Genetic mapping of Foxb1-cell lineage shows migration from caudal diencephalon to telencephalon and lateral hypothalamus

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Abstract

The hypothalamus is a brain region with vital functions, and alterations in its development can cause human disease. However, we still do not have a complete description of how this complex structure is put together during embryonic and early postnatal stages. Radially oriented, outside-in migration of cells is prevalent in the developing hypothalamus. In spite of this, cell contingents from outside the hypothalamus as well as tangential hypothalamic migrations also have an important role. Here we study migrations in the hypothalamic primordium by genetically labeling the Foxb1 diencephalic lineage. Foxb1 is a transcription factor gene expressed in the neuroepithelium of the developing neural tube with a rostral expression boundary between caudal and rostral diencephalon, and therefore appropriate for marking migrations from caudal levels into the hypothalamus. We have found a large, longitudinally oriented migration stream apparently originating in the thalamic region and following an axonal bundle to end in the anterior portion of the lateral hypothalamic area. Additionally, we have mapped a specific expansion of the neuroepithelium into the rostral diencephalon. The expanded neuroepithelium generates abundant neurons for the medial hypothalamus at the tuberal level. Finally, we have uncovered novel diencephalon-to-telencephalon migrations into septum, piriform cortex and amygdala.

Introduction

The hypothalamus is a brain region subserving vital functions, and alterations in its development can cause disease. Obtaining a complete description of how this complex structure is put together during embryonic and early postnatal stages will be helpful in understanding human pathological conditions (Michaud, 2001; Caqueret et al., 2005). The hypothalamus originates in the rostral diencephalon which, because of its situation between telencephalon and caudal diencephalon (including the thalamic region; Fig. 1), undergoes particularly complex patterning (Puelles & Rubenstein, 2003). The longitudinal axis of the neural tube divides the primordium into dorsal and ventral portions (Shimamura et al., 1995). The hypothalamus is subdivided into four areas (preoptic, anterior, tuberal and mammillary; Swanson, 1987; Simerly, 2004), of which the first two are dorsal and the last two are ventral (according to embryonic topology; Fig. 1), although they appear arranged rostrocaudally in the adult brain.

Birthdating shows that most of the hypothalamus develops by waves of neurogenesis from the rostral diencephalic neuroepithelium, followed by radially oriented, outside-in migration (Altman & Bayer, 1986), and gene expression studies confirm this general pattern (Alvarez-Bolado et al., 1995; Caqueret et al., 2006). However, cells from outside the hypothalamus (Muske & Moore, 1988; Schwanzel-Fukuda & Pfaff, 1989; Wray et al., 1989; Henderson et al., 1999) as well as tangential intrahypothalamic migrations (Alvarez-Bolado et al., 2000a) also have an important role. In addition, differential control of migration underlies important functional features such as the sexual dimorphism of some hypothalamic structures (Tobet, 2002). Neuroepithelial expansion is another mechanism resulting in increased cellular heterogeneity in brain regions. As the embryo grows, the neuroepithelium expands by symmetric (horizontal) mitosis (Rakic, 1988; Chen & McConnell, 1995). Differential expansion of neuroepithelial subpopulations could contribute to regionalization (Alvarez-Bolado et al., 1995). Alternatively, neuroepithelial cells can simply migrate inside the neuroepithelium (Fishell et al., 1993; Arnold-Aldea & Cepko, 1996; Golden & Cepko, 1996). The displaced neuroepithelial cells will generate cells for the region in which they settle. Ultimately, every forebrain region including the hypothalamus is a composite of cells from different origins (Marin & Rubenstein, 2003). Knowledge of the cell migrations involved is necessary for understanding forebrain development.

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Genetic neuroanatomy (Joyner & Zervas, 2006; Dymecki & Kim, 2007) is being successfully used to unravel the development of complex brain regions such as the cerebellum (Zervas et al., 2005; Sillitoe & Joyner, 2007). Similar approaches will be very useful for working out the different cell migrations and lineages in the hypothalamus. Here we have labeled the Foxb1 diencephalic lineage by crossing a Foxb1-Cre mouse line (Zhao et al., 2007) with reporter mouse lines. Foxb1 is a transcription factor gene widely expressed in the neural tube, with a rostral expression boundary between caudal and rostral diencephalon (Kaestner et al., 1996; Wehr et al., 1997; Alvarez-Bolado et al., 1999, 2000a) which makes this gene useful for studying cell migration into the hypothalamic primordium.

Materials and methods

Mouse lines

All experiments with animals were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and under authorization Az 32.22/Vo (Ordnungsamt der Stadt Göttingen).

