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Emx2 in the developing hippocampal fissure region

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Abstract

Mice deficient in transcription factor gene *Emx2* show developmental alterations in the hippocampal dentate gyrus. *Emx2*, however, is also expressed in the region around the developing hippocampal fissure. The developing fissure contains a radial glial scaffolding, and is surrounded by the outer marginal zone and the dentate marginal zone, which become specifically colonized by neurons and differentiate into stratum lacunosum-moleculare and molecular layer of the dentate, respectively. In this study we show that the *Emx2* mutant lacks the glial scaffolding of the fissure and has an outer marginal zone (precursor of the stratum lacunosum-moleculare), as well as a dentate marginal zone severely reduced in size while most of the *reelin (Reln)*-expressing cells that should occupy it fail to be generated. We have also identified a subpopulation of hippocampal *Reln*-expressing cells of the marginal zone, probably born in the hem, expressing a specific combination of markers, and for which *Emx2* is not essentially required. Additionally, we show differential mutant phenotypes of both *Emx2* and *Pax6* in neocortical vs. hippocampal *Reln*-expressing cells, indicating differential development of both subpopulations.

Introduction

Although it is known that the hippocampus of mice deficient in transcription factor Emx2 lacks the granule cell layer of the dentate gyrus (Pellegrini *et al*., 1996; Yoshida *et al*., 1997), defects in other hippocampal subdivisions have to our knowledge been unnoticed. We have reported observations suggesting that the morphogenesis of the hippocampal fissure could also be affected (Oldekamp *et al*., 2004). The marginal zone in close contact with the developing fissure (outer marginal zone or OMZ) is crucially important for hippocampal development, because it gives rise to a specific layer of the CA (Cornu Ammonis) region (the stratum lacunosum-moleculare) (Soriano *et al*., 1994). Therefore, investigating its development in the *Emx2* mutant could contribute to the understanding of the still unknown function of this gene. Although in adult brains the hippocampal fissure could be considered 'a virtual line' (Sievers *et al*., 1992), in the embryonic brain it is certainly a real space occupied by specific glial cells and their processes, as well as by blood vessels (Rickmann *et al*., 1987; Sievers *et al*., 1992). During development, the region around the hippocampal fissure could be considered as consisting of a central radial glial scaffolding (Rickmann *et al*., 1987; Sievers *et al*., 1992) and two apposed marginal zones – the OMZ and the dentate gyrus marginal zone (DMZ). These are surrounded by the inner marginal zone (IMZ). The OMZ, DMZ and IMZ arise as specializations of the telencephalic marginal zone, and each of them is colonized by specific neuronal populations and differentiates into a peculiar hippocampal layer, respectively, stratum radiatum, stratum lacunosum-moleculare and molecular layer of dentate gyrus (Soriano *et al*., 1994). Each of these layers has specialized cytoarchitecture, connectivity and functions (Amaral & Witter, 1995). In this work we will use the name 'developing hippocampal fissure region' to refer to the OMZ + DMZ. The developing hippocampal fissure region contains abundant *reelin* (*Reln*)-expressing neurons, some of which, like in the neocortical marginal zone, are called Cajal-Retzius cells (Soriano *et al*., 1994; Soriano & Del Rio, 2005). Here we show that, in the developing $Emx2^{-/-}$ hippocampal fissure region, the radial glial scaffolding is absent, the OMZ and DMZ are atrophic and *Reln*-expressing cells are missing, except a specific subpopulation. We have characterized this subpopulation by determining the expression or lack thereof of eight additional marginal zone markers. Finally, we compared the $Emx2^{-/-}$ region of the hippocampal fissure with that of *Small eye* mice, lacking transcription factor gene *Pax6* (Hill *et al*., 1991; Walther & Gruss, 1991), a functional antagonist of *Emx2* in neocortical development (Bishop *et al*., 2000, 2002; Muzio *et al*., 2002a,b; Muzio & Mallamaci, 2003). The differential phenotypes of *Emx2* as well as *Pax6* on both subpopulations are evidence of differential development of neocortical vs. fissural *Reln*-expressing cells of the marginal zone.

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Materials and methods

Animals

$Emx2^{/+}$ female mice of C57Bl6 genetic background (Pellegrini *et al*., 1996) were mated overnight with $Emx2^{/+}$ males and inspected for vaginal plug at 09.00 h on the following day; noon of this day was

considered embryonic day 0.5 (E0.5). Pregnant females were lightly anaesthetized with isoflurane vapours (IsoFlo-vet from Schering-Plough, New Jersey, USA), then killed by cervical dislocation. All adult animals used in this study were anaesthetized with isoflurane and killed by cervical dislocation. All embryos used were decapitated. Embryos were collected, polymerase chain reaction-genotyped (Savaskan *et al*., 2002), and further processed. *Small eye* (Pax6-deficient) mutant mice in the C56BL6/6J × DBA/2J background were similarly used. Adult animals were always manipulated under light anaesthesia with isoflurane vapors.

Experiments were carried out in accordance with the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC). The study was approved by the Service of Animal Protection of Lower Saxony.

BrdU injections

Pregnant mice from heterozygous crossings were intraperitoneally injected with BrdU (RPN201, Amersham Biosciences, Buckinghamshire, England) (50 μg/g of body weight), at 12.00, 15.00 and 18.00 h (Takahashi *et al*., 1993) on the appropriate pregnancy date (either at E10.5, E11.5, E12.5, E13.5 or E14.5). Embryos were collected at E12.5 and E14.5 (for E10.5 injections) or E18.5. Reelin-BrdU double-labelling studies were carried out on two *Emx2*$^{-/-}$ and two wild-type brains per injection age.

Immunohistochemistry

Embryos were fixed in 4% formaldehyde, embedded in paraffin and sectioned at 20 μm. Primary antibodies used: anti-nestin MAB353 (which is actually antibody 'Rat-401'; Hockfield & McKay, 1985) 1 : 5 and anti-reelin MAB5366 1 : 250 (both from Chemicon, Temecula, California, USA), anti-BrdU M0744 1 : 100 and anti-glial fibrillary acidic protein (GFAP) Z0334 1 : 1000 (both from Dakocytomation, Glostrup, Denmark), and anti-Ki-67 1 : 20 (BD Biosciences, Erembodegen, Belgium). We used biotinylated second antibodies, extravidin 1 : 100 and diaminobenzidine (both from Sigma, Saint Louis, Missouri, USA), or 'Alexa' fluorescent second antibodies (Molecular Probes, Portland, Oregon, USA). Heat-induced epitope retrieval was carried out in citrate buffer 0.1 M, with the help of a microwave oven. Two homozygous and two wild-type embryos were used per age.

Apoptosis detection (TUNEL)

The Apoptag kit (Chemicon) was used on formaldehyde-fixed, paraffin-embedded samples following the instructions of the manufacturer.

In situ *hybridization*

In situ detection of mRNAs with digoxigenin-labelled antisense riboprobes was performed on 20-μm cryostat sections, as described (Wilkinson & Nieto, 1993). We added an amplification step (tyramide), resulting in up to 100-fold increase in sensitivity (Adams, 1992; Yang *et al*., 1999). We used a GenePaint platform (Tecan Group, Maennedorf, Switzerland) so that prehybridization, hybridization, post-hybridization and detection were carried out automatically (Herzig *et al*., 2001). *In situ* hybridization was carried out on two wild-type and two homozygous brains (*Emx2* or *Pax6*) for every age. For Fig. 10, quantification was carried out by densitometric analysis of digital photography files with NIH-Image software (in the public domain). We measured average density per surface unit on identical square fields tightly fitting the hippocampal fissure region on four sections of each genotype at E18.5. 'Relative abundance values' were calculated by considering the highest value of each experiment as 100%. All sections compared had been automatically treated for *in situ* hybridization (see above) in the course of the same experiment.

