bHLH Gene Expression in the Emx2-deficient Dentate Gyrus Reveals Defective Granule Cells and Absence of Migrating Precursors

Dentate gyrus development is uniquely characterized by the existence of migrating precursors. The production of precursors by the neuroepithelium is regulated by the proneural cascade of bHLH genes, which show distinctive expression patterns in dentate. Mice carrying a mutation in Emx2, a neuroepithelial transcription factor, lack the granule cell layer which forms most of the dentate, although the corresponding neuroepithelium is correctly specified. To understand this phenotype, we have analyzed the expression of proneural genes (bHLH gene family) and other markers in Emx2-deficient dentate. Here we show that, in the mutant dentate, expression of bHLH genes, Tenascin C and GFAP is normally confined to the germinal layer, as are most neuronal and astrocytic precursors. Additionally, Mash1 expression (marker of migrating precursors) is lost during development. Mutant granule cells show arrested migration and lack NeuroD2 expression. These results are evidence that in Emx2 mutants, migrating precursors (secondary matrix) and astrocytes are absent, the radial migration substrate impaired and granule cells deficiently differentiated. Our analysis gives insight into how a general defect caused by the absence of Emx2 translates into the dentate-specific phenotype.

Keywords: differentiation, Mash1, migration, neurogenesis, proliferation, Prx1

Introduction

The dentate gyrus is a portion of the pallium forming a well-known macroscopic landmark (the dentate granule cell layer), whose morphology and development have been described in detail (Caviness, 1973; Stanfield and Cowan, 1979; Stanfield et al., 1979; Altman and Bayer, 1990a,b). Dentate development is uniquely characterized by the existence of precursor cells that at the same time divide and migrate towards the granule cell layer, generating postmitotic, immature granule neurons that migrate as well (Altman and Bayer, 1990a). In addition, dentate neuronal precursor cells produce functional neurons postnatally and in the adult (Altman and Das, 1965; Stanfield and Trice, 1988; van Praag et al., 2002) and can activate neuron production in response to stimuli (Kempermann et al., 1998).

The production of neuronal precursor cells by the neuroepithelium is initiated by the expression of Notch genes and their ligands (Delta genes) which gives rise to a genetic cascade of pro-neurogenic (Mash1, Ngn1, Ngn2) and anti-neurogenic (among them Hes5 and Id3) transcription factor genes, as well as neuronal differentiation-related genes (NeuroD1, NeuroD2), all of them belonging to the basic-helix-loop-helix family (Cau et al., 1997, 2000; Perron et al., 1999; Torii et al., 1999; Cai et al., 2000; Bertrand et al., 2002; Schuurmans and Guillemot, 2002). The expression pattern of such genes in developing dentate helps to understand how the dentate neuroepithelium generates neurons (Pleasure et al., 2000).

One important neuroepithelial transcription factor is Emx2, a vertebrate homeobox gene (Simeone et al., 1992a,b) related to the Droso phila gap gene ems (Dalton et al., 1989) whose detailed functions are not entirely clear (Gangemi et al., 2001; Heins et al., 2001; Galli et al., 2002). Emx2 is expressed in the telencephalon, including the dentate (Simeone et al., 1992a,b; Gulisano et al., 1996; Mallamaci et al., 1998). In addition to neocortical arealization defects (Bishop et al., 2000, 2002; Mallamaci et al., 2000a,b; Fukuchi-Shimogori and Grove, 2001, 2003; Muzio et al., 2002a,b; Muzio and Mallamaci, 2003), Emx2 mutant mice lack conspicuously a dentate granule cell layer (Pellegrini et al., 1996; Yoshida et al., 1997), which makes them a model for the study of the dentate neuroepithelium. The few granule cells produced in the mutant are poorly differentiated (Savaskan et al., 2002). The mutant dentate is correctly specified but suffers from a developmental problem affecting the entire medial cortex (Tole et al., 2000). Evidence suggests that this problem could be a defect in the cortical positional information signaling cascade mediated by Fgf8 (Fukuchi-Shimogori and Grove, 2001, 2003) and possibly also Wnt proteins (Muzio et al., 2002a; Ligon et al., 2003).

Here we have used in situ detection of bHLH genes and other markers to know more about the precise ways in which Emx2 deficiency translates into the dentate-specific phenotype.

Materials and Methods

Animal Husbandry and Embryo Harvesting

Emx2-/- female mice of mixed C129sv/J-C57Bl6 genetic background (Pellegrini et al., 1996) were mated overnight with Emx2-/- males of similar genetic background and inspected at 9:00 a.m. on the following day for the presence of vaginal plug. Noon of this day was assumed to correspond to embryonic day 0.5 (E0.5). At embryonic day 18.5 (E18.5) pregnant females were anesthetized with Isofluran (Baxter) and killed by cervical dislocation. Embryos were harvested, genotyped by PCR according to Savaskan et al. (2002) and further processed. The animals were treated in order to avoid any unnecessary suffering and in agreement with the German Law for Protection of Animals (Tierschutzgesetz).

