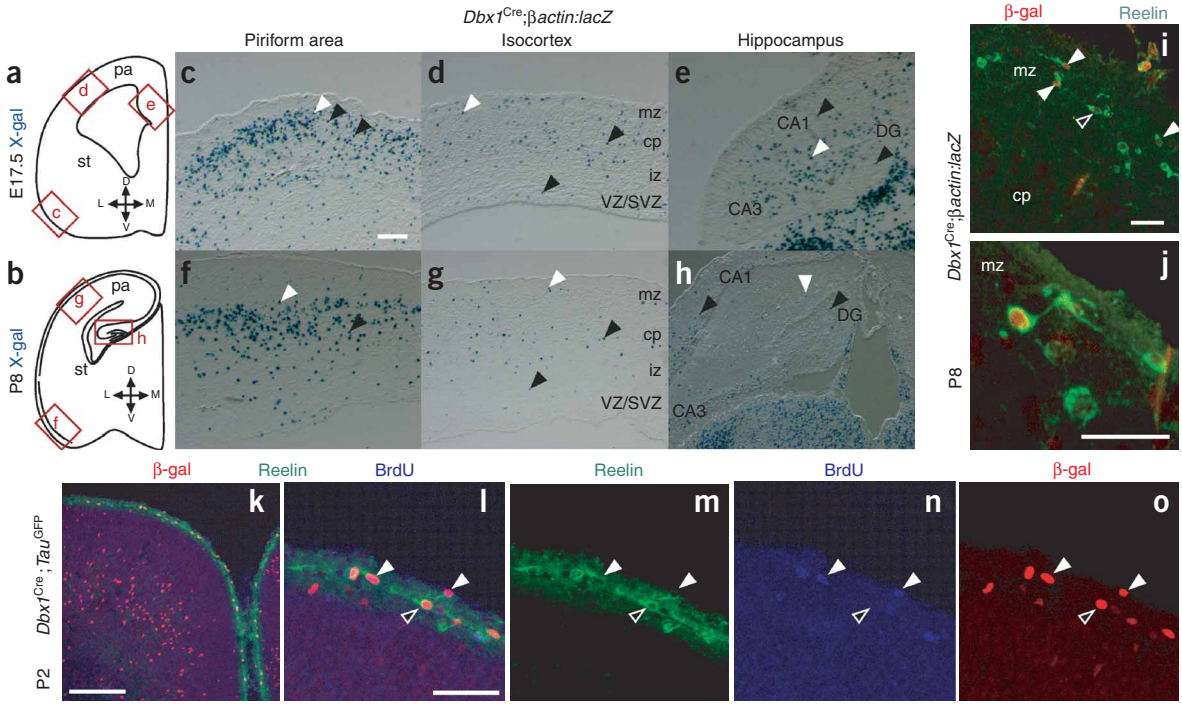

Figure 4 Populations of Cajal-Retzius neurons in layer I are derived from Dbx1$^+$ progenitors. (**a**,**b**) Location of areas enlarged in **c**–**h**. (**c**–**e**) X-gal staining on sections from the telencephalon of *Dbx1*$^{Cre/+}$;β*actin:lacZ* E17.5 embryos. (**f**–**h**) Same view as **c**–**e** in P8 mice. White arrowheads: β-gal$^+$ cells in the marginal zone. Black arrowheads: β-gal$^+$ cells in other cortical layers. (**d**,**g**) β-gal$^+$ cells (of which some were Reelin$^+$; data not shown) were also located in the other layers (ventricular zone (VZ)/subventricular zone (SVZ) and cortical plate (CP) of the isocortex of P8 *Dbx1*$^{Cre/+}$;β*actin:lacZ* animals. (**i**,**j**) Double labeling with Reelin and β-gal in *Dbx1*$^{Cre/+}$;β*actin:lacZ* P8 animals. CA1, CA3: Ammon's horn field 1 and 3; DG: dentate gyrus; pa: pallium; st: striatum; mz: marginal zone; cp: cortical plate; iz: intermediate zone; D, dorsal; L, lateral; M, medial; V, ventral. (**k**–**o**) Triple labeling with β-gal, Reelin and BrdU on cryostat sections of P2 *Dbx1*$^{Cre/+}$;*Tau*^{GFP} cingulate cortex after BrdU injection at E10.5 during gestation. **m**–**o** show single confocal images corresponding to merge in **l**: Reelin (**m**), BrdU (**n**) and β-gal (**o**). **l**–**o** show high-magnification view of layer 1 in **k**. Scale bars = 40 μm (**i**,**j**,**l**–**o**); 200 μm (**c**–**h**,**k**).