Results

The Emx2$^{-/-}$ *hippocampal fissure region lacks radial glial scaffolding*

In the *Emx2*$^{-/-}$ dentate gyrus the radial glial marker protein GFAP is very decreased, suggesting alterations of the glial scaffolding (Oldekamp *et al*., 2004). Here we wanted to investigate in detail the state of the radial glial fibres in the dentate of this mutant. Detection of nestin, an intermediate filament specifically expressed in radial glial cells (Hockfield & McKay, 1985), has been used to label the radial glia in cortex and hippocampus in foetal brains (Super *et al*., 2000; Alcantara *et al*., 2006). The anti-nestin antibody showed that in wild-type, the dentate migratory pathway at E18.5 was wide and formed by abundant fibres. Its two portions, radial and tangential, could be easily identified. The tangential portion ran parallel to the pia and abutted the tertiary matrix (Fig. 1A and C). In the mutant, however, the nestin-positive migratory pathway starting in the dentate neuroepithelium was abnormally sparse and short (Fig. 1B). Under high magnification, the mutant showed a thinned out radial pathway (arrows in Fig. 1D) followed by a short stretch that did not run tangentially but ended on the pia (Fig. 1D, arrowheads). Antibody detection of GFAP (another major radial glial marker) together with Ki-67 (a marker of dividing cells) (Gerdes *et al*., 1983) on wild-type revealed the scaffolding of the fissure (Fig. 2A, asterisk) as well as numerous progenitor cells migrating on GFAP-positive processes (Fig. 2A), as described (Eckenhoff & Rakic, 1984, 1988; Rickmann *et al*., 1987; Altman & Bayer, 1990a,b). In the mutant, the abortive radial glial fibres (arrow in Fig. 2B) supported the migration of only a few progenitors (arrowheads in Fig. 2B). These results indicate that dentate radial glial fibres are extremely reduced in number and have severely altered morphology in the *Emx2*$^{-/-}$ brain. Subpial migration of astrocyte precursors has a role in the formation of the normally developing fissure (Sievers *et al*., 1992). Accordingly, in the subpial region of the mutant we were able to observe GFAP- and Ki67-positive cells, although in very small numbers (Fig. 2C).

Emx2 *is specifically responsible for the growth of OMZ + DMZ (not IMZ)*

Next, we analysed the state of the marginal zones around the developing fissure (Fig. 2D) in the mutant. *Lhx6* is a LIM-homeobox gene (Grigoriou *et al*., 1998) expressed by a subpopulation of basal ganglia-born cells that migrate tangentially into the cortical marginal zone (Lavdas *et al*., 1999). In wild-type, *Lhx6*-expressing cells arrived at the hippocampal primordium by E15.5 (not shown). At E18.5, *Lhx6* mRNA was detected in the IMZ, and absent from the hippocampal fissure region (OMZ + DMZ) (Fig. 3A). In the *Emx2* mutant at that age, *Lhx6* mRNA was distributed in the IMZ (Fig. 3B and C). The neocortical marginal zone showed *Lhx6*-expressing cells and could be followed to the point (arrow in Fig. 3C) where it divides into *Lhx6*-

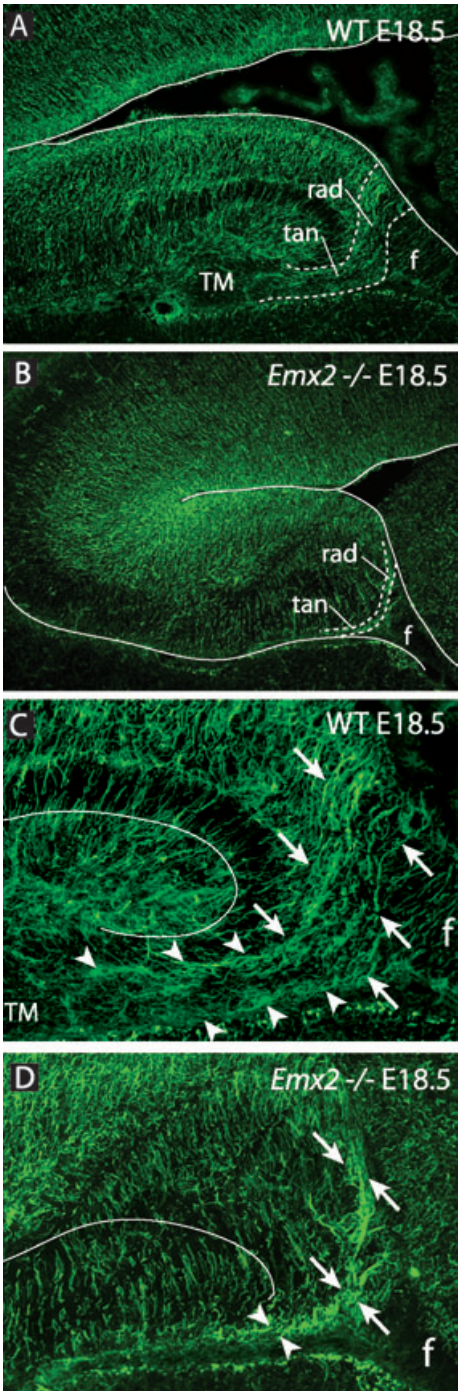

FIG. 1. Abnormal nestin expression in the $Emx2^{-/-}$ hippocampus. Anti-nestin antibody labelling on sagittal sections of E18.5 wild-type (A and C) and $Emx2^{-/-}$ (B and D) hippocampus. (C and D) High-magnification views of A and B, respectively. (A) Nestin fibres in wild-type hippocampus delineate the radial (rad) and tangential (tan) portions of the pathway (delineated with dotted lines) leading from the dentate neuroepithelium to the tertiary matrix (TM). (B) Nestin fibres in the $Emx2^{-/-}$ dentate are sparse and form only very shortened radial (rad) and tangential (tan) pathways (delineated with dotted lines). (C) High-magnification view of (A) showing the radial migration pathway of the dentate (arrows) followed by the tangential pathway (arrowheads); the fimbria (f) shows abundant parallel nestin-positive fibres. (D) High-magnification view of (B) showing the abnormally short and narrow nestin-positive migration pathway in the mutant (arrows, radial portion; arrowheads, tangential portion); the fimbria (f) is almost completely devoid of nestin-positive parallel fibres. A continuous line in (C) and (D) shows the ventral boundary of the CA pyramidal layer.

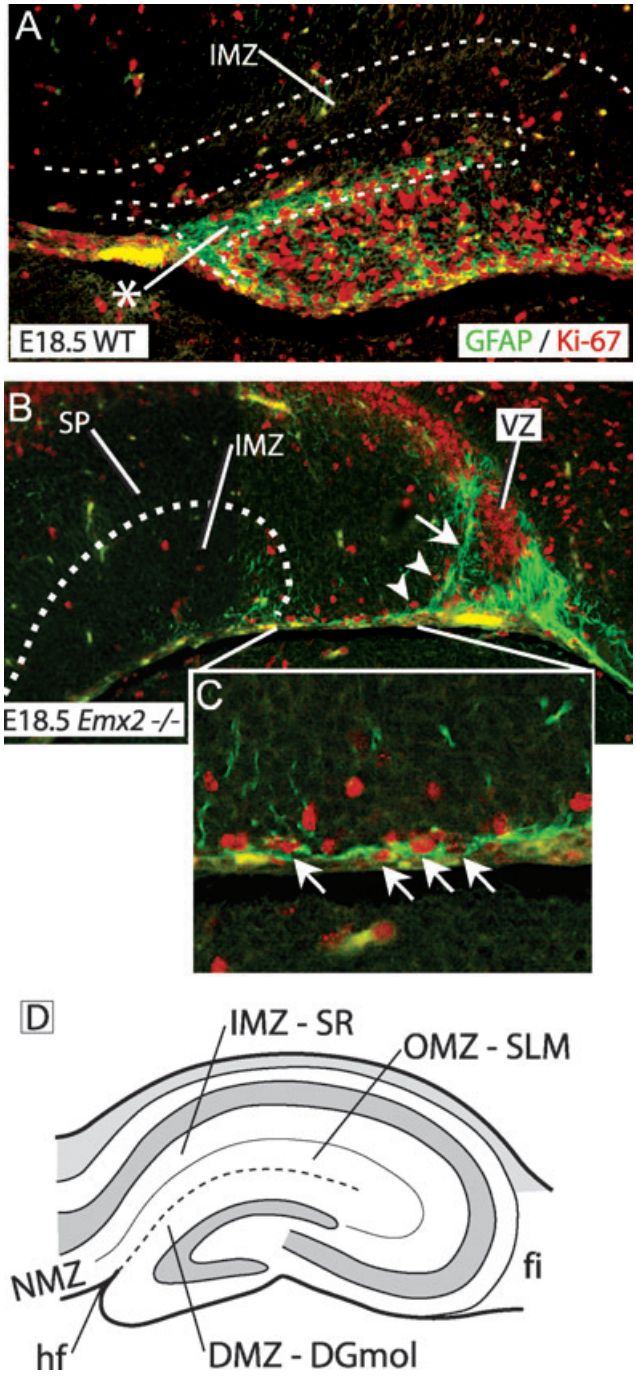

FIG. 2. Abnormal glial fibrillary acidic protein (GFAP) expression in the $Emx2^{-/-}$ hippocampus. (A–C) Antibody localization of GFAP (green) and Ki-67 (red) on E18.5 wild-type (A) and $Emx2^{-/-}$ (B and C) hippocampus. Asterisk in (A) points at fissure scaffolding. Notice the scarce progenitors (arrowheads) that migrate on the radial glial processes (arrow) of the mutant (B). (C) A magnified portion of B shows some subpial migrating GFAP-positive cells (arrows) in the mutant. (D) Diagram showing the marginal zones around the developing fissure region. Abbreviations: DGmol, molecular layer of dentate gyrus; DMZ, marginal zone of dentate gyrus; fi, fimbria; hf, hippocampal fissure; IMZ, inner marginal zone; NMZ, neocortical marginal zone; OMZ, outer marginal zone; SLM, stratum lacunosum-moleculare; SP, stratum pyramidale; SR, stratum radiatum; VZ, ventricular zone.