Age of Analysis

Our observations were made on 10 mutant brains age E18.5, three mutant brains age E14.5 and five mutant brains age E15.5, as well as equal numbers of wild-type littersmates. Other authors have described
the expression of bHLH markers in telencephalon at E14.5 in the mouse (Ma et al., 1997) or at the equivalent age E16 in the rat (Lindsell et al., 1996; Pleasure et al., 2000), making it an age of choice for analysis. E15.5 proved to be more interesting for us, since we found migration problems and the first migrations from the dentate are seen at E15.5 in our material. We also analyzed E18.5 brains because this is the oldest age reached by the Emx2 mutants (Pellegrini et al., 1996; Yoshida et al., 1997) and so it shows the dentate at the most developmentally advanced stage possible in these mice. By E18.5 all the dentate primordium subdivisions are present and different hippocampal fields can be specifically labeled by markers. Finally, the Emx2 dentate phenotype has been investigated at this age by other workers (Pellegrini et al., 1996; Yoshida et al., 1997; Tole et al., 2000).

Immunohistochemistry

Embryos were fixed in 4% formaldehyde, embedded in paraffin and sectioned at 20 µm. The following primary antibodies were used: monoclonal anti-Ki-67 (BD Biosciences), dilution 1:20; rabbit polyclonal anti-calretinin (Chemicon), dilution 1:500; and anti-GFAP (Dako Z0334), dilution 1:1000. The reactions were detected with biotinylated secondary antibodies, Extravidin (Sigma) 1:100 and diaminobenzidin (Sigma), or with fluorescent second antibodies (Molecular Probes). Before using anti-Ki-67 antibody, sections were left for 15 min in 0.1% Tween-20 in PBS, then treated for antigen retrieval (antigen unmasking) as follows: heated in microwave oven for 5 min in citrate buffer 0.1 M, then rapidly cooled down on ice; the heating–cooling cycle was repeated three times, each time using fresh citrate buffer.

In situ Hybridization

In situ detection of mRNAs with digoxigenin-labelled antisense riboprobes was performed on cryostat sections (20 µm thickness) essentially as described by Wilkinson and Nieto (1993). Our protocol additionally included a powerful signal amplification step (tyramide signal amplification), reported to result in up to 100-fold increase in sensitivity (Adams, 1992; Yang et al., 1999). We used a GenePaint platform (Tecan Group Ltd, Maennedorf, Switzerland) so that prehybridization, hybridization, post-hybridization and color detection reactions were carried out automatically (Herzig et al., 2001).

Results

Emx2 Expression in the Dentate Gyrus Primordium

We analyzed Emx2 dentate expression at E18.5 in order to know where to expect a cell autonomous phenotype. Our probe reproduced the reported neocortical distribution of Emx2 (not shown) (Simeone et al., 1992a,b; Gulisano et al., 1996; Mallamaci et al., 1998; Cecchi, 2002). Additionally, Emx2 was expressed in every subdivision of the dentate primordium (Fig. 1A). The distal part of the primordium showed a large number of heavily labeled cells following the characteristic shape of dorsal and ventral dentate blades, which by this age are starting to differentiate (Stanfield and Cowan, 1979). Closer examination of this area showed that the Emx2-expressing cells on the dorsal side corresponded...
approximately to the hippocampal fissure, suggesting that they were not granule cells. Through the hippocampal fissure, at this age the dorsal side is in contact with the cortical marginal zone, populated by Emx2- and calretinin-expressing Cajal–Retzius cells (Glezer et al., 1992; Mallamaci et al., 1998). Staining with anti-calretinin antibody (Fig. 1B) revealed that most of the Emx2-expressing cells in the dorsal side of the primordium were probably Cajal–Retzius cells of the hippocampal fissure, while the layer of calretinin-positive, differentiating granule cells immediately ventral to the fissure expressed lower levels of Emx2 (Fig. 1B). Nissl staining confirmed that the differentiating dorsal blade was intimately apposed to the longer and thicker hippocampal fissure, rich in cells but more loosely packed (Fig. 1C).

Emx2 was expressed at some level by cells at every subregion of the dentate primordium at this age; the strongest expression was in the radial and tangential migratory streams (as well as the hippocampal fissure), while tertiary matrix and differentiating granule cells, as well as ventricular and subventricular layers showed very low levels.