(**Fig. 4a**,**c**–**e**) and P8 (**Fig. 4b**,**f**–**h**) in *Dbx1*$^{Cre/+}$;β*actin:lacZ* animals. In order to determine the birthdates of Dbx1-derived cells present in the postnatal cortex, we analyzed β-gal$^+$ cells in *Dbx1*$^{Cre/+}$;*Tau*$^{GFP\text{-}IRESnlslacZ}$ animals at P2 after a single BrdU injection at E10.5 of gestation. Dbx1-derived β-gal$^+$ cells were observed as in P8 animals in the marginal zone/layer I and the CP (**Fig. 4k**). However, only β-gal$^+$ cells in the marginal zone/layer I coexpressed Reelin (**Fig. 4k**,**l**). In addition, cells in the marginal zone/layer I exclusively were labeled with BrdU, and some of the BrdU$^+$ cells in the marginal zone/layer I were βgal$^+$ and Reelin$^+$ (**Fig. 4l–o**). We conclude that β-gal$^+$/Reelin$^+$ cells present in the marginal zone/layer I were born at least in part at E10.5; this is consistent with the early birth date of Cajal-Retzius cells. β-gal$^+$ cells (of which some were Reelin$^+$) were also located in the other layers (ventricular zone (VZ)/subventricular zone (SVZ) and CP) of the isocortex of P8 *Dbx1*$^{cre/+}$;β*actin:lacZ* animals (**Fig. 4d**,**g** and data not shown). As Reelin$^+$ interneurons have been described in the postnatal cortical plate starting at P5 (refs. 10,11), β-gal$^+$/Reelin$^+$ cells in the CP at P8 are probably later-born Dbx1-derived interneurons. Moreover, β-gal$^+$ cells seemed to start decreasing in number in the isocortex and hippocampus at P8, consistent with the reported progressive disappearance of Cajal-Retzius cells from the marginal zone/layer I after P7 (**Fig. 4d**,**g**,**e**,**h**). Finally, some β-gal$^+$/Reelin$^+$ cells in the marginal zone/layer I of *Dbx1*$^{Cre/+}$;β*actin:lacZ* animals at P8 had the typical morphology, position and orientation of Cajal-Retzius neurons: at this stage their cell body had reached a final depth of about 20–30 μm, but they still had ascending branchlets that contacted the pial membrane (**Fig. 4i**,**j**).

We conclude that Dbx1$^+$ progenitors at the septum and PSB give rise to Reelin$^+$ *bona fide* postnatal Cajal-Retzius neurons on the basis of the following evidence: (i) the Reelin immunoreactivity of Dbx1-derived cells between E10.5 and E12.5 in the pallial preplate/marginal zone, (ii) the localization of these cells in the marginal zone/layer I of the isocortex, piriform and hippocampus at P8, (iii) their birth date at E10.5 in BrdU-injected P2 animals and (iv) their morphology.

Loss of Reelin$^+$ cells upon ablation of *Dbx1*-expressing cells

In order to analyze the effect of eliminating Dbx1 progenitors on Reelin$^+$ cell development, we inserted an *IRES-loxP-stop-pGKneo-loxP-DTA* (diphtheria toxin) cassette into the Dbx1 locus by homologous recombination (*Dbx1*$^{loxP\text{-}stop\text{-}loxP\text{-}DTA}$). A functional DTA is expressed exclusively upon Cre-mediated recombination. Mutant animals were crossed with a *Nes:Cre* mouse line which expresses the Cre recombinase ubiquitously in the neuroepithelium starting around E11.0 (ref. 32), allowing spatially and temporally restricted expression of the toxin.

Because of the multiple origins of Reelin$^+$ cells, we first analyzed *Reelin* mRNA expression using whole-mount *in situ* hybridization. Although Reelin is widely expressed throughout the telencephalic vesicles at E11.5, some areas seemed to be more intensively stained, namely the septum and the piriform territory (**Fig. 5b**,**c**). These same regions show stronger X-gal activity in E11.5 *Dbx1*$^{Cre/+}$;β*actin:lacZ* (**Fig. 5a**) and thus seem to correspond to areas where two of the migratory routes of Dbx1-derived cells are located. We observed a strong decrease in the number of Reelin-expressing cells in the most rostral and caudal piriform cortex in recombined *Dbx1*^{DTA};*Nes:Cre*