expressing IMZ and non-*Lhx6*-expressing OMZ + DMZ. *Lhx6* expression in the mutant, acting as a 'negative staining', defined the mutant OMZ + DMZ as a narrow band of cells between the IMZ

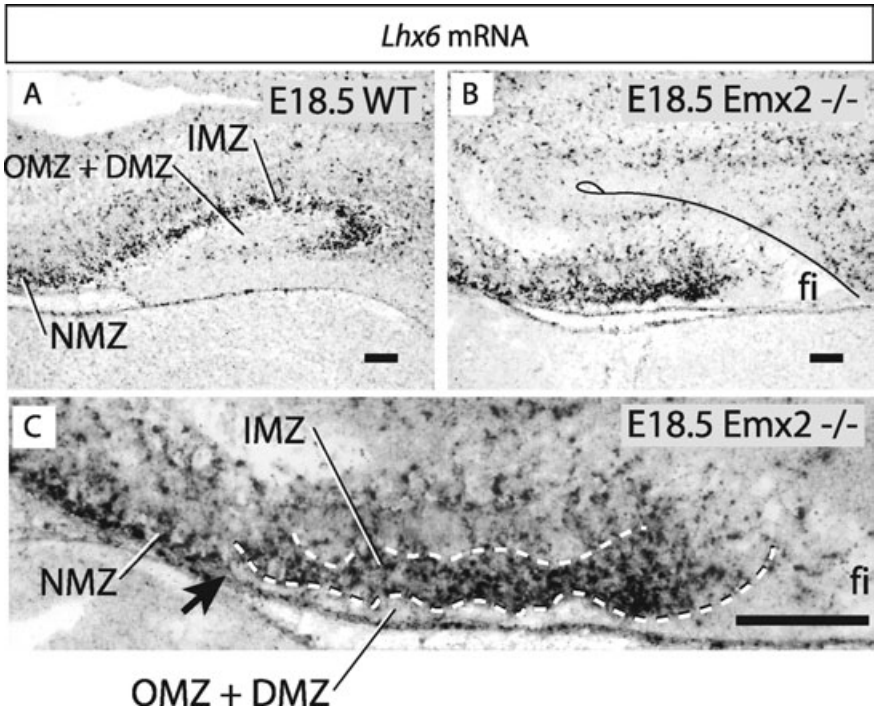

FIG. 3. Atrophic hippocampal fissure region in the $Emx2^{-/-}$ hippocampus. (A) By E18.5, *Lhx6*-expressing cells have entered the wild-type hippocampus and distribute along the inner marginal zone (IMZ) but not the outer marginal zone (OMZ). (B) In the E18.5 $Emx2^{-/-}$ hippocampus there is *Lhx6* expression in the IMZ. (C) A high-magnification view of (B), which shows that in the E18.5 $Emx2^{-/-}$ hippocampus, *Lhx6* expression in the IMZ (between dotted lines) acts as a 'negative staining' of the OMZ + dentate marginal zone (DMZ), which are devoid of *Lhx6* and appear very reduced. Arrow shows the 'entrance' to the hippocampal marginal zone, where NMZ divides into IMZ and OMZ. Abbreviations: fi, fimbria; NMZ, neocortical marginal zone. Scale bars: 100 μm.

(dorsally) and the pia (ventrally). The separation between both was remarkably well respected in the mutant (Fig. 3C).

These findings indicate that the marginal zones around the $Emx2^{-/-}$ hippocampal fissure region are correctly specified and located, and that at least the IMZ is populated by the appropriate cells. However, the mutant OMZ + DMZ are unable to grow to their normal size (they are atrophic).

Reln-*expressing cells in the* Emx2$^{-/-}$ *developing hippocampal fissure region*

Next we wanted to analyse the cells of the OMZ + DMZ. A very prominent cell group in these layers is the one formed by the *Reln*-expressing cells (Alcantara *et al*., 1998). We detected characteristically large amounts of *Reln*-expressing cells in wild-type marginal zone (Fig. 4A), while the $Emx2^{-/-}$ dentate (Fig. 4B) showed a very decreased number of them, located at the pial border in the presumptive localization of the hippocampal fissure (where the hippocampal primordium slightly folds in). Some *Reln*-expressing cells migrated dorsally into the IMZ, as observed also in wild-type (Fig. 4A and B).

We then analysed the expression of the LIM-homeobox gene *Lhx5*, a marker of the *Reln*-expressing cells of the marginal zone (Yamazaki *et al*., 2004) expressed in the developing hippocampus (Bertuzzi *et al*., 1996; Sheng *et al*., 1997; Hobert & Westphal, 2000). The brains of mice carrying null mutations of *Lhx5* show a hippocampal phenotype similar to that of $Emx2^{-/-}$, with loss of fissure region, dentate gyrus and *Reln*-expressing cells in the marginal zone (Zhao *et al*., 1999). We detected *Lhx5* mRNA in the wild-type neocortical marginal zone (not shown) and in the hippocampus (Fig. 4C), where abundant *Lhx5* cells were present in the OMZ + DMZ. This pattern was very similar to that of *Reln*, except that only very few *Lhx5* cells entered the wild-type IMZ (compare Fig. 4A to C). In the mutant, *Lhx5* expression disappeared from the neocortical marginal zone (not shown), but was conserved in the reduced OMZ + DMZ (Fig. 4D). The pattern of *Lhx5* in the mutant (Fig. 4D) was similar to that of *Reln* (Fig. 4B), with the only exception that (as in wild-type) very few *Lhx5*-expressing cells entered the IMZ (Fig. 4C). The results in Figs 3 and 4 show that, at the point where the neocortex meets the hippocampal primordium, the marginal zone divides into two regions (IMZ and OMZ + DMZ), differentially expressing *Lhx6 (*the IMZ; Fig. 3A) or *Lhx5 (*the OMZ + DMZ; Fig. 4C). The OMZ + DMZ are atrophic in the Emx2 mutant, and their *Reln*-expressing cells are severely decreased in number.

Reln-*expressing cells of the fissure region express* Emx2

Emx2 is a known marker of *Reln*-expressing cells of the marginal zone (Hevner *et al*., 2003). The fact that a certain number of *Lhx5*- and *Reln*-expressing cells remain in the *Emx2*-deficient fissure region could indicate the existence of some non-*Emx2*-expressing *Reln*-expressing cells in this area. In order to investigate this possibility, we used *in situ* hybridization for *Emx2* mRNA with a probe against the 3′ UTR of *Emx2* mRNA and were able to detect *Emx2* transcriptional activation (*Emx2*-TA) in homozygous brains. At E18.5, *Emx2* was strongly expressed in all portions of the dentate gyrus, and also in the OMZ, but not in the IMZ (Fig. 4E). In the mutant, *Emx2*-TA could be detected in a small group of cells (Fig. 4F) similar in size and position to that expressing *Reln* and *Lhx5*. A few labelled cells were inside the IMZ (arrowheads in Fig. 4F).