Migration of Proliferating Cells and Neurons is Differentially Affected in the Emx2 Mutant Dentate Gyrus

In the dentate primordium there are migrating immature neurons (postmitotic) as well as migrating proliferating (mitotic) cells which form the so-called `secondary matrix' (Altman and Bayer, 1990b). In order to determine how these different cell groups (mitotic and postmitotic) were affected by the Emx2 deficiency we used an antibody against antigen Ki-67, known to identify proliferating cells. At E18.5, precursor migration reaches its highest point in the dentate (Bagri et al., 2002). In wild-type, Ki-67 was abundantly expressed by cells not only in the ventricular layer (primary matrix), but also in the subventricular layer and migratory pathway (secondary matrix), in the tertiary matrix and in the hippocampal fissure (Fig. 2A,B). In the mutant dentate, the dentate primordium was a small, hook-shaped structure, characterized especially by a dramatically shortened tangential migration stream which abuts a cell-sparse area representing the presumptive hippocampal fissure (inexistent in the mutant) and stratum lacunosum-moleculare (Fig. 2C). Proliferating cells are dramatically decreased in the mutant primordium (Fig. 2D). Detailed examination of which (Fig. 2E) showed Ki-67 staining mostly in the ventricular layer. A few labeled cells could also be seen in the subventricular layer, in the migration stream (arrowheads in Fig. 2E) and in the presumptive fissure (arrows in Fig. 2E), indicating the existence of a very reduced secondary matrix and in shear contrast with the abundance of proliferative cells in the same regions of the wild-type (Fig. 2B). We then used an antibody against calretinin, a marker of neuronal differentiation. In the wild-type, calretinin-positive cells were found at the distal end of the tangential migratory stream as well as in the tertiary matrix (Fig. 2F). In the mutant, calretinin-positive cells were dramatically reduced in number, and present in the tangential migratory stream (Fig. 2G). In summary, Emx2 deficiency in the dentate affected the mitotic and postmitotic migrating subpopulations differentially: while proliferating cells were arrested in the ventricular layer or its immediate vicinity, at least some immature neurons were able to migrate part of the way towards the presumptive location of the granule cell layer, before being arrested in the tangential migration stream.

Delayed and Transient Expression of Mash1 in the Emx2 Mutant Dentate

The dentate migrating precursors have been shown to express transcription factor genes Mash1 and Notch1 and at least some of them additionally express Delta1, Id3 and Hes5 (Pleasure et al., 2000). We wanted to investigate the expression of Mash1 in the mutant dentate, since it did not seem to have migrating precursors. At E14.5, Mash1 expression formed a patch in the dentate neuroepithelium (Fig. 3A), as described by Pleasure et al. (2000). Mash1 was not expressed at all in mutant dentate at the same age (Fig. 3B), although normal expression could be detected on the same sections in structures not expressing Emx2, like the basal ganglia (BG in Fig. 3B).

By E15.5, Mash1 expression was restricted to the neuroepithelium immediately adjacent to the fimbria (fimbrial neuroepithelium) and to a few radially migrating cells starting to form the secondary matrix (Fig. 3C). In the mutant, there was a small patch of Mash1-expressing neuroepithelium at the very end of the primordium (arrowhead in Fig. 3D, corresponding presumably to the fimbrial neuroepithelium of the wild-type), but no cells were detected migrating from it.

By E18.5 there was expression of Mash1 in the ventricular and subventricular zones as well as in the migration stream (arrows in Fig. 3E). The region of the fissure (asterisk in Fig. 3E) was also labeled by the marker. At this age, expression of Mash1 had completely disappeared from every subregion of the mutant dentate primordium (Fig. 3F), with exception of the mutant presumptive fissure region, which was consistently labeled (asterisk in Fig. 3F).

Notch1 and Delta1 are Abnormally Confined to the Germinal Layers in the Emx2 Mutant Dentate

Notch1 is specifically expressed in dentate migratory precursors (Pleasure et al., 2000). We detected incipient Notch1 expression as early as E14.5 in wild-type neuroepithelium (Fig. 4A), in coincidence with published data (Lindsell et al., 1996; Pleasure et al., 2000). No difference between both genotypes was seen at this age (Fig. 4A,B). However, at E15.5, there was Notch1 expression in the wild-type hippocampal ventricular zone, including dentate. The signal was particularly strong close to the ventricle and diminishing towards the subventricular layer (Fig. 4C). Labeled cells could be seen in the radial migratory stream as well as in the tangential stream, with a few cells scattered between dentate and Ammon’s horn neuroepithelium (arrowheads in Fig. 4C). The mutant showed expression in the germinal layers of the hippocampus and only very weak signal in the dentate neuroepithelium (arrowhead in Fig. 4D). At E18.5, Notch1 was expressed homogeneously in all subregions of the wild-type dentate, from ventricular zone to tertiary matrix (Fig. 4E). In Emx2 mutants, at this age, a small but clearly visible fimbria was visible. The mutant fimbrial neuroepithelium did not express Notch1. In the rest of the mutant hippocampal anlage, Notch1 expression was restricted to the ventricular zone, while no hybridization signals could be observed in the migratory stream (Fig. 4F).