In the Foxb1<sup>Cre</sup> mouse line (Zhao et al., 2007; kept in the C57BL/6 background), the Foxb1 coding sequence was replaced by the Cre re-

Fig. 1. Subdivisions of the diencephalon in the E12.5 mouse embryo.

Fig. 2. Transient expression but extended lineage of Foxb1 in the diencephalon. (A) Foxb1 whole-mount ISH on E8.5 mouse embryo showing expression in diencephalon, not telencephalon (separated by a straight line). (B) β-galactosidase detection on Foxb1<sup>Cre</sup>-ROSA26R embryo at the beginning of neurulation. (C) shows the same embryo as in (A) from the top. (D) View from top of the embryo in B showing the basal plate of the presumptive diencephalon. Inset shows section through the dotted line in B. Foxb1-expressing cells are found in the diencephalic ventral midline and adjacent to it. Asterisks mark the rostral tip of expression in the ventral midline of D and inset. (E, F) At E9.5, Foxb1 mRNA had disappeared from the ventral rostral diencephalon (E), while lineage labeling shows the ventral midline labeled up to eye level (asterisk in F). For abbreviations in this and subsequent Figures see main list.
Lineage labeling

In the ROSA26R animals, the reporter gene β-galactosidase was inserted in the constitutively active ROSA locus downstream of a floxed stop codon. Upon Cre-mediated recombination, the stop codon was deleted and β-galactosidase was constitutively produced. Therefore, this reporter is a lineage marker: in mice carrying both the Foxb1-Cre and the ROSA26R alleles, cells expressing Foxb1 and any cell derived from them will permanently express β-galactosidase. Similar principles apply to the use of Z/AP mice, which carry human placental alkaline phosphatase (hPLAP) as reporter. As hPLAP attaches to axonal membranes it is a very good marker of axons of lineage-labeled neurons (Fields-Berry et al., 1992; Gustinich et al., 1997; Leighton et al., 2001).

The expression of lineage markers is ‘cumulative’ (Louvi et al., 2007), as the lineage domain enlarges as progressively more cells start expressing the marker (here Foxb1). In addition, when lineage labeling appears beyond the expression domain of the marker it is indicative of migration.

We analyzed the Foxb1 lineage in ROSA26 crossings at the following ages: embryonic day (E)8.75, E9.5, E11.5, E12.5, E14.5, E15.5, E16.5, E18.5 and postnatal day (P0); in Z/AP crossings the ages were E12.5, E13.5 and P0. For every embryonic age up to E12.5 we examined two to five litters, and from E15.5 on we examined two to four brains for each age. Both Figs 6 and 7 show material from one brain, for consistency. We identified structures in the brain according to current reference works (Alvarez-Bolado & Swanson, 1996; Paxinos & Franklin, 2001).

β-Galactosidase activity detection

β-Galactosidase activity was detected as described (Koenen et al., 1982). Embryos from timed pregnancies were collected, washed with cold PBS and fixed for 30–50 min in 1% paraformaldehyde, 0.2% glutaraldehyde and 0.02% NP40 in PBS. The embryos were then rinsed and incubated in staining solution [1 mg/mL Xgal, 2 mM MgCl2, 5 mM K3Fe(CN)6 and 5 mM K4Fe(CN)6] in PBS overnight in the dark at RT. For animals older than E12.5, the brains were dissected out, fixed in 4% paraformaldehyde for 60 min at 4°C, embedded in agarose and cut into sections (150 μm). The sections were fixed on ice for 30 min, washed with PBS, incubated with staining solution and fixed again in 4% paraformaldehyde for 60 min.

Alkaline phosphatase activity detection

Material was collected, fixed in 4% paraformaldehyde on ice for 60 min, embedded in agarose and cut into sections (150 μm). The sections were fixed again in 4% paraformaldehyde with 0.2% glutaraldehyde on ice for 60 min, rinsed, incubated for 30 min at 72°C to inhibit endogenous phosphatase activity, rinsed in alkaline phosphatase buffer (100 mM Tris–Cl, pH 9.5, 100 mM NaCl and 10 mM MgCl2), incubated with staining solution (250 μL nitro-blue-tetrazolium and 187.5 μL 5-bromo-4-chloro-3-indolyl phosphate per 50 mL in alkaline phosphatase buffer) overnight at 4°C, and fixed in 4% paraformaldehyde for 60 min at 4°C (Lobe et al., 1999).