The labelling of *Reln*, *Lhx5* and *Emx2* expression in the mutant (Fig. 4) provides a 'positive staining' of the OMZ + DMZ matching the 'negative staining' of these layers imparted by IMZ-marker *Lhx6*

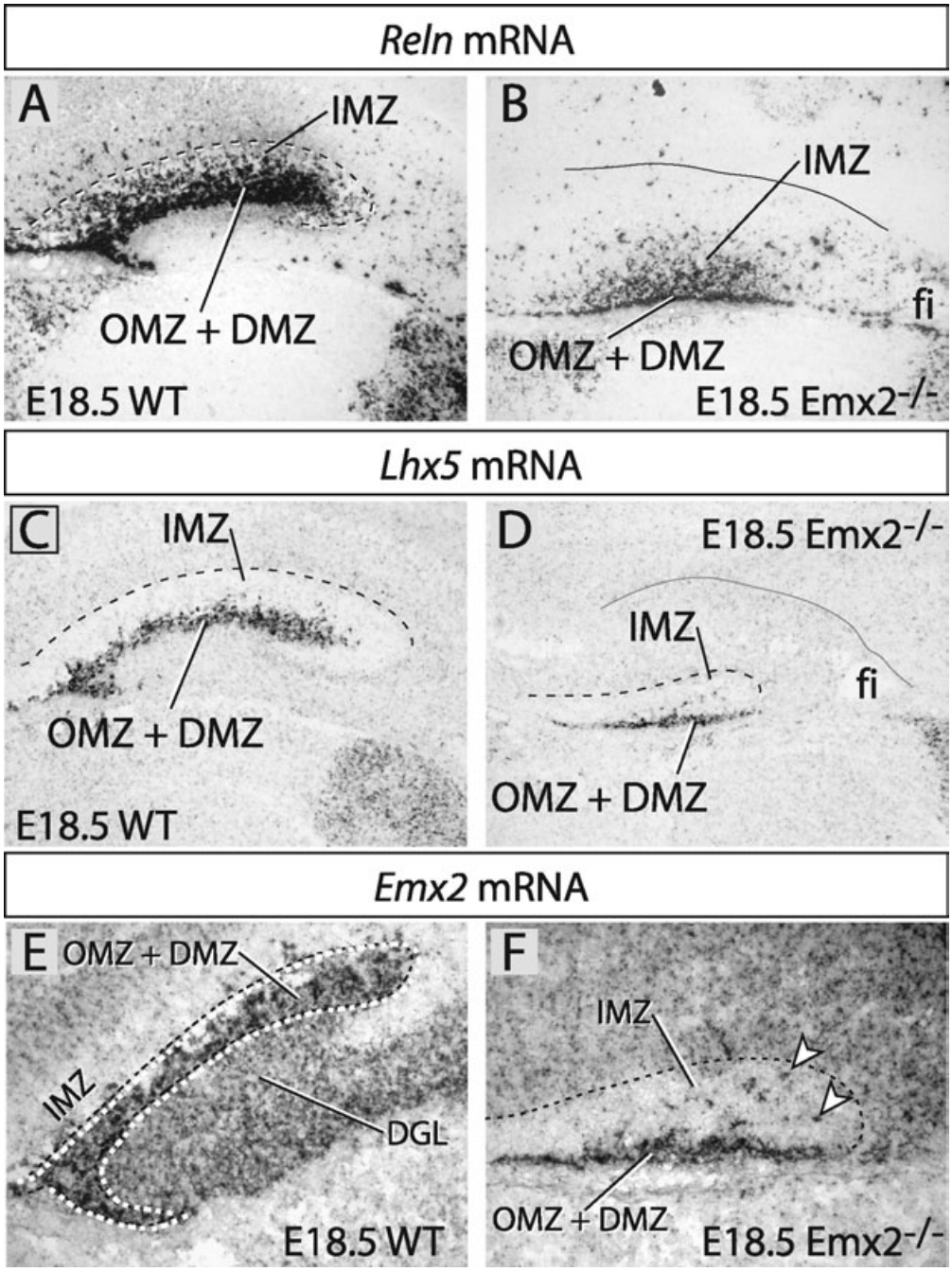

FIG. 4. *Reln*-expressing cells are very reduced in *Emx2*$^{-/-}$ hippocampus. (A and B) *reelin* mRNA in wild-type (A) and *Emx2*$^{-/-}$ (B) E18.5 hippocampus. (C and D) *Lhx5* mRNA in wild-type (C) and *Emx2*$^{-/-}$ (D) E18.5 hippocampus. (E, F) *Emx2* mRNA in wild-type (E) and *Emx2*$^{-/-}$ (F) E18.5 hippocampus. Abbreviations: DGL, dentate granular layer; DMZ, dentate marginal zone; fi, fimbria; IMZ, inner marginal zone; OMZ, outer marginal zone.

(Fig. 3). Furthermore, our data indicate that the few *Reln*-expressing cells of the mutant hippocampal fissure region do express *Emx2* (i.e. show *Emx2*-TA) and that, however, they do not need the Emx2 protein to be produced, correctly localized and express at least some of their specific markers.

Further characterization of the Reln*-expressing cells in the* Emx2$^{-/-}$ *fissure*

At this point we wanted to characterize further this population of *Reln*-expressing cells of the fissure region, which express *Emx2* but, as opposed to the rest, do not need this gene as an essential requirement. Subdivision of marginal zone cells in the neocortex has been tried by different methods, each with advantages and disadvantages (see Discussion). We decided to use *in situ* hybridization to characterize the expression by these cells of five additional marker genes known to be expressed in the marginal zone. Calretinin (*Calb2*) is a gene expressed by all reelin-expressing cells of the marginal zone from early on in development (Takiguchi-Hayashi *et al*., 2004); expression of this gene can be used for instance to identify Cajal-Retzius cells in reeler mutants, which do not express reelin (Coulin *et al*., 2001). We found abundant *Calb2* mRNA in the wild-type fissure (Fig. 5A) as expected, and we could ascertain expression of *Calb2* also in the mutant fissure