Notch ligand Delta1 was expressed by individually recognizable cells in the E14.5 wild-type germinal layer (Fig. 4G), particularly in the fimbrial neuroepithelium (Pleasure et al., 2000). The same region showed only few labeled cells in the mutant (Fig. 4H). Delta1 was still expressed by a few cells in the E15.5 wild-type germinal layer, including the fimbrial neuroepithelium, although at this age no migrating cells expressed the
Figure 2. Proliferating cells and neurons arrested at different levels in the Emx2 mutant dentate gyrus. (A, B) Comparison of Nissl-stained (A) anti-Ki-67-labeled (B) wild-type dentate shows that proliferating cells can be found in every subregion, including the hippocampal fissure (outlined and asterisk). (C, D) The Nissl-stained mutant primordium (C) is a hook-shaped area formed by ventricular and subventricular zones, a radial migration stream and a very shortened tangential migration stream. A cell-sparse area (asterisk in C, D, E, G) corresponds to the presumptive hippocampal fissure. Proliferating cells (D) are found mainly in ventricular and subventricular zones and some scattered ones in the radial and tangential migration streams. (E) High-magnification image of (D) shows very sparse proliferating cells in the migration stream (arrowheads) and in the aborted hippocampal fissure region (arrows), which is in register with a pial region of increased proliferation (white arrowhead). (F, G) Neuronal cells (as labeled by anti-calretinin antibody) in wild-type (F) and Emx2 -- (G) E18.5 dentate. The abundant labeled cells in the wild-type contrast with the few calretinin-positive neuronal cells in the mutant (G), present only along the tangential migratory stream (arrows). Abbreviations: asterisk, region of the hippocampal fissure; fi, fimbria; rad, radial migration stream; sub, subventricular layer; tan, tangential migration stream; ter, tertiary matrix; ven, ventricular layer.
marker (Fig. 4I). The mutant dentate showed very few to no Delta1-expressing cells in the dentate primordium at this age (Fig. 4J). By E18.5, there was signal in the wild-type radial and tangential stream, as well as in the tertiary matrix (Fig. 4K), suggesting Delta1 could label a subgroup of migratory precursors. Delta1 was abnormally restricted to the ventricular zone in the mutant dentate (Fig. 4L).

**Figure 3.** Proneural gene Mash1 expression disappears in the Emx2-deficient dentate gyrus. (A, B) Mash1 expression forms a patch in the neuroepithelium of wild-type (A) E14.5 dentate (arrowhead). In the mutant (B) no expression could be observed at this age (arrowhead). (C, D) In wild-type E15.5 dentate (C), Mash1 is expressed in the fimbrial neuroepithelium (arrowhead); a few labeled cells have started migrating (rad). In the mutant (D) a smaller but correctly placed patch of Mash1 expression could be seen (arrowhead). (E, F) In wild-type E18.5 (E), Mash1 is expressed by cells in the migratory stream (arrow) and in the hippocampal fissure area (asterisk). No hybridization for Mash1 is present in the mutant dentate (F; outlined). The presumptive region of the fissure (asterisk) expresses low levels. Abbreviations: asterisk, region of the hippocampal fissure; BG, basal ganglia; chp, choroid plexus; fi, fimbria; rad, radial migration stream; sub, subventricular zone; tan, tangential migration stream; ter, tertiary matrix; ven, ventricular layer. Scale bars = 200 µm.

Id3, Hes5 and Ngn2 are Abnormally Confined to the Germinal Layers in the Emx2 Mutant Dentate