Immunohistochemistry

Paraffin sections (15 μm) of P0 Foxb1Cre/ROSA26R mouse brains were dewaxed, preincubated in PBT with 10% fetal calf serum and incubated overnight at 4°C in antibody. Alexa (Molecular Probes-Invitrogen, Karlsruhe, Germany) fluorescent secondary antibodies were used for visualization (1 : 500). Antibodies were: anti-MCH (rabbit, 1 : 100), Phoenix Pharmaceuticals, Burlingame, CA, USA; anti-orexin (mouse, 1 : 10), R&D Systems GmbH, Wiesbaden, Germany; anti-GAD ‘pan’ antibody (rabbit, 1 : 100), Abcam, Cambridge, UK; anti-calbindin (rabbit, 1 : 100), Chemicon-Millipore GmbH, Schwabach, Germany; anti-calretinin (rabbit, 1 : 200), Swant, Bellinzona, Switzerland; anti-β-galactosidase (chicken, 1 : 100), Abcam; anti-glial fibrillary acidic protein (GFAP; rabbit, 1 : 200), DakoCytomation; anti-neurofilaments (2H3, 1 : 5; this mouse monoclonal antibody, developed by T. M. Jessell and J. Dodd, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA, USA).

Microscopy

Leica DMR and MZ APO microscopes (Leica Mikrosysteme, Wetzlar, Germany), Olympus DP50 cameras (Olympus, Tokyo, Japan) and CELL-F 2.6 software (Olympus Soft Imaging Solutions GmbH, Münster, Germany) were used for analysis and photography. Image contrast was enhanced by applying Photoshop 7.0 (Adobe Systems Inc., San José, CA, USA) software tools to one whole image file at a time.

Results

Lineage-labeling with the Foxb1-Cre mouse line

The Foxb1-Cre mouse line (Zhao et al., 2007) carries the recombinase Cre under the control of the regulatory sequences of Foxb1. To use this line as a marker of the Foxb1 lineage, we crossed it...
with reporter mouse lines engineered to carry inactive reporter genes (e.g. easily detected enzymes such as β-galactosidase or alkaline phosphatase) in every cell. In the mouse progeny that we obtained upon crossing the Foxb1-Cre mouse line with a reporter mouse line, every cell expressing Foxb1 carried a reporter gene made permanently active by the Cre recombinase. As the active reporter gene is inherited by any cells derived from Foxb1-expressing neuroepithelium, the full Foxb1 lineage was labeled (see Materials and methods). The mice showed otherwise normal appearance and behavior.

To identify cell migration by lineage labeling, we compared the location of cells expressing Foxb1 with the location of Foxb1-lineage cells: of necessity, any lineage-labeled cells found beyond the Foxb1 expression boundary had either migrated from the Foxb1-expressing domain or were derived from mitotic neuroepithelium that had expanded from the Foxb1-expressing domain. For this reason, we first recorded the expression domains of Foxb1 in the early diencephalon (Figs 2 and 3), using the nomenclature summarized in Fig. 1 (Puelles & Rubenstein, 2003).

**Fig. 3.** Foxb1 expression in the caudal diencephalon peaked early and disappeared. (A, C) Foxb1 whole-mount ISH on hemisected embryonic mouse heads. (B, D) β-Galactosidase activity detection on hemisected embryonic Foxb1-Cre/ROSA26R mouse heads. (A) At E11.5, Foxb1 expression peaked in thalamus, prethalamus and midbrain. Rostral to the mammillary body there was no expression. (B) Foxb1 lineage labeling at E11.5 occupied the ventral midline of the entire neural tube with a rostral limit at the level of the lamina terminalis (asterisk). Arrow points at Foxb1-lineage cells in the basal ganglia region. The thalamus, prethalamus, mammillary area and part of the tuberal area are also labeled. White arrowhead, zona limitans. (C) At E12.5, Foxb1 expression in the caudal diencephalon was disappearing. White arrowhead, zona limitans. (D) At E12.5, the Foxb1 lineage formed most of the dorsal and ventral caudal diencephalon, including part of the prethalamus (ventral thalamus). The mammillary and part of the tuberal areas are labeled. White arrowhead, zona limitans. Asterisk marks rostralmost extent of Foxb1 lineage; scattered cells were found more dorsally (arrows). (E) Transverse section through the caudal diencephalon of an E11.5 embryo. Arrowheads, zona limitans. (F) Diagram summarizing the distribution of Foxb1-lineage cells in the diencephalon at E12.5.