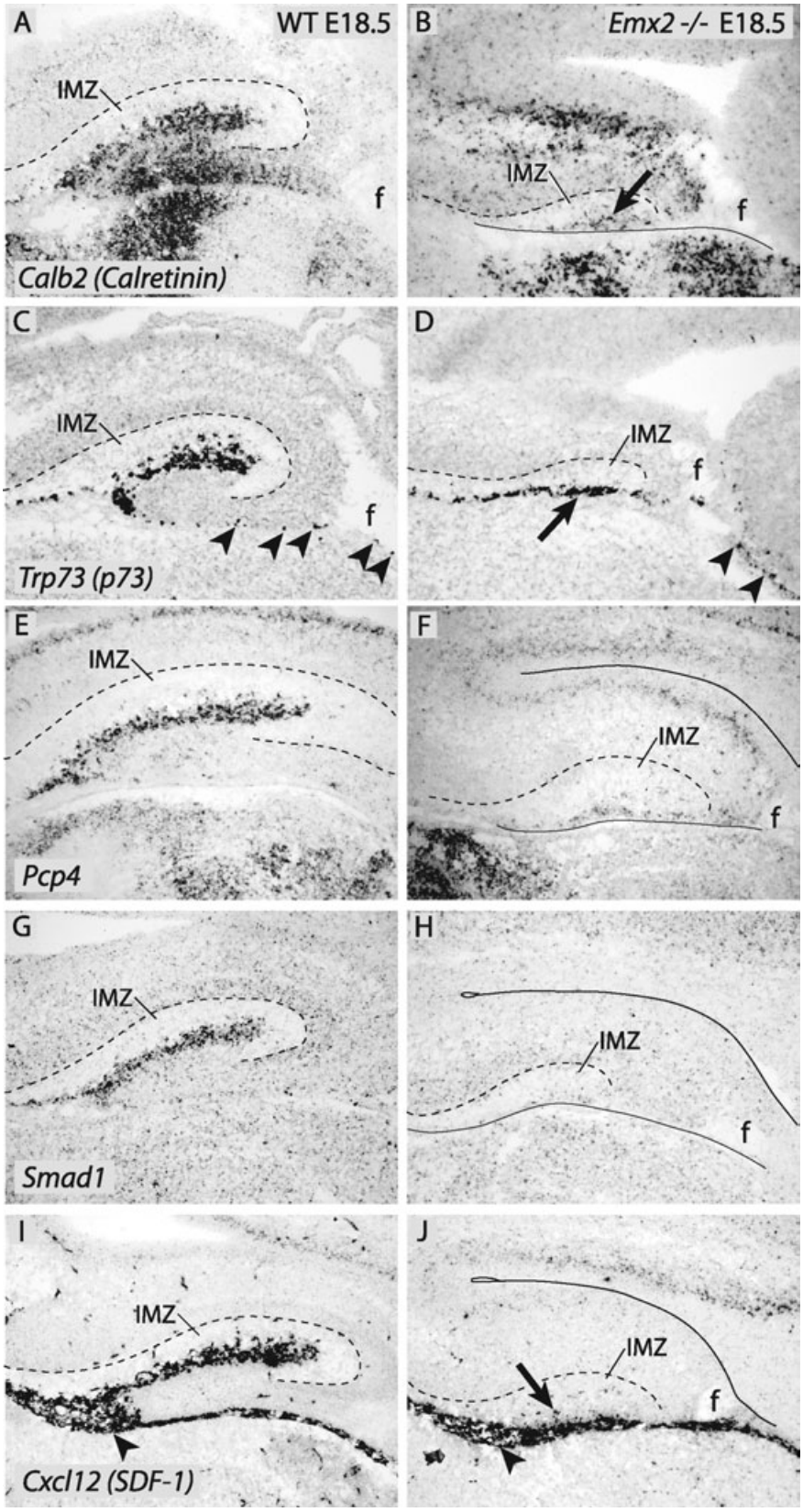

FIG. 5. Genetic marker expression in the *Emx2–/–* hippocampus. (A and B) Detection of *Calretinin* (*Calb2*) mRNA in the E18.5 wild-type (A) and $Emx2^{-/-}$ (B) hippocampus. Arrow in (B) points at remnants of *Calb2* expression in the mutant. (C and D) Detection of *Trp73* mRNA in the E18.5 wild-type (C) and $Emx2^{-/-}$ (D) hippocampus. Arrowheads in C and D show a trail of Trp73 expression from the cortico-choroid region to the hippocampus. Arrow in D shows Trp73 expression in the mutant hippocampal marginal zone. (E and F) Detection of *Pcp4* mRNA in the E18.5 wild-type (E) and $Emx2^{-/-}$ (F) hippocampus. (G and H) Detection of *Smad1* mRNA in the E18.5 wild-type (G) and $Emx2^{-/-}$ (H) hippocampus. (I and J) Detection of *Calretinin* (*Calb2*) mRNA in the E18.5 wild-type (I) and $Emx2^{-/-}$ (J) hippocampus. Arrowhead in I and J points at pial expression. Arrow in J shows a few labelled cells in the mutant. Abbreviations: f, fimbria; IMZ, inner marginal zone.

(Fig. 5B), consistently in a pattern similar but not exactly matching that of *Reln*, *Lhx5* or *Emx2* (Fig. 4B, D and F). Expression of transcription factor gene *p73* (*Trp73*) together with *Reln* is considered characteristic of Cajal-Retzius cells (Abraham *et al*., 2004). We found strong expression of *p73*/Trp73 in the wild-type hippocampal marginal zone as expected (Fig. 5C). A trail of labelled cells could be seen apparently originated in the former 'hem' region (cortico-choroidal border) (arrowheads in Fig. 5C). In the mutant, labelled cells formed a compact, strongly expressing group (arrow in Fig. 5D), with the same position and appearance as markers *Reln*, *Lhx5* and *Emx2* (Fig. 4B, D and F). A trail of cells from the former 'hem' could be followed also in the mutant (arrowheads in Fig. 5D). *Pcp4* encodes a modulator of calcium-signalling cascades (Slemmon *et al*., 1996) and has been recently identified as a marker of *Reln*-expressing cells of the marginal zone (Yamazaki *et al*., 2004). We detected it in the wild-type developing fissure region (Fig. 5E), but it was virtually absent or only very weakly expressed from the mutant hippocampal marginal zone (Fig. 5F). *Smad1* is a transcription factor gene essential for the transduction of the BMP signalling pathway (Angley *et al*., 2003) as well as a key player in angiogenesis (Goumans *et al*., 2003). It has also been identified as a marker of *Reln*-expressing cells of the marginal zone (Yamazaki *et al*., 2004). Accordingly, we found *Smad1* expression in the wild-type foetal fissure (Fig. 5G), but completely absent in the mutant (Fig. 5H). Finally, *Cxcl12*/SDF-1 is a cytokine gene essential in hippocampal development (Bagri *et al*., 2002) and in angiogenesis (Mirshahi *et al*., 2000; Salcedo & Oppenheim, 2003), and a marker of *Reln*-expressing cells of the marginal zone (Yamazaki *et al*., 2004). We detected its expression in the fissure region of the wild-type foetus (Fig. 5I), as well as in pial structures of vascular appearance (arrowhead in Fig. 5I). It was still present in the mutant pia (arrowhead in Fig. 5J), but absent from the hippocampus except for a few isolated cells (arrow in Fig. 5J).

Our results at this point suggested that the *Reln*-expressing cells remaining in the mutant hippocampus belong to a group normally expressing *Reln*, *Lhx5*, *Emx2* and *p73*/Trp73, but not *Smad1*, *Pcp4* or *Cxcl12*/SDF-1. Additionally, the presence of *p73*/Trp73 expression in the mutant, as well as in a trail of cells starting in the cortico-choroidal region, suggests that the 'survivor' cells are originated in the hem.

Development of Reln*-expressing cells in the* Emx2$^{-/-}$ *hippocampal marginal zone*

We then analysed the development of *Reln*-expressing cells of the marginal zone in the hippocampal region with anti-reelin antibodies in wild-type and mutant (Fig. 6). The distribution of *Reln*-expressing cells was similar in wild-type and mutant at E12.5. The hippocampal primordium showed only a few *Reln*-expressing cells, situated on the pial side (arrows in Fig. 6A and B). At E14.5, *Reln*-expressing cells were more abundant in the hippocampal primordium of the wild-type than in that of the mutant (Fig. 6C and D). In the mutant, the medial neocortical primordium (asterisk in Fig. 6D) showed at this age an abnormal and transient accumulation of neocortical *Reln*-expressing cells, which has been described (Mallamaci *et al*., 2000). We did not observe this transient accumulation in the hippocampus, which is the object of our study (separated from the neocortex by a dotted line in Fig. 6D). Later on, as in wild-type the hippocampal fissure started to develop, numerous *Reln*-expressing cells began to accumulate in it (Fig. 6E). In the mutant, however, only a few labelled cells were found. They were aligned in a row in the place where the pallium folds in slightly, indicating the region of the abortive hippocampal fissure (Fig. 6F). This region showed in the mutant already at this age the same appearance as later at E18.5 (compare Fig. 6F with Fig. 4B). In order to quantify the reduction in *Reln*-expressing cells in the mutant, we counted the *Reln*-expressing cells in the hippocampal marginal zone in all sections of two wild-type and two mutant brains at E18.5. The average number of reelin-expressing cells in the mutant hippocampal marginal zone at this age was less than 20% of the number in the wild-type (Fig. 6G). Thus, in the *Emx2*$^{-/-}$ hippocampal primordium, *Reln*-expressing cells are sparse from early on, gradually becoming rarer except for one group that forms in the vestige of the hippocampal fissure.

Reln*-expressing cells of the hippocampal fissure are born at normal ages but in reduced numbers in the* Emx2$^{-/-}$ *mutant*

Our data at this point showed a loss of *Reln*-expressing cells in the *Emx2* mutant marginal zone (except for a specific subpopulation). We asked what could be the mechanism of that loss. Cells can be reduced in number either because of reduced proliferation, or because of increased cell death, or because they migrate away and are not to be found where usually expected. In the neocortex of the *Emx2*$^{-/-}$ mutant there is a loss of *Reln*-expressing cells (Mallamaci *et al*., 2000; Shinozaki *et al*., 2002), although the mechanism is still not clear. In addition, and contrary to the situation in the hippocampus, the mutant neocortex does not show any specific remnant population of *Reln*-expressing cells.

First of all, to investigate hippocampal *Reln*-expressing cell proliferation in wild-type and mutant, we injected pregnant mice with BrdU at different gestation ages. Previous work (Takiguchi-Hayashi *et al*., 2004) has shown that *Reln*-expressing cells of the marginal zone of the cortex are born from E10.5 to E13.5, so we injected BrdU at these ages. We then used antibodies to detect reelin (cytoplasm) and BrdU (nucleus) in *Reln*-expressing cells of this region at E18.5 (Fig. 7A and B). At E18.5, both wild-type and *Emx2*$^{-/-}$ hippocampal fissure region (Fig. 7C) contain *Reln*-expressing cells born at E11.5, E12.5 and E13.5. Surprisingly, counting cells at E18.5 we did not find *Reln*-expressing cells labelled at E10.5. No *Reln*-expressing cells of this region were born at E14.5 (not shown). However, consistent with our previous results (Fig. 6), the total number of *Reln*-expressing cells in the mutant fissure region was much smaller than in wild-type. One *Emx2*-expressing neuroepithelial region characterized as a major source of *Reln*-expressing cells and immediately adjacent to the hippocampus is the cortical hem (Shinozaki *et al*., 2002; Takiguchi-Hayashi *et al*., 2004). Therefore, our result suggests that reduced production of *Reln*-expressing cells by the *Emx2*$^{-/-}$ cortical hem is the main cause of the absence of these cells in the mutant hippocampal primordium. This points to a possible answer to the question of the mechanism of disappearance of *Reln*-expressing cells in the *Emx2*$^{-/-}$ hippocampus.