Id3 was not expressed by the wild-type dentate at E14.5 (Fig. 5A), but the mutant dentate consistently showed an Id3-expressing patch of neuroepithelium at that age (Fig. 5B). Id3 however has been reported as showing expression in the dentate gyrus of the rat (Pleasure et al., 2000) at a develop-
Figure 4. Expression of Notch1 and Delta1 is abnormally restricted to ventricular and subventricular zones in Emx2 mutant dentate gyrus. (A, B) At E14.5, both wild-type (A) and mutant (B) dentate neuroepithelium show weak Notch1 expression (arrowheads). (C, D) At E15.5, the wild-type dentate (C) shows intense Notch1 expression in the ventricular layer and less intense in the subventricular; numerous labeled cells form a radial migratory stream (rad); isolated labeled cells can also be seen (arrowheads). The mutant (D) dentate germinal layer expresses Notch1 (arrowhead), but no labeled migrating cells can be seen. (E, F) Presence of numerous cells expressing Notch1 in the wild-type migratory stream at E18.5 (E). In the Emx2 mutant, Notch1 is expressed only in the neuroepithelium (F). (G, H) Delta1 is expressed by a large number of individual cells in the E14.5 wild-type dentate neuroepithelium (arrowhead in G), while in the mutant only a few cells express Delta1 (arrowhead in H). (I, J) By E15.5, Delta1 expression has decreased in the wild-type (I) and mutant (J) dentate neuroepithelium. (K, L) Cells expressing Delta1 can be seen in the migratory stream at E18.5 in wild-type (K). In the mutant at E18.5 (L) no labeled migrating cells could be seen, and expression was confined to the germinal layers. Abbreviations: asterisk, region of the hippocampal fissure in the mutant; BG, basal ganglia; chp, choroid plexus; rad, radial migratory stream; sub, subventricular layer; tan, tangential migration stream; ter, tertiary matrix; ven, ventricular layer. Scale bars = 200 µm.
Figure 5. Id3 and Hes5 are confined to the neuroepithelium in the mutant Emx2 dentate. (A, B) Id3 is not expressed at E14.5 in wild-type dentate (arrowhead in A), but shows a patch of premature expression in the Emx2-deficient dentate neuroepithelium (arrowhead in B). (C, D) Id3 is expressed in the hippocampal germinal layers of the E15.5 wild-type (C), particularly in the fimbrial neuroepithelium (arrowhead), as well as in few radially migrating cells (rad). Id3 is expressed at this age in the mutant (arrowhead in D), but no migrating cells can be seen. (E, F) At E18.5, Id3 is expressed throughout the wild-type (E) developing dentate gyrus subregions. In the mutant (F), Id3 is restricted to the germinal layer (arrowhead). (G, H) At E14.5, Hes5 is expressed by both wild-type (arrowhead in G) and mutant (arrowhead in H) dentate neuroepithelium. (I, J) Hes5 is expressed in the wild-type germinal layers and in radially migrating cells at E15.5 (I), while in the mutant (J) seems confined to the neuroepithelium. (K, L) By E18.5 Hes5 expression can be found in every subregion of the dentate (K), but is still confined to the neuroepithelium in the mutant (L). Abbreviations: asterisk, region of the hippocampal fissure in the mutant; BG, basal ganglia; chp, choroid plexus; fi, fimbria; rad, radial migration stream; sub, subventricular layer; tan, tangential migration stream; ven, ventricular layer. Scale bars = 200 µm.
mental stages (E16–17) in principle comparable to mouse E14.5. The difference is probably due to the fact that it is not possible to match the ages of the two species so precisely. Additionally, there could be subtle temporal differences in the development of rat and mouse dentate. The premature expression of \( \text{Id3} \) in Emx2-deficient dentate was found consistently in our data set.

\( \text{Id3} \) was expressed in the germinal layers of the wild-type hippocampus at E15.5, particularly on the fimbrial neuroepithelium (Fig. 5A). A few expressing cells could be seen starting to migrate down the radial stream (Fig. 5C). In the mutant, \( \text{Id3} \) expression was similar, but no labeled migrating cells were seen (Fig. 5D).

In E18.5 wild-type, \( \text{Id3} \) was intensely expressed in the ventricular layer as well as in cells along the migratory pathway (Fig. 5E). The intensity of \( \text{Id3} \) expression and the number of cells expressing this gene increased distally, reaching a maximum in the tertiary matrix (Fig. 5E). In the Emx2 mutant dentate no cells expressing \( \text{Id3} \) were seen in the migratory stream, while the well-defined patch of \( \text{Id3} \) expression in the germinal layers appeared wider and more intensely labeled than in the wild-type (Fig. 5F).

\( \text{Hes5} \) was already expressed by the wild-type and mutant dentate at E14.5 (Fig. 5G,H). Early expression in wild-type dentate neuroepithelium has been reported (Pleasure et al., 2000). Abundant labeled cells formed a wide radial migration stream in the wild-type at E15.5 (Fig. 5I). In the mutant at the same age no \( \text{Hes5} \)-expressing migrating cells could be observed (Fig. 5J).

At E18.5, \( \text{Hes5} \) was expressed in ventricular zone, migratory stream and tertiary matrix (Fig. 5). Its expression intensity did not increase distally as for \( \text{Id3} \), but was the same through the primordium. In the mutant E18.5 dentate, \( \text{Hes5} \) was restricted to the ventricular zone (Fig. 5). \n
\( \text{Ngn2} \) is another proneural gene expressed in the mouse (Sommer et al., 1996) and rat (Pleasure et al., 2000) hippocampus. Comparison of dentate expression at E14.5, E15.5 and E18.5 in wild-type (Fig. 6A,C,F) and mutant (Fig. 6B,D,F) showed that \( \text{Ngn2} \) seemed correctly expressed in the mutant ventricular and subventricular layers. However, the labeled migrating cells that in the wild-type appeared as soon as E15.5 were absent in the mutant both at E15.5 and E18.5. The fact that at E18.5, \( \text{Ngn2} \) was intensely expressed in wild-type hippocampal neuroepithelium and also (although at very low levels) in the migratory stream (Fig. 6E), raised the possibility that \( \text{Ngn2} \) is a marker for a small subgroup of migrating precursors.

These results suggested that precursor cells going through all the stages of bHLH gene expression were present in the mutant primordium, but abnormally restricted to the germinal side.