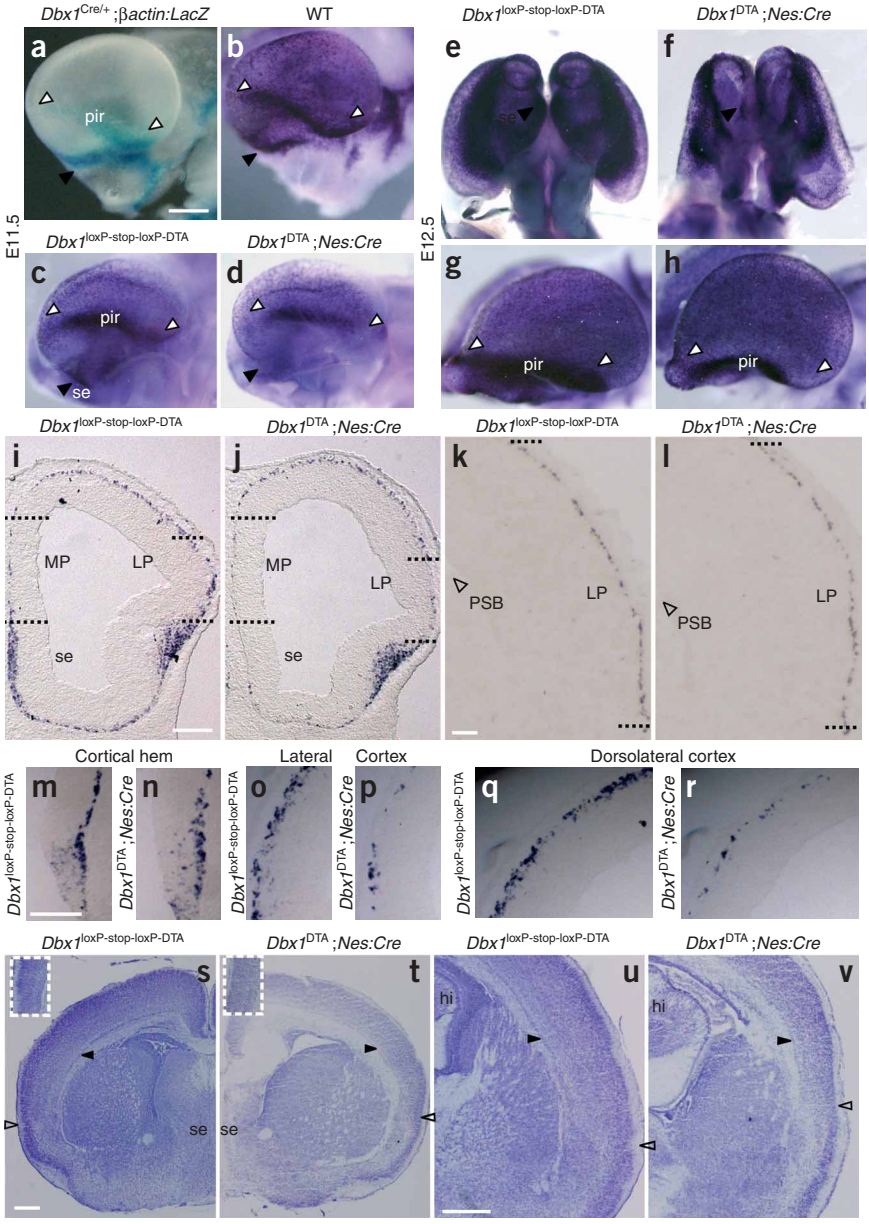

Figure 5 Ablation of Dbx1-derived cells results in loss of Reelin$^+$ cells in different cortical regions and in cortical defects. (**a**) Whole-mount X-gal staining (blue) in E11.5 *Dbx1*$^{Cre/+}$;β*actin:lacZ* telencephalon. (**b**–**h**) Whole-mount *in situ* hybridization with a *Reelin* mRNA probe (purple) in E11.5 wild-type (*Dbx1*$^{LoxP\text{-}Stop\text{-}LoxP\text{-}DTA}$; **b**,**c**), E11.5 *Dbx1*^{DTA};*Nes:Cre* (**d**), E12.5 wild-type (**e**,**g**) and E12.5 *Dbx1*^{DTA};*Nes:Cre* (**f**,**h**) embryos. White arrowheads: rostral and caudal piriform areas. Black arrowheads: septum. (**i**,**j**,**m**–**r**) *In situ* hybridization with a *Reelin* mRNA probe on sections of E12.5 wild-type (**i**,**m**,**o**,**q**) and *Dbx1*^{DTA};*Nes:Cre* (**j**,**n**,**p**,**r**) embryos at rostral (**i**,**j**) and caudal (**m**–**r**) levels. **m**–**r** show high-magnification views of different telencephalic regions. (**k**,**l**) *In situ* hybridization with a *Reelin* mRNA probe on rostral sections of E14.5 wild-type (**k**) and *Dbx1*^{DTA};*Nes:Cre* (**l**) embryos at level equivalent to **i**,**j**. High-magnification view of the lateral pallium. LP: lateral pallium; MP: medial pallium; se: septum; pir: piriform cortex. (**s**–**v**) Cresyl violet staining of P0 wild-type (**s**,**u**) and *Dbx1*^{DTA};*Nes:Cre* (**t**,**v**) telencephalons at rostral (**s**,**t**) and caudal levels (**u**,**v**) showing differences in cortical cytoarchitecture. Insets in **s**,**t**: high-magnification view of the cingulate cortex. White arrowheads: position of rhinal fissure. Black arrowheads: position of Layer VIb. hi: hippocampus. Scale bars = 1 mm (**a**–**h**), 500 μm (**i**,**j**,**s**–**v**), 50 μm (**k**,**l**–**r**).

embryos at E11.5 (**Fig. 5d**, white arrowhead) as well as an almost complete loss in the septum (**Fig. 5d**, black arrowheads). At E12.5, we detected a strong loss of Reelin staining in the rostral piriform, caudal piriform/amygdaloid complex and septum as well as a significant reduction in the 'intermediate rostrocaudal' piriform cortex (**Fig. 5e**–**h**). An overall reduction of the rostrocaudal axis of the telencephalic vesicles and of the olfactory bulb was also observed. Using a TUNEL reaction we showed that Dbx1-derived cells start to die around E11.0 in the septum and at E11.5 at the PSB (**Supplementary Fig. 2**). Therefore, it is possible that some early Dbx1-derived cells are spared from cell death and thus account for some of the Reelin$^+$ neurons still present in the piriform cortex and in the most rostrodorsal cortex. Notably, a strong reduction of Reelin expression was detected in the septum as well as in the dorsolateral, medial and caudal piriform cortex in E12.5 *Dbx1*^{DTA};*Nes:Cre* embryo sections (**Fig. 5i**,**j**,**o**–**r** and data not shown). No differences were observed between wild-type mice and mutants in the cortical hem (**Fig. 5m**,**n**). In contrast, when Reelin expression was analyzed at E14.5, no significant differences were observed between the wild-type and ablated cortex (**Fig. 5k**,**l**), suggesting that Reelin$^+$ cells from other sources rapidly cover up the regions deprived of Dbx1-derived Cajal-Retzius cells. Since these animals die at birth, to study the effect of Dbx1-derived cell ablation on later cortical development, we analyzed P0 *Dbx1*^{DTA};*Nes:Cre* animals. Differences were consistently observed in the cytoarchitecture of the cerebral cortex between wild-type and mutant animals at both rostral and caudal levels (**Fig. 5s**–**v**; n = 3). Defects were more pronounced in the lateral regions of the cortex. The thickness of the cingulate cortex appeared fairly normal (compare insets in **Fig. 5s**,**t**). As expected from the rapid repopulation of the ablated cortex with Cajal-Retzius cells from other sources, the mutant cortex did not show a Reeler phenotype, which is characterized by failure of preplate splitting, disorganized cortical plate and cell-dense layer I. Indeed, a cell-poor marginal zone and a distinguishable Layer VIb were present in the mutant cerebral cortex (**Fig. 5s**–**v**), suggesting a normal splitting of the preplate. We conclude that ablation of Dbx1 progenitors starting at E11.0 results in (i) loss of Cajal-Retzius cells in distinct regions of the developing cortex, in particular the medial and dorsolateral pallium, and (ii) alteration of the early postnatal cortical cytoarchitecture.