An early-born/early-transient population of Reln*-expressing cells in wild-type and* Emx2$^{-/-}$ *hippocampus*

Surprisingly, and in contrast to reliable reports establishing that cortical *Reln*-expressing cells of the marginal zone originate from E10.5 on (Alcantara *et al*., 1998; Mallamaci *et al*., 2000; Takiguchi-Hayashi *et al*., 2004), we did not observe in the E18.5 fissure any *Reln*-expressing cells born at E10.5 either in wild-type or in mutant. Besides, the mutant neocortex at E11.5 shows abundant *Reln*-expressing neurons (Mallamaci *et al*., 2000), presumably born on E10.5. Therefore, to examine this question in more detail, we injected pregnant mice with BrdU at E10.5, and collected the embryos at E12.5 and E14.5. Double-labelled cells (BrdU and reelin) were consistently detected in the fissure region of wild-type and mutant at E12.5 (Fig. 8A and B) but not at E14.5 (Fig. 8C and D). Counting BrdU-

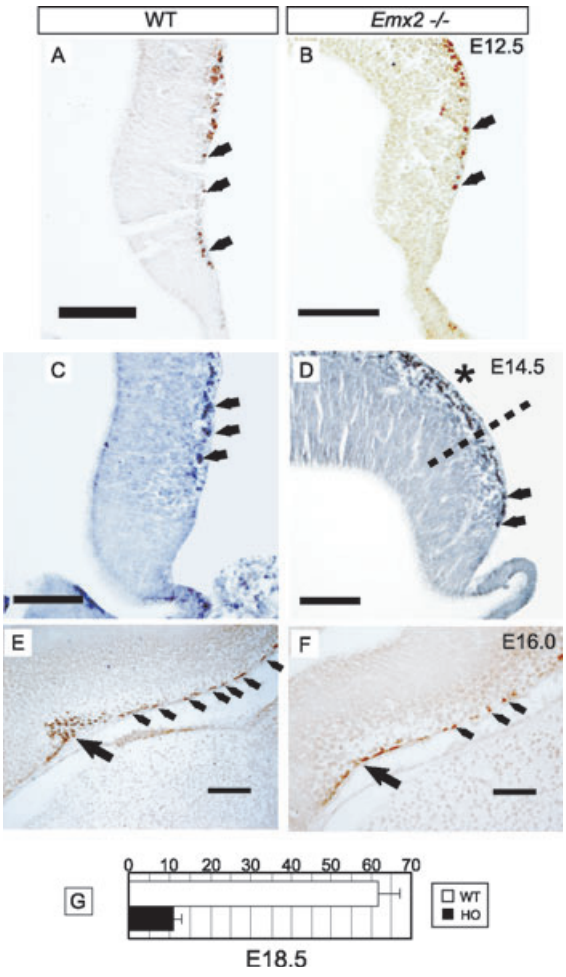

FIG. 6. Development of *Reln*-expressing cells in *Emx2*$^{-/-}$ hippocampus. Antibody localization of reelin in the wild-type (A, C and E) and *Emx2*$^{-/-}$ (B, D and F) medial cortex at E12.5 (A and B), E14.5 (C and D) and E16.0 (E and F). (A and B) At E12.5 both wild-type (A) and mutant (B) show scattered *Reln*-expressing cells (arrows) in presumptive hippocampus. (C and D) At E14.5, the wild-type hippocampus (C) shows a row of *Reln*-expressing cells (arrows), while the mutant hippocampus (D) shows some scattered *Reln*-expressing cells (arrows); an accumulation of *Reln*-expressing cells can be seen in the neocortex (asterisk), separated from hippocampal primordium (no accumulation) by a dotted line. (E and F) At E16.0, the wild-type hippocampus (E) shows a row of *Reln*-expressing cells (small arrows) and a larger group accumulating in the developing fissure region (large arrow). In the mutant hippocampus (F) *Reln*-expressing cells are less and scattered (small arrows), they do not accumulate in the fissure region (large arrow) and remain close to the pial border. (G) Reelin cells of the fissure per section of E18.5 brain in wild-type (white column) or *Emx2*$^{-/-}$ (black column).

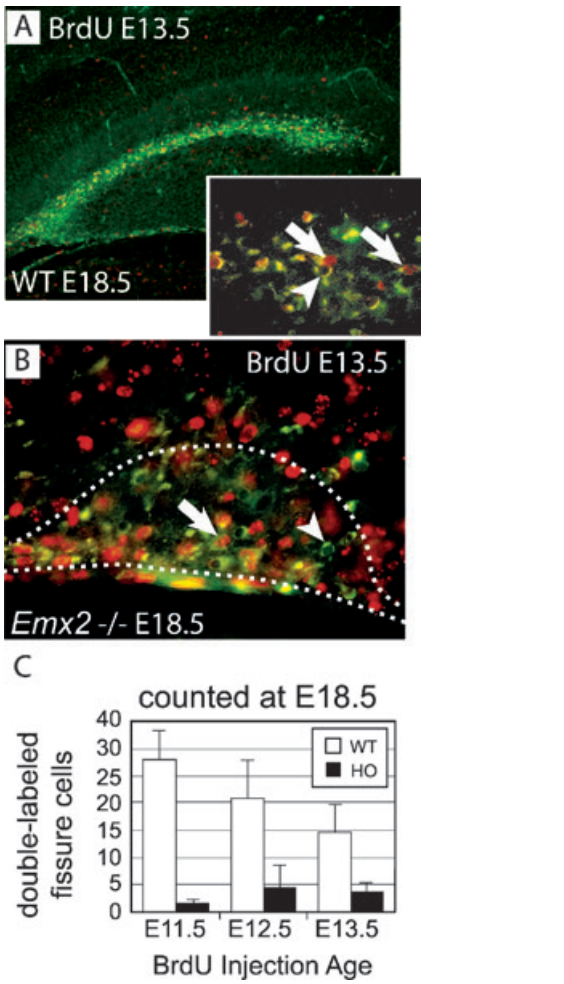

FIG. 7. Late-born *Reln*-expressing cells in the *Emx2*$^{-/-}$ hippocampus. (A and B) Examples of antibody detection of BrdU and reelin in hippocampal fissure region at E18.5 in wild-type (A) and *Emx2*$^{-/-}$ (B). (A) The entire fissure region, inset shows a high-magnification detail. Numerous cells are labelled green for reelin (arrowheads), of which some show red nuclei for BrdU (arrows). (C) Number of BrdU-reelin double-labelled cells in the hippocampal fissure per section (both sides) found at E18.5 after BrdU injections either at E11.5, E12.5, E13.5 or E14.5. White bars, wild type; black bars, *Emx2*$^{-/-}$. Error bars: standard deviation.

labelled neurons at E12.5 and E14.5 in the whole pallial marginal zone (presumptive cortex and hippocampus) revealed a decrease of about 60% both in wild-type and in mutant (Fig. 8E), which can be attributed to 'dilution' in a rapidly growing brain. The decrease was, however, particularly dramatic for *Reln*-expressing cells, which almost completely disappear from the telencephalic marginal zone by E14.5 (Fig. 8F). Finally, we were not able to find any E10.5-born *Reln*-expressing cell in the hippocampal anlage at E14.5, either in wild-type or in mutant (Fig. 8G). We concluded that, in the wild-type as well as in the *Emx2*$^{-/-}$ brain there is an early-born and early-transient population of *Reln*-expressing cells. This population is born at about E10.5 and not detectable any more at E14.5, either because the cells migrate elsewhere or downregulate reelin expression (see Discussion).

No increase in cell death in the Emx2$^{-/-}$ *marginal zone*

In search of a mechanism for the reduced amount of *Reln*-expressing cells in the hippocampal marginal zone of this mutant, we reasoned that increased cell death could concur with reduced production to decrease the number of *Reln*-expressing cells. To investigate this, we performed TUNEL staining on wild-type and mutant brain sections at different ages (Fig. 9). Our analysis at E14.5, E16.5 (Fig. 9A–D) (and also at E12.5 and E18.5, not shown) revealed only a small number of cell death profiles in wild-type and mutant hippocampi, and failed to reveal any difference between the two genotypes. This is consistent with decreased production as the main cause of the *Reln*-expressing cell deficiency in the *Emx2*$^{-/-}$ hippocampal fissure region.