**Transient expression but extended lineage of Foxb1 in the diencephalon**

Aspects of the Foxb1 expression pattern have been reported, particularly at late prenatal–postnatal stages (Kaestner et al., 1996; Alvarez-Bolado et al., 1999). For our lineage analysis we needed a complete and systematic exploration of the dynamic changes in the rostral expression boundary, not yet available in the literature.

Foxb1 is expressed in the neural plate from diencephalon to spinal cord (Fig. 2A; also Ang et al., 1993; Zhao et al., 2007). Early Foxb1-Cre/ROSA26R embryos stained in toto for β-galactosidase activity showed a general distribution of labeling similar to that of Foxb1 mRNA (Fig. 2B). Relatively weak and transient expression in the early diencephalon makes it difficult to assess the distribution of Foxb1 mRNA in the open neural tube (Fig. 2C). Lineage labeling, however, yielded a clear picture, although it appeared with a short time lag after Foxb1 expression (after the Foxb1 locus becomes active, the Cre recombinase has to be produced at high enough levels to render the reporter gene active, and the reporter enzyme has to be
synthesized). Careful examination revealed Foxb1 lineage-labeled cells in the ventral midline of the presumptive diencephalon and midbrain (Fig. 2D), as was confirmed by sectioning (inset in Fig. 2D).

At E9.5, Foxb1 expression was disappearing from the ventral side of the diencephalon and the ventral midline was no longer labeled (Fig. 2E). Lineage labeling showed a faithful record of previous Foxb1 expression in the ventral midline (cumulative labeling; Louvi et al., 2007), which was labeled up to eye levels (asterisk in Fig. 2F). The ventral portion of the diencephalon, including the infundibular region, was labeled as well. At this age, Foxb1 expression was high in the dorsal portion of the neural tube, including caudal diencephalon (thalamic region) and midbrain (Fig. 2E). Accordingly, lineage labeling began to appear in these regions (Fig. 2F). At E9.5 the diencephalon was free from Foxb1 expression (Fig. 2E) and Foxb1-lineage cells (Fig. 2F).

**Foxb1 expression in the caudal diencephalon peaked early and disappeared**

At E11.5, expression of Foxb1 reached its peak of intensity in the caudal diencephalon, including a large thalamic (dorsal thalamic) domain and a small prethalamic (ventral thalamic) domain (Fig. 3A). Expression in the rostral diencephalon (including the hypothalamic primordium) strongly and specifically labeled the mammillary body (Fig. 3A). Horizontal sections, however, demonstrated that at this stage Foxb1 was expressed in the mantle but was no longer expressed in the neuroepithelium (not shown). In the caudal diencephalon, thalamus and tegmentum were heavily labeled while the prethalamus showed labeled and unlabeled cells. In the rostral diencephalon, the dorsal portion did not show any Foxb1-lineage cells. In the ventral portion, the mammillary area and adjacent regions were labeled, as well as some cells in the tuberal area (Fig. 3B). The Foxb1 lineage in the ventral midline extended rostrally into the roof of the ventral diencephalon and reached the level of the lamina terminalis (asterisk in Fig. 3B). At this point, in some cases, the labeled neuroepithelium extended into the basal ganglia (arrow).

At E12.5 the major brain regions are recognizable by specific gene expression (Shimamura et al., 1995). At this age, Foxb1 expression had almost completely vanished from the caudal diencephalic neuroepithelium, except for very weak domains in the thalamus and prethalamus (Fig. 3C), separated by a clear boundary corresponding to the zona limitans interthalamica (arrowhead in Fig. 3C). In the rostral diencephalon, the mantle layer (postmitotic neurons) of the mammillary body was also very strongly labeled at this age (Fig. 3C). Lineage labeling at E12.5 covered the thalamus as well as part of the prethalamus in the caudal diencephalon (Fig. 3D). The ventral portion of the caudal diencephalon was also completely labeled (Fig. 3D). In the rostral diencephalon, mammillary area labeling covered a domain larger than the actual Foxb1 expression in the mammillary body. Numerous labeled cells were also present in the tuberal area (Fig. 3D). The labeled ventral midline seemed to have ceased expanding rostrally, and its rostral end was at this stage caudal to eye levels (asterisk in Fig. 3D). However, scattered labeled cells were found at the level of the lamina terminalis (arrows in Fig. 3D).

In transverse sections, the thalamic neuroepithelium was intensely labeled while the prethalamic neuroepithelium showed labeled and unlabeled cells. The two domains were clearly separated by a sharp boundary (zona limitans; arrowheads in Fig. 3E). Figure 3F summarizes our findings.