Expression of Astroglial Precursor Marker Tenascin C and of GFAP is Restricted in the Mutant Dentate

Our results to this point suggested that perhaps the major defect in Emx2 mutant dentate was not in the ability of precursors to express bHLH genes, but in their capacity to migrate. Radial glial cells are a key element in the histogenesis of the dentate gyrus. Their processes provide a scaffolding on which developing dentate cells migrate; later many of them migrate distally, giving rise to astrocytes and contributing to the formation of the granule cell layer (Eckenhoff and Rakic, 1984; Rickmann et al., 1987). Therefore we analyzed the status of astrocytes and their precursors in developing wild-type and mutant dentate. Tenascin C (Chiquet and Fambrough, 1984; Chiquet-Ehrismann et al., 1986), a glia-produced extracellular matrix protein (Bartsch et al., 1992a,b), is a marker of the migratory astroglial precursors of the dentate gyrus (Bartsch et al., 1992a,b; Yuasa, 2001a,b). Tenascin C was specifically expressed in the wild-type dentate neuroepithelium at E14.5 (Fig. 7A), but not yet in the mutant, where onset of expression suffers a delay (Fig. 7B). In wild-type E18.5 dentate (Fig. 7C), strong expression of Tenascin C could be observed in the ventricular and subventricular layers and at every level of the primordium. In the mutant, however, Tenascin C was expressed only in the germinal zone and in very few cells of the radial migration stream (Fig. 7D). Therefore, astroglial migratory precursors are mostly confined to the germinal layers in the Emx2 mutant, in agreement with our results for bHLH genes. Glial fibrillary acidic protein (GFAP) is a protein abundantly expressed by radial glial cells and astrocytes (Eckenhoff and Rakic, 1984). In the neuroepithelium and secondary matrix, it colocalizes with Tenascin C (Yuasa, 2001a). GFAP expression could be detected in the E18.5 wild-type brain in every dentate subregion (Fig. 7E), while in the mutant expression was restricted to the neuroepithelium (Fig. 7F). Immunocytochemical detection of GFAP in wild-type and mutant hippocampus at E18.5 (Fig. 7G,H) was consistent with the \( \text{in situ} \) findings. While the wild-type hippocampus showed robust GFAP immunoreactivity (Fig. 7G), the GFAP scaffolding in the mutant was clearly defective (Fig. 7H) and only very few GFAP-containing cells were found distal to the mutant radial migration stream (arrowhead in Fig. 7H).

Granule Cells Stop Migrating and Accumulate in the Tangential Migratory Stream of the Mutant Dentate

Despite the absence of migrating precursors, calretinin expression showed (Fig. 2G) that some granule cells were still produced in the mutant. This was in agreement with previously reported results (Tole et al., 2000) using other markers. We asked if these mutant granule cells were correctly specified. \( \text{Prox1} \) is a homeobox transcription factor gene (Oliver et al., 1993) specifically expressed by dentate granule cells (Elliott et al., 2001). In wild-type, \( \text{Prox1} \) was weakly expressed in the hippocampal neuroepithelium at E14.5 (Fig. 8A), but completely absent in the mutant at that age (delayed onset; Fig. 8B). At E18.5, the wild-type germinal ventricular and subventricular zones were no longer labeled, but \( \text{Prox1} \) was intensely expressed in the tangential migratory stream and in the tertiary matrix (Fig. 8C). In contrast, Emx2 mutants abnormally showed strong expression of \( \text{Prox1} \) in ventricular and subventricular zones at E18.5 (Fig. 8D). Besides, \( \text{Prox1} \)-expressing cells, which were not seen at this age in the mutant radial migration stream, formed abnormally large, strongly expressing groups in the tangential migration stream (Fig. 8D), suggesting arrested migration.

\( \text{NeuroD1} \) and \( \text{NeuroD2} \) are bHLH genes without proneural function (Bertrand et al., 2002). \( \text{NeuroD1} \) is expressed in postmitotic, immature neurons of the dentate as an early neuronal differentiation marker. When the differentiation into dentate granule cells is complete (i.e., in the tertiary matrix and early granule layer), \( \text{NeuroD2} \) is also expressed (Pleasure et al., 2000). In E18.5 wild-type dentate, \( \text{NeuroD1} \) showed very strong expression in the subventricular layer, migration stream...
NeuroD1 was strongly expressed but confined to subventricular zone and radial migratory stream, barely entering the tangential stream (Fig. 8F). NeuroD2, the marker of recently differentiated cells, was completely absent from the mutant primordium (Fig. 8H). These observations suggest that the Emx2 mutant dentate is only able to produce a small number of immature neurons which start down the differentiation pathway, although they do not reach full differentiation.

Our results with NeuroD1, NeuroD2 and Prox1 are evidence of abnormal granule cell differentiation and migration arrest. Table 1 summarizes genetic expression in the wild-type and mutant dentate.

Discussion
Why the Emx2 mutant mouse lacks a dentate granule cell layer (Pellegrini et al., 1996; Yoshida et al., 1997) is still not known. Given the expression pattern of Emx2 in the pallium, the hypothesis that this gene has one single function responsible for neocortical and dentate phenotypes is the most parsimonious (Tole et al., 2000). The aim of our work was to characterize the ways in which a general cortical maturation problem could translate into absence of a dentate granular layer in the Emx2 mutant.