VP but not septum-derived Reelin$^+$ cells express calretinin

A high proportion, but not all, of the Reelin$^+$ neurons in the marginal zone express calretinin[11]. In order to determine if Dbx1-derived cells give rise to calretinin$^+$/Reelin$^+$ cells, triple immunolabeling using antibodies against β-gal, Reelin and calretinin were performed on coronal

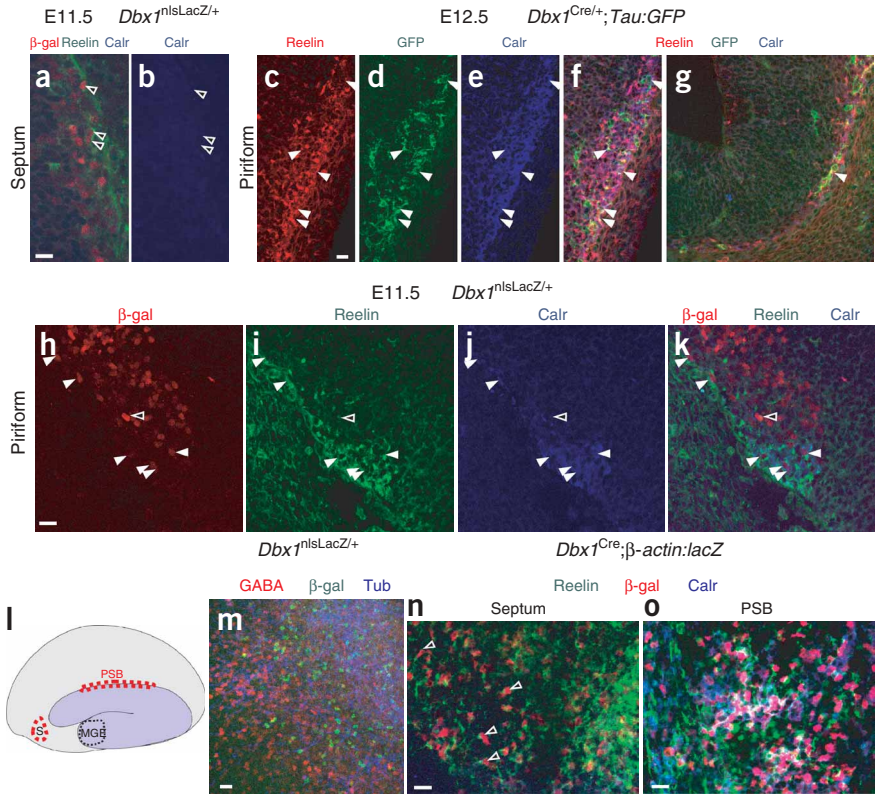

Figure 6 Dbx1-derived Reelin$^+$ cells from the PSB express calretinin, but those from the septum do not. Confocal microscope images of triple labeling with β-gal (red), Reelin (green) and calretinin (blue) in E11.5 *Dbx1*$^{nlsLacZ/+}$ (**a**,**b**,**h**–**k**) and Reelin (red), GFP (green) and calretinin (blue) in E12.5 *Dbx1*$^{Cre/+}$;*Tau*^{GFP} telencephalon (**c**–**g**). **g** shows ventral telencephalic wall including the ventral piriform territory. White arrowheads: triple-labeled β-gal$^+$/Reelin$^+$/calretinin$^+$ cells. Black arrowheads: Reelin$^+$/calretinin$^+$-only cells. Young β-gal$^+$ (β-gal^{high}) cells in the VZ of the septum and VP are Reelin$^-$/calretinin$^-$. (**l**) Schematic of the areas dissected as explants of the septum and PSB in **m**–**o**. Explants were dissected from E12.5 *Dbx1*$^{nlsLacZ/+}$ embryos and kept in culture for 2 d in **m** and from E11.5 *Dbx1*$^{Cre/+}$;β*actin:lacZ* and for 3 d in **n**,**o**. **m** shows triple labeling with GABA (red), β-gal (green) and β-tubulin (blue). **n**,**o** show triple labeling with Reelin (green), βgal (red) and calretinin (blue). Scale bars = 20 μm.

sections of E11.5 *Dbx1*$^{nlslacZ/+}$ telencephalon. In the presumptive piriform cortex marginal zone, almost all (95%) β-gal$^+$ cells were Reelin$^+$, of which 40–50% were calretinin$^+$ (**Fig. 6h–k**). We were surprised to find that at the pial surface in the septum 95–98% of β-gal$^+$ cells were Reelin$^+$, but none of these β-gal$^+$/Reelin$^+$ cells coexpressed calretinin (**Fig. 6a**,**b**). β-gal$^+$/Reelin$^+$/calretinin$^-$ cells were also located ventrally and dorsally to the septum. Consistent with these results, in E12.5 *Dbx1*$^{Cre/+}$;*Tau*^{GFP} telencephalon, mediorostral cortical GFP$^+$/Reelin$^+$ cells very rarely coexpressed calretinin (5–10%) whereas 43–55% of piriform and isocortical marginal zone GFP$^+$/Reelin$^+$ cells expressed calretinin (**Fig. 6c–g**). At later stages (E14.5), likely because of the extensive migration from their multiple sites of origin, Reelin$^+$/calretinin$^+$ or Reelin$^+$/calretinin$^-$ cells intermingle in different cortical regions.