**Neuroepithelial migration from diencephalon into the early telencephalon**

Foxb1 has been reported as a diencephalic marker, not expressed in the early telencephalon of the mouse or zebrafish (Kaestner et al., 1996; Wehr et al., 1997; Varga et al., 1999). We confirm this (Figs 2E and 3A and C), indicating that any Foxb1-lineage cell found in the telencephalon would have migrated from diencephalic levels. As labeled cells could have been overlooked due to the evagination of the telencephalic vesicles after E10.5, we examined lineage-labeled, transversely sectioned E11.5 brains (Fig. 4A–D). Surprisingly, at the level where diencephalon and telencephalon are continuous, a trail of labeled neuroepithelial cells entered the cortex (arrowheads in Fig. 4A–D). The apparent origin of the migrating neuroepithelial cells was in the prethalamus (Fig. 4B–D). No labeled cells were found in the telencephalon at levels rostral or caudal to the ones shown in Fig. 4A or Fig. 4D, respectively.

**Extension of axons into hypothalamus before the beginning of migration**

To explore the relation of the Foxb1 lineage with the early thalamic axons we used the Z/AP reporter mouse line, which carries as reporter an enzyme that attaches to axonal membranes, efficiently labeling the Foxb1 lineage axons (see Materials and methods). At ~E12.5, the thalamocortical projection (internal capsule) was visible (Fig. 4E and F). More caudally, we observed an axonal bundle directed ventrally towards the hypothalamus (Fig. 4G). At this age, the region of the hypothalamus receiving the axons was devoid of Foxb1-lineage cells (Figs 3C and D and 4F and H). As development proceeded, the thalamocortical and thalamohypothalamic Foxb1-lineage bundles elongated and became increasingly distinct (Fig. 4I–K).

**Migratory routes into ventral diencephalon and telencephalon**

Mapping the relation between the Foxb1-lineage domains and the spatiotemporal pattern of Foxb1 expression in the forebrain (Figs 2 and 3) until it becomes stable or disappears was the prerequisite to the analysis of cell migration into the hypothalamic primordium. From this point on, detection of Foxb1 lineage-labeled cells rostral to the boundary would be indicative of migration. At E14.5, Foxb1-lineage cells apparently from the prethalamus started migrating in the rostral and ventral direction (arrowheads in Fig. 4L and M). The migrating cells were associated with thalamohypothalamic axons (arrow in Fig. 4M) and not with the internal capsule (ic in Fig. 4L and M). This phenomenon became more clear at E15.5, when the migrating cells reached the ventralmost level of the hypothalamus (Fig. 4N and O). At E18.5 the migrating cell group appeared completely established (Fig. 4P and Q).

On sagittal sections at different ages (Fig. 5A–S) we detected two additional migratory routes. Cells from the prethalamus extended rostrally into the presumptive septum on medial levels from E14.5 (arrow in Fig. 5A) through E15.5 (arrows in Fig. 5D and F) to E18.5 (arrows in Fig. 5L and M). The migration into the hypothalamus (see above, Fig. 4) was also evident on sagittal sections at E14.5 (black arrowhead in Fig. 5C), E15.5 (black arrowheads in Fig. 5F–I) and E18.5 (black arrowheads in Fig. 5N and O). In a third migration route, labeled cells from the caudal hypothalamus migrated into ventral levels of the telencephalon by taking advantage of the ventral diencephalon–telencephalon continuity. This migration became apparent at E15.5 (white arrowheads in Fig. 5I and K) and was very substantial at E18.5 (white arrowheads in Fig. 5P–S).
Rostral expansion of caudal diencephalic neuroepithelium

We went on to map the Foxb1 lineage onto the hypothalamus and telencephalon at postnatal P0, when regionalization and most mantle formation are over (Figs 6 and 7).

Sagittal sections through the midline showed that the boundary of the most intensely labeled neuroepithelium (white dotted line in Fig. 6A) was broadly similar to that found at earlier stages, immediately after Foxb1 expression had disappeared from the caudal diencephalon (Fig. 3D). However, we observed several intriguing departures from this pattern, i.e., labeled cells located rostral to the boundary, in keeping with the migrations observed at earlier stages (Figs 4 and 5).

A region of neuroepithelium rostral to the boundary was labeled with β-galactosidase (asterisks in Fig. 6A); this was much more...
rostrally positioned than any Foxb1-expressing cell had been, and than any Foxb1-lineage cell that could be seen at E12.5 (compare with Fig. 3D). These cells demonstrate a rostral expansion of the caudal diencephalic neuroepithelium (dorsally) and of the mammillary neuroepithelium (ventrally). Transverse sections confirmed this expansion (asterisks in Fig. 7F–J).