Following an analysis of the expression of genes of the proneural cascade as well as other markers in the mutant dentate, we have uncovered alterations in the precursor cells, in the granular cells and in the glial scaffolding. (i) Remarkably, mutant neuroepithelial precursors express Notch1, Delta1 and the genes of the bHLH cascade almost with no alteration. (ii) However, the subventricular layer or secondary matrix (Altman and Bayer, 1990a) is drastically reduced in the mutant dentate. (iii) The few granule cells formed in the mutant are not correctly differentiated (do not express NeuroD2) and are not able to migrate beyond the tangential...
Figure 7. Astrocytes and their precursors show arrested migration in the Emx2 mutant dentate. (A, B) At E14.5, Tenascin C is expressed in the wild-type (arrowhead in A) but not the mutant (arrowhead in B) dentate neuroepithelium. (C, D) At E18.5, Tenascin C is expressed at every level of the wild-type dentate (C), but mostly confined to the germinial layers (and scattered cells in the radial stream) of the mutant dentate (D). (E, F) GFAP is expressed at every level of the wild-type E18.5 dentate (E), but confined to the ventricular layer in the mutant (arrowhead in F). (G, H) GFAP protein is abundant in the wild-type dentate (G), but scarce in the mutant (H). Arrowheads in (H) and inset show the position of the astrocyte found in the most distant position to the mutant neuroepithelium. Abbreviations: asterisk, region of the hippocampal fissure; BG, basal ganglia; chp, choroid plexus; fi, fimbria; hf, hippocampal fissure; rad, radial stream; sub, subventricular layer; tan, tangential stream; ven, ventricular layer. Scale bars = 200 µm.
Figure 8. Prox1-expression in the neuroepithelium and migration stream of Emx2 mutant dentate gyrus. (A, B) Prox1 is expressed weakly by the E14.5 neuroepithelium, but absent in the mutant (arrowhead in B). (C, D) By E18.5 in wild type (C) Prox1 is expressed in tangential migratory stream and tertiary matrix and absent from the hippocampal fissure region (outlined). In the mutant (D) Prox1 expression is abnormally present in the ventricular and subventricular zones. Labeled cells accumulate in the tangential stream. (E, F) In E18.5 wild-type (E), many NeuroD1-expressing cells are seen in all dentate gyrus subregions. In the mutant (F), NeuroD1-expressing cells are present in the radial and tangential migratory stream. (G, H) In E18.5 wild-type (G) dentate, NeuroD2 is expressed only in the tertiary matrix; in the mutant (H), NeuroD2 expression is completely missing. Abbreviations: asterisk, region of the hippocampal fissure; BG, basal ganglia; chp, choroid plexus; fi, fimbria; rad, radial migration stream; sub, subventricular layer; tan, tangential migration stream; ter, tertiary matrix; ven, ventricular layer. Scale bars = 200 µm.
stream, where they seem to accumulate. (iv) The GFAP-expressing radial glial scaffolding of the mutant dentate is almost inexistent, suggesting that migration problems are an important part of the phenotype.

**Expression of Precursor Markers in the Emx2-deficient Dentate**

Our data on Tenascin C and bHLH gene expression in wild-type mouse developing dentate match those reported by others (Lindsell et al., 1996; Sommer et al., 1996; Ma et al., 1997; Pleasure et al., 2000; Yuasa, 2001a). Our central finding in the mutant is that the Emx2-deficient dentate neuroepithelium is able to express the bHLH gene cascade in a way that matches the wild-type almost completely, suggesting that basic genetic control of neurogenesis is maintained in the dentate in the absence of Emx2. We have found however in the mutant what appear to be minor alterations in the spatio-temporal expression patterns: delayed onset and premature conclusion of expression of proneural gene Mash1; premature expression of Id3, an antineural gene (Benezra et al., 1990; Ellis et al., 1990; Garrell and Modolell, 1990); the number of Delta1-expressing cells seems considerably reduced in the mutant. Additionally, expression of Tenascin C seems also delayed. These changes could indicate a certain disregulation in the early steps of the neurogenic cascade, perhaps ultimately in relation with the inability to establish a secondary matrix.

**Emx2 Function in the Telencephalon and in the Dentate Gyrus**

There is evidence that Emx2 exerts its functions in the telencephalon by antagonizing the Fgf8 (Fukuchi-Shimogori and Grove, 2001, 2003) and Wnt (Ligon et al., 2003) signaling systems. At the cellular level, deficiency of Emx2 in the telencephalon alters the positional information grid (Bishop et al., 2000, 2002) at least partially through alteration of tangential and radial neuronal migration (Mallamaci et al., 2000b; Shinozaki et al., 2002; Ligon et al., 2003). In agreement with this, the development of radial glial cells is known to be altered in Emx2 mutants (Mallamaci et al., 2000b). Both positional information and migration are important in establishing and implementing the cortical protomap (Rakic, 1991; Muzio et al., 2002a; Muzio and Mallamaci, 2003). Perturbances of positional information and neuronal migration affecting the entire medial cortex could then translate into a specific dentate defect by altering a dentate-specific process like the generation of a migrating secondary matrix. As a parallel process, abnormal radial glial cells fail to migrate themselves and to properly support migration of the precursors.