To test whether the lack of calretinin expression in Dbx1-derived septal Reelin$^+$ cells was not just a temporal delay, we cultured PSB and septum explants of E11.5 *Dbx1*$^{Cre/+}$;β*actin:lacZ* embryos in collagen for 3 DIV and immunolabeled for β-gal, Reelin and calretinin. PSB explants ($n = 3$) contained many β-gal$^+$/Reelin$^+$ cells, and most of them also expressed calretinin (**Fig. 6o**). Very few β-gal$^+$/Reelin$^+$/calretinin$^-$ cells were observed in these explants compared with the piriform territory *in vivo* (**Fig. 6f**,**k**). These results suggest that β-gal$^+$/Reelin$^+$/calretinin$^-$ cells in the VP of E11.5 *Dbx1*$^{nlslacZ/+}$ and E12.5 *Dbx1*$^{Cre/+}$;*Tau*^{GFP} telencephalons are likely to express calretinin at later stages rather than representing a real ability of VP Dbx1 progenitors to give rise to two distinct Reelin$^+$ cell populations. On the contrary, explants dissected from the septum ($n = 3$) contained many β-gal$^+$/Reelin$^+$ cells which were mostly calretinin$^-$ (**Fig. 6n**) after 3 DIV. These results suggest that the ventral pallium and the septum are the origin of β-gal$^+$/Reelin$^+$/calretinin$^+$ and β-gal$^+$/Reelin$^+$/calretinin$^-$ cells, respectively, and that the lack of calretinin expression in septum-derived cells is not due to a developmental delay. We conclude that two subpopulations of Cajal-Retzius neurons with distinct characteristics (origin, onset of appearance, migration route, destination and gene expression profile) derive from *Dbx1*$^+$ progenitors of the septum and the ventral pallium.

DISCUSSION

Using mouse genetics we have identified two previously unknown sites of origin of Cajal-Retzius cells: the septum and the PSB. Two distinct subsets of Cajal-Retzius cells are generated during early development from Dbx1$^+$ septal and PSB progenitors and differ in their site of origin, onset of appearance, migration routes, destination and expression of molecular markers. We propose that distinct subpopulations of Cajal-Retzius cells originate from at least three distinct focal areas, including the caudomedial hem[17], at the borders of the developing pallium. Different cortical regions are populated by specific combinations of these Cajal-Retzius subtypes.

Genetic tracing of cell populations derived from *Dbx1*$^+$ progenitors

Morphological and immunohistological studies have been used to describe populations of cells according to their position, morphology and gene expression profile. Nevertheless, they have limits in determining the relationship between the cell types observed at different stages and their sites of origin and migration routes. We have used a knock-in strategy at the *Dbx1* locus to follow the fate of Dbx1-derived cells. *Dbx1* is expressed by progenitors in restricted domains of the telencephalon: the VP at the PSB[22,26,27], the septum and the AEP/POA. Anatomical studies of often transient and non-overlapping expression profiles in progenitors or differentiated cells had suggested that the VP gives rise to part of the claustroamygdaloid complex[27]. However, the transient expression of *Dbx1* in the ventricular zone has thus far prevented from tracing the derivatives of this domain. Moreover, because of the lack of specific molecular markers, derivatives of the septum have not yet been analyzed in detail. The analysis of *Dbx1*^{nlslacZ} and *Dbx1*$^{Cre/+}$;*reporter:LacZ/GFP* animals has permitted us to follow the progeny of Dbx1$^+$ progenitors through their entire lifespan from the ventricular zone to their adult location and thus analyze cell identity, migration routes and final location of Dbx1$^+$ progenitors-derived cells. Indeed, we were able to genetically trace the progeny of Dbx1 progenitors and to identify them as the first Reelin$^+$ neurons to appear in the preplate of the septum and of the VP.

Migration routes of Dbx1-derived neurons

Subpallial regions have been shown to be the source of tangentially migrating cells into the cortex[3,31]. We show that tangential migration also occurs between different pallial territories and that Cajal-Retzius cells migrate from focal progenitor sites at the border of the pallium to different regions of the developing cortex. Indeed, septal Cajal-Retzius cells are clearly pallial, as they coexpress Emx1 (A.P., unpublished results), and the expression of the *Dbx1* gene in the lateral wall corresponds to the ventral pallium ventricular zone[26,27].

DiI has been used to study migration of interneurons from the subpallium to the cortex (for review see refs. 3,31). However, migration from the PSB has been particularly difficult to address, as the PSB does not correlate with morphological landmark (angle) and is crossed by many cells from the subpallium migrating towards the cortex. Previous studies using DiI labeling near the PSB suggested that subpallial cells cross the PSB to form the piriform area[29], but the origin and molecular identity of these cells was not determined. Moreover, DiI labeling close to the PSB was reported in the rat[16,33]. However, these studies failed to identify the PSB as the source of dorsally migrating cells. In addition, it has been suggested[16] that Reelin$^+$ but calretinin$^-$ Cajal-Retzius cells migrate like interneurons from the MGE to the pallium through the marginal zone. We suggest that these Cajal-Retzius cells traverse the MGE by rostrocaudal and ventrodorsal migration but were born in the septum or AEP/POA. Thus, DiI labeling has fallen short in determining the progenitor sites for some migrating cells.