Migration stream from prethalamic levels into the lateral hypothalamus
In the mantle layer, we observed a striking departure from the early Foxb1 expression and lineage boundaries. A large group of cells was positioned between the zona incerta (prethalamus or ventral thalamus) and the hypothalamus (arrow in Figs 6C and 7F–H). This cell group seemed the result of the migration stream detected at E14.5 (Figs 4L and N and 5) and it was sharply delimited lateromedially (compare Fig. 6C to B and D) and rostrocaudally (arrows in Fig. 7F–H), ending in the lateral hypothalamus at anterior levels.

The lateral hypothalamus at the tuberal level
More caudally (tuberal level), the medial and lateral hypothalamus showed abundant scattered Foxb1-lineage cells (Fig. 7I and J). These cells must have either migrated caudorostrally through the mantle layer (presumably from the mammillary area) or were generated in the expanded neuroepithelium (asterisks in Figs 6A and 7I and J). A small
and compact group of labeled cells was found consistently in the lateral hypothalamus at this level (arrowhead in Fig. 7I).

**The mantle in the mammillary region**

We have mentioned the expansion of the labeled mammillary neuroepithelium (see above). In the rostral diencephalic mantle, the entire mammillary body was formed by cells of the *Foxb1* lineage (Figs 6B–D and 7K), in agreement with the *Foxb1* expression pattern (Fig. 3C). The posterior hypothalamus was also formed by expanded mammillary neuroepithelium, as could be expected from the lineage labeling at E12.5 (Fig. 3C and D).

**Diencephalon-to-telencephalon migrations, medial and lateral**

We established that the early expression domain of *Foxb1* did not reach the telencephalon (Figs 2–4). However, we found a number of *Foxb1* lineage-labeled cells in the telencephalon at P0. The presence of such labeled cells must have been the result of migration from caudal levels or cell division from migrated neuroepithelium (Fig. 4). At medial levels, labeled cells formed a migration stream into the posterior septum (septofimbrial nucleus and triangular nucleus of the septum) which was clear on sagittal sections (Fig. 6A and B) as well as in transverse sections (Fig. 7A–C). At lateral levels, abundant *Foxb1*-lineage cells migrated from the thalamic region into the amygdala, globus pallidus and piriform cortex (Figs 6E and F and 7A–C).

**Telencephalic settling places of Foxb1-lineage cells**

To confirm the identity of the telencephalic regions receiving *Foxb1*-lineage cells, we labeled transverse sections of *Foxb1Cre-ROSA26R* neonatal brains (P0) with antibody against β-galactosidase and against glutamic acid decarboxylase (GAD). The distribution of GAD in cell bodies and axon terminals has a well-known and characteristic regional pattern (see for instance the Allen Brain Atlas:...
http://mouse.brain-map.org) allowing for the identification of the major areas of the forebrain (Fig. 8). The results confirmed our previous detection of Foxb1-lineage cells in the globus pallidus, amygdala and lateral hypothalamus (Figs 5 and 6).

**Characterization of Foxb1-lineage cells in telencephalon and hypothalamus**

We then carried out an initial characterization of Foxb1-lineage cells with antibodies against different neuronal and glial markers (Fig. 9). In the cortex, Foxb1-lineage cells were not abundant, and most of them expressed GAD (Fig. 9A–C). Calretinin was a less frequent marker (Fig. 9D and E), and we could not detect colocalization with calbindin in any case (Fig. 9F). Foxb1-lineage cells in the amygdala colocalized mostly with GAD (Fig. 9G and H). Two very specific markers of cell populations in the lateral hypothalamus are hypocretin (also known as orexin) and melanin concentrating hormone (MCH; see for instance Cvetkovic et al., 2004). Some of the lateral hypothalamic Foxb1-lineage cells coexpressed hypocretin/orexin (Fig. 9I) or, more often, MCH (Fig. 9J and K). None of the Foxb1-lineage cells coexpressed GFAP, a glial marker. The exception was the lining of the third ventricle, where some radial glial cells (which also express GFAP) were double-labeled (Fig. 9L).
agreement with our detection of Foxb1 lineage in the hypothalamic neuroepithelium (Fig. 7F–J).