**Drastic Reduction of the Secondary Matrix in the Emx2 Mutant**

From around E14.5 to E15.5, a group of dividing precursors born in the dentate neuroepithelium migrates away from the ventricular zone and establishes a mitotic center, the subventricular zone or secondary germinal matrix (Altman and Bayer, 1990a). The secondary matrix gives rise to more precursors which eventually form a migratory stream of mitotic cells which will finally produce the granule cells (Altman and Bayer, 1990a,b). We have been able to follow the formation of the secondary matrix in the wild-type dentate at E15.5 by labeling it with probes to Notch1, Delta1 and bHLH genes. At this age, migrating cells expressing Ngn2 or Hes5 are the most numerous, while Notch1-expressing migrating cells and especially those expressing Mash1 or Id3 seem scarce, and almost none expresses Delta1. The different amounts of radially migrating cells labeled with each of the probes suggests the existence of different subpopulations of migrating precursors (Pleasure et al., 2000).

In the Emx2-deficient dentate, in contrast, the secondary matrix is extremely reduced, to judge by the very small number of proliferating cells in the mutant migratory stream, the fact that migrating precursor markers Id3, Notch1, Hes5, Delta1 and Ngn2 are restricted to the neuroepithelium ever since the earliest stages, and the premature loss of migrating precursor marker Mash1. Staining with the anti-Ki67 antibody, however, shows a very small number of cells forming a subventricular layer in the mutant, indicating that in the absence of Emx2 a vestigial secondary matrix can still be formed. Given that the dentate neuroepithelium (primary matrix) gives origin to the granule cells only through the secondary matrix (Altman and Bayer, 1990a), the almost total absence of a secondary matrix is the cause of the dramatic reduction in the number of granule cells in the mutant.

**The Glial Scaffolding in the Emx2-deficient Dentate**

The neuroepithelium not only produces secondary precursor cells, but provides them with the first substrate on which they can migrate, i.e. the radial glial processes (Eckenhoff and Rakic, 1984; Rickmann et al., 1987). Any alteration able to

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**Table 1**

Confinelement of markers to the proximal side of the mutant dentate primordium

<table>
<thead>
<tr>
<th>marker</th>
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<td>+</td>
<td>+</td>
<td>Mash1</td>
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<td>NeuroD2</td>
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</tbody>
</table>

Expression of genetic markers in the wild-type and Emx2-deficient developing dentate subregions. Very low, low, medium and high expression intensities are represented by (+), +, ++ and +++. ‘No expression’ is represented by –
modify the ability of the neuroepithelium to generate a secondary matrix could also translate into perturbation of this key geniculate element. The long processes of the radial glia contain GFAP and are used by precursors as migration substrate (Rickmann et al., 1987). Tenascin C-expressing astrocyte precursors use the same route to reach the presumptive region of the granule cell layer, to whose histogenesis they contribute (Yuasa, 2001a,b). Abnormal radial glial cells and altered neuronal migration have been indeed described in the Emx2 mutant cortex (Mallamaci et al., 2000b; Shinozaki et al., 2002). Accordingly, our data show dramatic reduction in the radial glial scaffolding of the mutant dentate. The radial glial scaffolding is impaired and the number of astrocytes decreased in the Emx2 mutant according to our GFAP immunocytochemistry data, although in situ hybridization shows that GFAP-expressing precursors exist in the mutant neuroepithelium. A hypothetical scenario can be envisaged in which an initial failure of the neuroepithelial cells to produce the right number of properly differentiated radial processes would lead to inability of the astrocyte precursors to reach the granule cell layer region and, in parallel, histogenetic failure of the granule cell layer.

Granule Cells in Emx2 Mutant Dentate are Few and Incompletely Differentiated

Absence of migration could impair the function of precursors for instance by preventing them to enter into contact with signaling molecules that would normally be found in the migration pathway. We have previously shown in vitro that the granule cells in this mutant are incorrectly differentiated (Savaskan et al., 2002). Accordingly, in the present study we have found delayed expression of granule cell marker Prox1. Absence of NeuroD2, a marker of recently differentiated granule cells, confirms that these neurons do not reach terminal differentiation. From our data it cannot be known if the differentiation problems are cell-autonomous or secondary to differentiation problems in the precursor cells. Intriguingly, the few granule cells (and astrocytes) that are formed in the mutant are able to migrate as far as the tangential migratory stream, maybe because their substrate requirements are different from those of the precursors.

Notes

J.O. and N.K. contributed equally to this study. G.A.B. and T.S. contributed equally to this study. This work was supported by DFG SK 49/3-3, the European Union and the German Ministry of Research. Norah Szabó helped with the immunohistochemistry.

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