Both Pax6 *and* Emx2 *are needed in the development of the* Reln*-expressing cells of the hippocampal fissure region*

The previous results suggested that the formation of the hippocampal fissure region and its colonization by *Reln*-expressing cells are specific

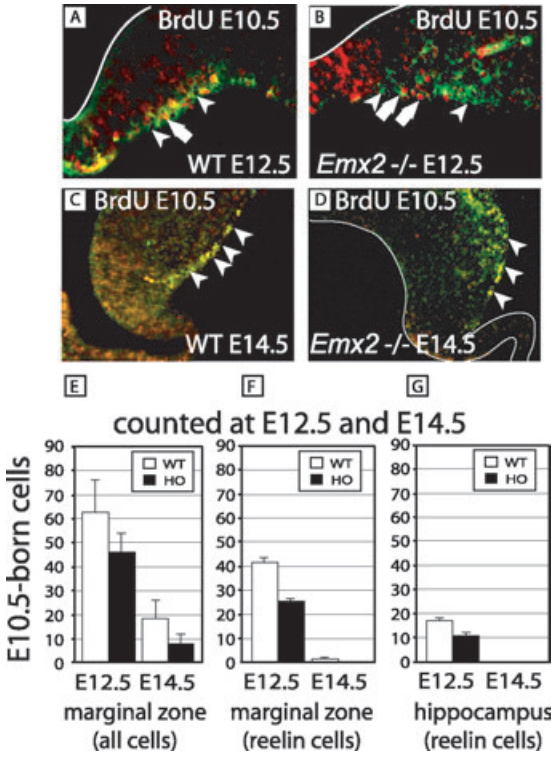

FIG. 8. Early-born *Reln*-expressing cells in the *Emx2*$^{-/-}$ hippocampus. (A and B) Antibody detection of BrdU (injected E10.5) and reelin in hippocampus at E12.5 in wild-type (A) and *Emx2*$^{-/-}$ (B). Numerous cells are labelled green for reelin (arrowheads), of which some show red nuclei for BrdU (arrows). (C and D) Antibody detection of BrdU (injected on E10.5) and reelin in hippocampus at E14.5 in wild-type (C) and *Emx2*$^{-/-}$ (D). Of the cells labelled green for reelin (arrowheads), none shows red nuclei for BrdU (injected on E10.5). (E–G) Number of E10.5-born cells (detected by BrdU antibody) per histological section (both sides) at E12.5 and E14.5 in wild-type and *Emx2*$^{-/-}$. (E) Countings including the total marginal zone or in the case of E12.5 the outer border of the pallium. (F) Only double-labelled cells have been counted. (G) Only double-labelled cells of the hippocampal primordium have been counted. Error bars: standard deviation.

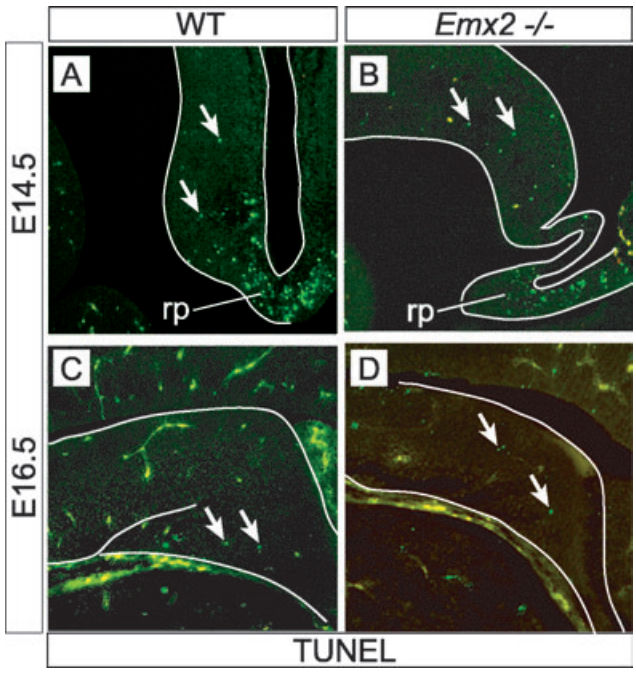

FIG. 9. Cell death in *Emx2*$^{-/-}$ hippocampus is unchanged. TUNEL-stained sections of wild-type (A and C) and *Emx2*$^{-/-}$ (B and D) hippocampal area at E14.5 (A and B) and E16.5 (C and D). Cell death rates are low in both wild-type and mutant at every age investigated. The telencephalic roof (rp in A and B) shows a characteristically large number of dying cells. Arrows show some typical cell death images.

regionalization processes regulated by *Emx2*. *Pax6* is a key developmental transcription factor that functionally antagonizes *Emx2* in pallial regionalization (Bishop *et al*., 2000, 2002). To investigate a possible role of *Pax6* in the formation of the hippocampal fissure we looked at the distribution of *Reln*-expressing cells in the fissure region of foetuses carrying the *Pax6* null allele *Small eye* (*Sey*) (Hill *et al*., 1991). The *Pax6*$^{Sey/Sey}$ neocortical marginal zone is thicker than in wild-type, containing abnormally large numbers of *Reln*-expressing cells (Stoykova *et al*., 2003). We confirmed an increase in *Reln*-expressing cells in the *Pax6*$^{Sey/Sey}$ neocortical marginal zone (Fig. 10A and B). However, we detected a decrease in *Reln*-expressing cells in *Pax6*$^{Sey/Sey}$ hippocampal fissure region as compared with wild-type (compare Fig. 10C and D). The enlarged *Pax6*$^{Sey/Sey}$ neocortical marginal zone (arrowheads in Fig. 10D) consistently showed a narrowing in the immediate vicinity of the hippocampal fissure region (arrow in Fig. 10D), i.e. a region of transition where the neocortical marginal zone kept its normal width in the *Pax6*$^{Sey/Sey}$ mutant. Intriguingly, the reduction in *Reln*-expressing cells in *Pax6*$^{Sey/Sey}$ was limited to the OMZ, while the DMZ seemed unaffected (Fig. 10E and F). We confirmed the reduction in *Reln* mRNA in the *Pax6*$^{Sey/Sey}$ OMZ by measuring the intensity of *Reln* expression in neocortical marginal zone and OMZ in wild-type and mutant (Fig. 10G and H). These results show that *Pax6* has differential and opposed effects on neocortical vs. fissural *Reln*-expressing cells, and that both *Emx2* and *Pax6* are needed in order to obtain an OMZ of normal size and with its full complement of *Reln*-expressing cells.

Discussion

We have investigated for the first time developmental anomalies of the *Emx2*-deficient hippocampal marginal layers. In particular, we have focused on the involvement of *Emx2* in hippocampal folding (fissure formation) and in the development of the OMZ (precursor of the stratum lacunosum-moleculare). Our findings include: (i) *Emx2* is essential for the development of the radial glial scaffolding of the hippocampal fissure; (ii) the marginal zone immediately apposed to the *Emx2*$^{-/-}$ hippocampal fissure region (OMZ) is atrophic and reduced to a vestigial size – it can be said that the mutant lacks a stratum lacunosum-moleculare; (iii) the *Reln*-expressing cells that should occupy the OMZ (as well as the DMZ) are severely decreased in the *Emx2*$^{-/-}$ because of decreased production; (iv) we have identified a subpopulation of hippocampal *Reln*-expressing cells, probably born in the hem, expressing a specific combination of markers, and for which *Emx2* is not essentially required; (v) finally, the function of *Emx2* and *Pax6* in the formation of the presumptive stratum lacunosum-moleculare is similar, although they functionally antagonize each other in the neocortical marginal zone.

Finally, all the structures affected by the *Emx2*$^{-/-}$ phenotypes described here (fissure region *Reln*-expressing cells, dentate neuroepithelium, neocortical hem) express *Emx2* (the present work and Oldekamp *et al*., 2004), suggesting that these mutant phenotypes could be to a large degree cell autonomous.

Glial scaffolding and Reln*-expressing cells in the fissure region*

Radial glial cells of the dentate ventricular layer give rise to two systems of processes (Rickmann *et al*., 1987; Sievers *et al*., 1992; Yuasa, 2001).