The combination of genetic tracing and DiI labeling has allowed us to unequivocally determine the routes of migration of genetically labeled progenies of Dbx1 progenitors. We provide evidence that populations of Reelin$^+$ Cajal-Retzius cells in the marginal zone of the cortex are derived from progenitors in the VP and septum. We describe ventral and a dorsal migration trajectories from the VP ventricular zone and the septum (**Supplementary Fig. 3**). If the ventral migration from the VP has already been suggested and might correspond to the lateral/ventral migratory stream[29–31], the others had not been reported previously. Three streams of migration for interneurons, similar to the ones we describe in this work, have been identified[34], beginning at E11.5 in the mouse. The authors suggested that an interplay might occur between Cajal-Retzius cells and interneurons to ensure proper cortical integration. Dbx1-derived Cajal-Retzius cell migration is in place earlier than that of interneurons from the subpallium and correlates with the time of appearance of the first-born neurons in the preplate. Thus, they may release cues (if any exist) along these early migration paths (E10.5), which could influence the migration of later-born cells, including interneurons.

Regional differences in Dbx1-derived Cajal-Retzius subtypes

Our data show that PSB-derived cells migrate dorsally up to the isocortex and ventrally to the piriform cortex. Dbx1-derived cells from the septum reach the medial cortex dorsally and at least as far as the piriform region ventrolaterally. The piriform cortex might, therefore, be of mixed origin with respect to Cajal-Retzius cells and might be populated by cells derived from the VP and the septum. Taking into account recent results[17], we believe that at rostral levels, an early Dbx1-derived population of Cajal-Retzius cells generated from the septum will preferentially populate the frontomedial cortex, whereas populations of Cajal-Retzius cells derived from the hem and the PSB (Dbx1-derived) will colonize the caudomedial/dorsal and lateral cortex, respectively (**Supplementary Fig. 3**). Because of the high motility of Dbx1-derived Cajal-Retzius cells *in vitro* and *in vivo* and that of hem-derived Reelin$^+$ cells *in utero*[17], classes of Cajal-Retzius cells generated at different sites have the capacity to intermingle in certain regions of the developing cortex. Our genetic ablation experiments of Dbx1-derived Cajal-Retzius cells using DTA confirm the tracing studies and show a differential loss of Reelin$^+$ cells in cortical regions. Thus, cortical territories are populated by different combinations of molecularly distinct Cajal-Retzius subtypes from early stages of development, when regionalization takes place, and this might contribute to rendering these territories molecularly distinct.

Septum and VP-derived Cajal-Retzius subtypes are distinct

Our results suggest that two subpopulations of Cajal-Retzius neurons with distinct characteristics (origin, onset of appearance, migration route, destination and gene expression profile) derive from *Dbx1*$^+$ progenitors of the septum and the ventral pallium. First, the onset of generation of *Dbx1*-derived cells in the VP seems to be later than that in the septum and correlates precisely with that of Reelin$^+$ cells. VP and septum-derived Reelin$^+$ cells migrate along distinct routes to different regions of the embryonic pallium and are observed in the marginal zone of their postnatal derivatives. Septum- and VP-derived cells preferentially populate rostral and lateral cortical territories, respectively, and some seem to have reached their final destination very early during development. Finally, *Dbx1*-derived cells of the VP and septum differ in expression of calretinin *in vivo* and *in vitro*. These results strongly suggest that two distinct populations of Reelin$^+$ cells are derived from Dbx1$^+$ progenitors: a calretinin$^+$ population from the VP and a calretinin$^-$ population from the septum.

The origins of Cajal-Retzius cells have been a long unresolved question. The olfactory primordium was proposed as a source of Cajal-Retzius cells in macaque monkeys[5]. Our results show that the ventral pallium is the source of a calretinin$^+$ population of Cajal-Retzius neurons invading the mouse isocortical and piriform marginal zone. However, β-gal$^+$/Reelin$^+$/calretinin$^-$ cells are also present in the piriform cortex and the isocortex at E12.5. These cells might have been generated in the VP and/or the septum, or alternatively might acquire calretinin expression later. The retrobulbar area was also proposed as the source of a population of Cajal-Retzius neurons[4] and calretinin$^-$ Cajal-Retzius neurons were described in the marginal zone of the hippocampus[10]. We show that the septum, close to the retrobulbar area, is a source of calretinin$^-$ Cajal-Retzius neurons, invading the rostral cortex and possibly the marginal zone of the hippocampus by rostrocaudal migration. The two subpopulations of Cajal-Retzius neurons (septum- and VP-derived) that we describe are likely to intermingle in the dorsal pallium (**Supplementary Fig. 3**), consistent with the previous observation in the isocortex of a few calretinin$^-$ and a majority of calretinin$^+$ Cajal-Retzius neurons[11]. Recently, the caudomedial wall of the telencephalic vesicles, including the hem, has been reported to be a site of origin of Reelin$^+$ neurons[17]. Calretinin is expressed in the majority of these cells at late stages of development. These cells migrate extensively throughout the neocortical marginal zone with a caudomedial-rostrolateral gradient and are likely, therefore, to provide Cajal-Retzius calretinin$^+$ cells to dorsal cortical regions. Our experiments do not exclude that additional sites of origin for Cajal-Retzius cells might exist and that some Cajal-Retzius cells could also be generated in the dorsal pallium ventricular zone, as it has been shown for humans[5]. However, the three focal sites (hem[17], septum and ventral pallium) account for a vast proportion of Cajal-Retzius cells in the mouse, and molecular evidence is lacking that the dorsal pallium neuroepithelium does produce Cajal-Retzius cells under normal conditions in the mouse. The relative importance of focal sources and local production of Cajal-Retzius cells by the dorsal pallium could distinguish primates and rodents, as in the case of interneurons, and may represent a mechanism to ensure cell

diversity and increase complexity required for the evolution of the human cortex.