Axon-dependent migration into the lateral hypothalamus

Intriguingly, the migration from prethalamus into the lateral hypothalamus was tangential (rostrocaudal; see Fig. 1), suggesting that axons rather than radial glia could be the substrate (Fig. 10A). In brains from Foxb1-Cre/ZAP crossings, we detected an important axonal bundle apparently coursing from the prethalamic region into the lateral hypothalamus (Fig. 10B). The β-galactosidase domain and the alkaline phosphatase domain coincided in size, shape and position (Fig. 10A and B), suggesting the axon-dependent nature of this migration. By using antineurofilament antibody (monoclonal antibody 2H3, green in Fig. 10C and D) and anti-β-galactosidase antibody (red in Fig. 10C and D) we confirmed the existence of this axonal bundle.

Diverse migration strategies into the tuberal portion of the lateral hypothalamus

At tuberal levels, the lateral hypothalamus showed abundant labeled cells. In contrast to the compact arrangement of Foxb1-lineage cells at anterior levels (Fig. 10A and B), here labeled cells were mostly scattered (Fig. 10E). Close inspection of the settling patterns suggested that these cells could have reached their positions in the medial and lateral hypothalamus according to different strategies. In the medial hypothalamus, labeled cells were usually radially arranged and in some cases their trail could be followed to the labeled neuroepithelium (Fig. 10E and G). In the lateral hypothalamus, however, labeled cells were not disposed in radial columns and lacked an obvious connection to the neuroepithelium, suggesting a tangential migration from caudal levels (Fig. 10E and F). Finally, another group without obvious relation to the neuroepithelium at this level was consistently found in the lateral hypothalamus, ventral to the cerebral peduncle (Fig. 10H). Our results are summarized in Fig. 11.

Discussion

Because of the position of its rostral expression boundary, Foxb1 lineage-labeling can detect tangential migrations from caudal levels into the rostral diencephalon and the telencephalon. One caveat is that inside the rostral diencephalon there is a source of Foxb1-lineage cells, the mammillary area, which contributes cells to more rostral regions (caudorostral intrahypothalamic migration). Foxb1-lineage mapping reveals overall cell migrations but is not appropriate for detailed mechanistic analysis of specific migrating cohorts whose settling point is known (see for instance Henderson et al., 1999).

Tangential migration in the hypothalamus

Hypothalamic migration is mostly radial (Altman & Bayer, 1986), which does not exclude tangential migration, as most of the forebrain shows a mixed pattern of radial and nonradial migration (Balaban et al., 1988). Tangential migration uses axons as a substrate (axonophilic/neuronophilic migration; Rakic, 1990; Golden et al., 1997), but neurons can migrate through a permissive corridor of membrane-attached molecules (Wichterle et al., 2003) or even precede the axons (Lopez-Bendito et al., 2006). Several functionally important neuronal groups enter the hypothalamus following nonradial trajectories. Gonadotrophin releasing hormone-expressing neurons migrate from nasal epithelium into hypothalamus following tangential axons (Muske & Moore, 1988; Schwanzel-Fukuda & Pfaff, 1989; Wray et al., 1989) in a precisely regulated manner (reviewed in Schwarting et al., 2007). A unique radial-to-tangential migration stream follows radial processes from the lateral ventricle neuroepithelium (telencephalon), entering the diencephalon tangentially to settle in the medial hypothalamus, ventral to the anterior commissure (Henderson et al., 1950 T. Zhao et al. © The Authors (2008). Journal Compilation © Federation of European Neuroscience Societies and Blackwell Publishing Ltd European Journal of Neuroscience, 28, 1941–1955
1999). This estrogen-controlled migration results in sexual dimorphism in the preoptic/anterior area (Wolfe et al., 2005; Knoll et al., 2007).

**Tangential migration of Foxb1-lineage cells into the lateral hypothalamus**

Here we uncover a group of Foxb1-lineage cells extending from the prethalamus into a restricted lateral hypothalamic region as the result of tangential, axonophilic migration. Although we still do not have a full neurochemical characterization of hypothalamic Foxb1-lineage cells, we show that some Foxb1-lineage lateral hypothalamic neurons express specific lateral hypothalamic markers (hypocretin or MCH). Developmental, connectional and neurochemical heterogeneity of the MCH-expressing neurons has been reported (Brischoux et al., 2001, 2002; Cvetkovic et al., 2004), but to our knowledge the hypocretin/orcin-expressing population has until now been considered homogeneous (Amiot et al., 2005).