Several reports have described differences between cortical regions that are consistent with the existence of distinct Cajal-Retzius populations and with the fact that the presence of different Cajal-Retzius subtypes correlates with region-specific properties. In Tbr1 mutant animals[35] the piriform and isocortex are hypocellular, but the hippocampus is not. This correlates with a decrease of Reelin expression in Cajal-Retzius cells in the lateral and piriform cortex but not in the medial cortex. In these animals, differences in lamination are also observed in different cortical territories. Moreover, the differences in the phenotype in the medial versus the lateral cortex in Emx1/2 single and double mutants suggest the existence of Cajal-Retzius populations with different origins[13,36,37]. Notably, we have detected Emx1 expression in Dbx1-derived cells in the septum but not at the PSB at E11.5 (A.P., unpublished results) suggesting that septum- and PSB-derived Cajal-Retzius cells might differ in expression of Emx genes. These results, together with the role of Cajal-Retzius cells in maintenance of the radial glia phenotype[8] and in axonal growth[38], strongly suggest additional functions of Cajal-Retzius cells besides their general role in lamination and strongly supports the notion of functional heterogeneity of Cajal-Retzius cells.

What is the purpose of subtypes of Cajal-Retzius cells in the cortex? Together with the data from previous reports[11,17,39], our results show that molecularly distinct Cajal-Retzius subtypes migrate tangentially from at least three focal sources at the borders of the pallium and populate different cortical territories at early stages of development. Even if these three populations of Cajal-Retzius cells intermingle afterwards, cortical territories will differ in the percentage of distinct Cajal-Retzius subtypes, and this might contribute to determine region and/or area-specific properties. Notably, in animals with mutated p73 (a gene expressed in the caudal hem and tenia tecta), hem-derived Cajal-Retzius cells are lost, and an expansion of calretinin expression is detected in dorsal cortical regions[15]. These data are consistent with our results that show the existence of a rapid compensation mechanism between distinct classes of Cajal-Retzius cells. In addition, a dorsal shift of the entorhinal cortex and a transformation of occipital and posterior temporal areas into an enthorhinal-like cortex is observed in the p73 mutants[40]. Consistent with these findings, lamination also differs between cortical regions, and the presence of Cajal-Retzius subtypes correlates with differences in the numbers of cell layers (three in allocortical regions, such as the hippocampus or the piriform cortex, four in mesocortical regions and six in the isocortex), and thus it is possible that distinct Cajal-Retzius classes might have a role in region-specific lamination. Notably, Dbx1 expression is conserved in the septum of the chick telencephalon but not in the PSB (VP) and correlates with the medial cortex being a laminated region in this species, unlike the dorsal ventricular ridge, a derivative of the VP/LP (lateral pallium) in birds and reptiles[41]. In addition, the presence of an anterior piriform cortex in this species is consistent with a contribution from the septum as we have suggested in the mouse. Thus, the PSB expression of Dbx1 might have been recruited during evolution to support a function or functions specific to mammals.

METHODS

Generation of Dbx1 mutant mice. The *Dbx1*^{nlsLacZ} mutant mouse line was generated by replacing the *Dbx1* gene coding sequence with an *nlslacZ/pGK-neo* cassette as previously reported[24]. In this construct, the *lacZ* gene coding for a nuclear β-galactosidase protein is translated at the first ATG of the *Dbx1* gene. *Dbx1*$^{loxP\text{-}stop\text{-}loxP\text{-}DTA}$ animals were generated by inserting an *IRES-loxP-stop-pGK-neo-loxP-DTA* cassette into the *Bam*HI restriction site present in the 3′ UTR of the *Dbx1* gene. In this cassette, the open reading frame of the diphtheria toxin gene (*DTA*)[42] is interrupted by a *pGK-neo* cassette (for selection in embryonic stem cells) flanked by *loxP* sites[43]. The cassette is preceded by an IRES (internal ribosome entry site). *Dbx1*^{Cre} animals were generated by inserting an *IRES-CRE-pGK-Hygro*^{r} cassette into the *Bam*HI site present in the 3′ UTR of the *Dbx1* gene. Recombination was achieved in two steps using the I-SceI-induced gene replacement system developed previously[44]. Both the *Dbx1*^{Cre} and *Dbx1*$^{loxP\text{-}stop\text{-}loxP\text{-}DTA}$ were constructed by inserting an IRES cassette in the same 3′ UTR site; therefore, expression from the recombinant loci are expected to be very similar. Differences in labeling in *Dbx1*$^{nlslacZ/+}$ and *Dbx1*$^{Cre/+}$;*βactin:lacZ* embryos were observed. These are likely to correspond to a delay in the recombination and expression of the reporter gene in *Dbx1*$^{Cre/+}$;*βactin:lacZ* embryos, and thus the earliest Dbx1-derived cells are not labeled in these animals. Use of mice in this study was approved by Veterinary Services of Paris.

Animal strains. *βactin:loxP-stop-loxP-lacZ* reporter animals were a gift from D. Anderson, (California Institute of Technology, Pasadena, California)[28]. In this transgenic line, the *lacZ* gene under the control of the chick β-actin promoter is preceded by a transcription-translation stop cassette surrounded by two *loxP* sites.

Tau$^{loxP\text{-}stop\text{-}loxP\text{-}MARCKSeGFP\text{-}IRES\text{-}nlslacZ}$ was obtained by replacing the coding sequence of the *Tau* gene (microtubule associated protein) with a sequence coding for a MARCKS (myristoylated alanine-rich C-kinase substrate) protein fused to green fluorescent protein (GFP) and followed by an IRES*nlslacZ* cassette[45]. The gene coding for the MARCKS protein is preceded by a transcription-translation stop cassette surrounded by two *loxP* sites. All animals are kept in a C57B6 background. The deleter *Nes:Cre* animals expressing the Cre recombinase under the control of the Nestin promoter were previously described and were a gift from F. Tronche[32] (College de France, Paris).

Embryos and postnatal animals were genotyped by PCR using primers specific for the different alleles (Cre, lacZ, GFP, Dbx1 and DTA). *Dbx1*^{Cre};*Tau*^{GFP} recombined embryos were sorted directly with a fluorescence binocular lens.

***In situ* hybridization, X-gal staining and immunocytochemistry.** For staging of embryos, midday of the vaginal plug was considered as embryonic day 0.5 (E0.5). Embryos for immunocytochemistry were fixed at 4 °C using 4% paraformaldehyde (PFA) in 0.1 phosphate buffer (PB) pH 7.3 for 2 h; rinsed in PBS for 2 h; cryoprotected overnight using 30% sucrose, 0.1 M PB and embedded in O.C.T. compound (Sakura). Embedded tissue was sectioned on a cryostat with a 12 μm step. β-gal activity was revealed by incubating sections or whole-mount embryos for 3 h to overnight at 37 °C in a 600 μg/ml X-gal solution in 0.1 M PB, 2.0 mM $MgCl_2$, 0.01% sodium desoxycholate, 0.02% NP40, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$. *In situ* hybridization on sections and antibody staining was performed as previously described[24]. *In situ* probes were mouse Reelin[46], chick Dbx1 (ref. 23) and mouse Dbx1 (ref. 47). Whole-mount *in situ* hybridization was performed according to a previously described protocol[48]. Immunohistochemistry on sections and explants were performed as previously described[23]. Primary antibodies were rabbit anti-β-galactosidase (Rockland; 1:1,000), G10 mouse anti-Reelin (1:1,000; gift of A. Goffinet, University of Louvain Medical School, Brussels), goat anti-calretinin (Swant; 1:500); rat anti-BrdU (Accurate Chemical; 1:400). All fluorescent secondary antibodies were purchased from Jackson ImmunoResearch. TUNEL was performed according to the supplier's protocol (Roche).

Postnatal animals at P2 and P8 were perfused using 4% PFA. *Dbx1*^{Cre};*Tau*^{GFP} P2 animals and E10.75–E11.0 embryos were obtained from females injected intraperitoneally with a single dose of BrdU (15 mg/kg) at E10.5 of gestation.

Explant and slice cultures and DiI injection. After removal from the placenta, embryos were maintained in PBS containing 50 U/ml penicillin G (Invitrogen), 50 μg/ml streptomycin sulfate (Invitrogen) and 6 mg/ml glucose at 0 °C. The same conditions were used for explant and slice cultures. For explant cultures, the head was dissected, the meninges were removed and explants of PSB and septum were isolated from the telencephalic vesicles. After polymerization of a 20 μl layer of collagen (90% Vitrogen 100, 10% 5× culture medium (DMEM 2.5×/F12 2.5× (Invitrogen), 0.15% $NaHCO_3$) in a well 15 min at 37 °C, explants were immersed in a second 20 μl drop of collagen and polymerized 45 min at 37 °C before adding 500 μl of the culture medium. For slice cultures, the dissected telencephalon was embedded in 3% low melting point agarose (Invitrogen) in L15 (Invitrogen) supplemented with 50 U/ml penicillin G,

50 μg/ml streptomycin sulfate and 5.4 mg/ml glucose at 37 °C, chilled at 0 °C, and coronal sections of 250 μm were prepared using a vibratome. Sections were positioned on a Millicell membrane (0.4 μm, Millipore) laid on 1.1 ml of culture medium in one well of Nunclon (6 wells, Nunc). The culture medium for both explant and slice cultures was prepared as follow: DMEM 0.5×, F12 0.5×, 2 mM L-glutamine, 6 mg/ml glucose, 0.075% $NaHCO_3$, 10 mM HEPES, 500 U/ml penicillin G, 500 μg/ml streptomycin sulfate, 1× B27 supplement (Invitrogen). DiI crystals (Molecular Probes) were inserted into the septum or PSB of coronal slices at different rostrocaudal levels of *Dbx1*$^{Cre/+}$;*Tau*^{GFP} embryos. Slices and explants were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 for 1 and 2–4 d, respectively. We analyzed GFP and DiI colabeling using a confocal microscope, and as DiI strong spots could have bled into the green channel, we considered DiI$^+$/GFP$^+$-only cells in which the GFP staining was homogeneously distributed along all the processes and cell body. Thus, it is likely that we underestimated the number of colabeled cells.

Images acquisition. Pictures were acquired using a digital camera coupled to a fluorescence binocular lens or a confocal microscope (Leica TCS Sp2).

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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