Other descriptions of hypothalamic development contain data compatible with this migration. Expression of calretinin shows an early caudal diencephalic domain later extending into the lateral hypothalamus (Abbott & Jacobowitz, 1999), possibly representing a migration stream similar to the one we describe here. Fate-mapping studies of the avian neural plate can provide evidence of longitudinal cell migration. In chicken, tissue grafted in the vicinity of the thalamic eminence and prethalamus generates cells for the lateral hypothalamus (experiment QFM-38 in Cobos et al., 2001) or dorsal hypothalamus and preoptic area (experiment R-173 in Garcia-Lopez et al., 2004). While our mouse data agree in general with these results, it is not immediately obvious whether they describe comparable phenomena.

Additionally, the lateral hypothalamus (tuberal level) contains many labeled cells not radially arranged that could originate in the mammillary area then migrate caudorosally. One example is the caudal-to-rostral migration from the mammillary body settling in a circumscribed area of the lateral hypothalamus (Fig. 7I). As these cells express Foxb1 (Alvarez-Bolado et al., 2000a), it is in principle possible that they do not migrate but appear to do so.

**Neuroepithelial migration into cortex**

Part of the lateral hypothalamic neuroepithelium actually migrates from midbrain levels (Manning et al., 2006). Tangential migration
of neuroepithelial cells inside the diencephalon is a known but infrequent phenomenon (Arnold-Aldea & Cepko, 1996; Golden et al., 1997). We show that a reduced number of neuroepithelial cells migrates early from caudal diencephalon into telencephalon (Fig. 4A–D), presumably originating the rare Foxb1-lineage cells that we have found in the cortex and which are often interneurons (coexpressing GAD). As far as we know, this would be the first report of neuroepithelial migration from diencephalon into cortex giving rise to interneurons. In the mouse, as a rule, cortical interneurons are generated in the basal ganglia (Marin & Rubenstein, 2003).

Additionally, we show what appears to be clonal expansion of the neuroepithelium in a very specific hypothalamic region giving rise to cells for the tuberal level.
Different types of radial migration in the hypothalamus

Tuberal nuclei (ventromedial and arcuate) are generated locally by the neuroepithelium of the third ventricle (McClellan et al., 2008). However, some radially migrating Foxb1-lineage cells seem to contribute to the arcuate, suggesting a contribution from ‘expanded’ neuroepithelium. Radial migration from neuroepithelium which has previously expanded tangentially can be considered a special case of the general radial pattern found in hypothalamus. Other specific hypothalamic subpopulations show characteristic variations on radial migration, for instance the parvicellular endocrine neurons migrate tangentially but do not follow the outside-in rule (Markakis & Swanson, 1997).

Diencephalic migration into the telencephalon

No clearcut boundary separates diencephalon from telencephalon (Inoue et al., 2000; Puelles et al., 2000; Trujillo et al., 2005), allowing for migration between them (Mitrofanis, 1994; Henderson et al., 1999; Letinic & Rakic, 2001; Morante-Oria et al., 2003). We have detected a novel migration stream from the dorsal diencephalon to the posterior septum and possibly the globus pallidus, as well as a migratory route from the caudal hypothalamus into piriform cortex and amygdala. Two caveats apply here. A subpopulation of piriform cortex neurons expresses Foxb1 at a later stage of development (~E14.5; Alvarez-Bolado et al., 1999), so they may not be related to more caudal neuroepithelium by lineage. Besides, a reduced number of Foxb1 lineage-labeled ventral midline cells reach the lamina terminalis and enter the basal ganglia (Fig. 3B and D), and some prethalamic neuroepithelial cells migrate into the telencephalon (Fig. 4A–D); therefore, some of the labeled cells in septum and pallidum could originate in migrated neuroepithelium (as opposed to tangentially migrating through the mantle).

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Abbreviations

3V, third ventricle; AHA, anterior hypothalamic area; AMY, amygdala; ARH, arcuate nucleus; CTX, cortex; DI, diencephalon; E, embryonic day; GAD, glutamic acid decarboxylase; GFAP, glial fibrillary acidic protein; GP, globus pallidus; hPLAP, human placental alkaline phosphatase; HYP, hypothalamus; ic, internal capsule; inf, infundibulum; ISH, in situ hybridization; LGl, lateral geniculate dorsal; LHA, lateral hypothalamic area; LM, lateral mammillary nucleus; LV, lateral ventricle; MAM, mammillary area; MBO, mammillary body; MCh, melanin-concentrating hormone; os, optic sulcus; P, postnatal day; PBS, phosphate-buffered saline; P BT, phosphate-buffered Tween; PH, posterior hypothalamus; PIR, piriform cortex; PRO, preoptic area; PT, pretectum; PTh, parahippocampal area; TH, thalamus (dorsal thalamus); TL, telencephalon, TUB, tuberal area; ZI, zona incerta.

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