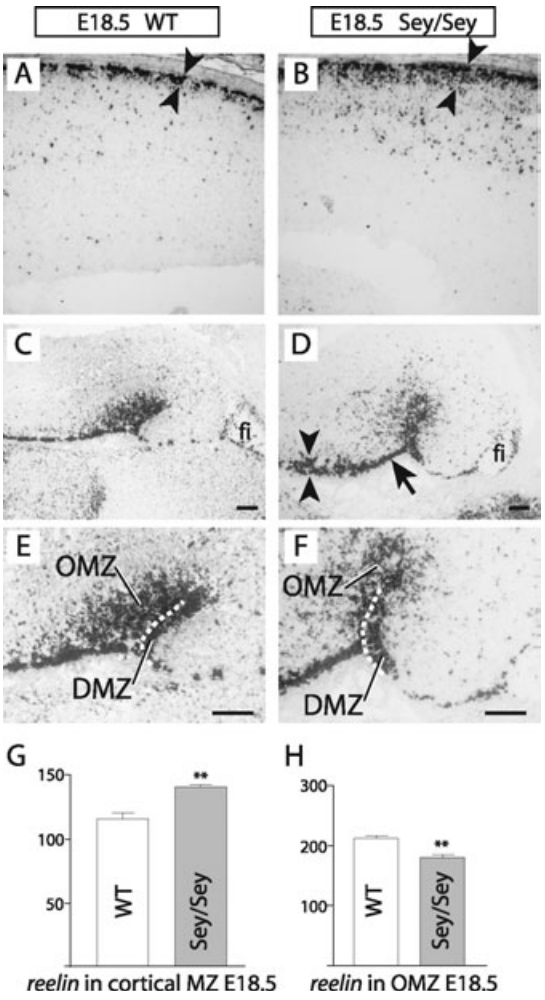

FIG. 10. Both *Emx2* and *Pax6* are involved in outer marginal zone (OMZ) formation. (A and B) *In situ* hybridization for *Reln* mRNA on E18.5 wild-type (A) and $Pax6^{Sey/Sey}$ mutant (B) neocortex. Pairs of arrowheads show the marginal zone thicker in (B) ($Pax6^{Sey/Sey}$) than in (A) (wild-type). (C) *Reln* expression on a sagittal section of E18.5 wild-type brain. (D) *Reln* expression on a sagittal section of E18.5 $Pax6^{Sey/Sey}$ brain. Arrowheads show the thickened neocortical marginal zone in the mutant. Arrow points at narrow band of mutant marginal zone immediately adjacent to hippocampal fissure. (E) High-magnification detail of C with a dotted line separating the labelled dentate marginal zone (DMZ, right side) from the labelled OMZ (left side). (F) High-magnification detail of D showing that the reduction in reelin expression is mostly on the OMZ side. (G and H) Densitometric analysis of *Reln* expression intensity confirms significant increase in $Pax6^{Sey/Sey}$ neocortical marginal zone (G) and significant decrease in $Pax6^{Sey/Sey}$ hippocampal fissure (H). Error bars: standard deviation. Abbreviations: fi, fimbria; WT, wild type. Scale bars: 100 μm. $^{**}P < 0.01$.

One of them follows a straight course immediately adjacent to the fimbria towards the pial side (radial migratory pathway, Fig. 1A and C) and then turns in the direction of the future dentate granular layer (tangential migratory pathway, Fig. 1A and C). The second system of processes courses towards the developing fissure, whose scaffolding it forms. As a third glial component, GFAP-expressing astrocytes able to divide migrate tangentially under the pia, enter the developing fissure and spread along its scaffolding forming two–three cell rows (Sievers *et al*., 1992). Our results here show that in the $Emx2^{-/-}$ dentate the various glial components are differentially altered. The long radial glial processes forming the scaffolding of the fissure are completely missing (Fig. 2A and B); the processes forming the migratory pathway are present but dramatically reduced in number and length (Figs 1B and D, and 2B). Finally, some of the subpial migratory-dividing astrocytes are present in the mutant, although they fail to form or colonize the fissure region, remaining close to the pia (Fig. 2C).

Our analysis emphasizes the existence of trophic interactions between the different elements of the fissure region. Although *reeler* mutants are able to develop a hippocampal fissure (Hamburgh, 1963), reelin is essential for the proper development of the radial glia of cortex and cerebellum (Soriano *et al*., 1997; Hartfuss *et al*., 2003). Because *Emx2* is expressed both by *Reln*-expressing cells and dentate gyrus neuroepithelium, probably in the $Emx2^{-/-}$ brain both elements are altered, and later these defects compound.

Reln-*expressing cells and hippocampal folding*

The OMZ + DMZ of the $Emx2^{-/-}$ is extremely reduced in size (Fig. 3). Neither reports by other authors nor our own observations (not shown) have found comparable atrophy in the $Emx2^{-/-}$ neocortical marginal zone. This suggests specific trophic effects of *Reln*-expressing cells on the growth of the marginal zones around the fissure region. Because the proper growth of these marginal zones would lead to hippocampal folding, maybe this observation can be put in the context of other mutant phenotypes, including at least the *Lhx5* (Zhao *et al*., 1999) and *p73* mutants (Meyer *et al*., 2004), characterized by a decrease in *Reln*-expressing cells together with failure of hippocampal folding. This adds to growing evidence of a role of the *Reln*-expressing cells in cortical folding involving probably other molecules than reelin (Alcantara *et al*., 2006).

A specific subpopulation of Reln-*expressing cells in the hippocampus does not require* Emx2

The marginal zone contains a morphologically heterogeneous population of *Reln*-expressing cells, which has been subclassified by using the Golgi method (Marin-Padilla, 1998) and by means of antibodies against calbindin and calretinin (reviewed in Soriano & Del Rio, 2005). More recently, detection of gene expression by *in situ* hybridization has revealed that different groups of *Reln*-expressing cells of the marginal zone express specific markers, perhaps revealing their different points of origin in the telencephalon (Lavdas *et al*., 1999; Hevner *et al*., 2003; Yamazaki *et al*., 2004). We have used nine marker genes known to be expressed at least in some reelin-expressing cells of the marginal zone in order to characterize the unique group of *Reln*-expressing cells, located in the hippocampus, which are preserved in the *Emx2* mutant. These cells express indeed *Reln* and *Emx2*, as well as *Lhx5* and *p73/Trp73* (Figs 3–5). A few of them seem to weakly express *Calb2* (*calretinin*) and *Cxcl12/*SDF-1 (Fig. 5B and J). Finally, none of them expresses *Pcp4* or *Smad1* (Fig. 5F and H). *Cxcr4* encodes the receptor for cytokine *Cxcl12* and is also expressed in the hippocampal fissure region (Lu *et al*., 2002; Yamazaki *et al*., 2004). However, our data (not shown) indicate that it is also expressed abundantly in the IMZ, making it difficult to assess in the mutant hippocampus. Expression of *Reln* and *p73/*Trp73 in the marginal zone has been considered a defining trait of Cajal-Retzius cells (Meyer *et al*., 2002, 2004; Abraham *et al*., 2004); therefore, at least some of the 'survivor' cells in the mutant hippocampus can be considered Cajal-Retzius cells.

Mechanism of disappearance of Reln-*expressing cells of the hippocampal fissure in the* Emx2 *mutant*

Several mechanisms can contribute to the absence of a certain cell population from the place where it would usually be expected: reduced

proliferation, increased cell death, abnormal migration or a combination thereof. According to our results, it would seem that the decrease in *Reln*-expressing cells in the *Emx2* mutant hippocampal marginal zone is due to a decrease in production. Most, if not all, *Reln*-expressing cells of the marginal zone of the medial cortex (including hippocampus) originate in the immediately adjacent cortical hem (Shinozaki *et al*., 2002; Takiguchi-Hayashi *et al*., 2004; Yoshida *et al*., 2006), an *Emx2*-expressing neuroepithelial area severely decreased in size in the $Emx2^{-/-}$ brain (Shinozaki *et al*., 2002, 2004). Accordingly, the distribution of reelin in the developing hippocampus (Fig. 6) plus the birthdate labellings (Fig. 7) point to important reduction in *Reln*-expressing cell production in $Emx2^{-/-}$ brains. The second important mechanism that could contribute to a reduction in the number of *Reln*-expressing cells could be increased cell death. Our results with TUNEL labelling (Fig. 9) on wild-type brains agree with reports that most developmental cell death in wild-type rodent pallium is postnatal (Ferrer *et al*., 1990; Gould *et al*., 1991), and that prenatal cell death in the wild-type marginal zone is negligible (Thomaidou *et al*., 1997). In addition, we found no change in the $Emx2^{-/-}$ brain, excluding cell death as the mechanism of disappearance. Finally, the *Reln*-expressing cells of the *Emx2* mutant could migrate away from the telencephalon to some other place. However, the $Emx2^{-/-}$ cortex has demonstrable tangential migration defects (Shinozaki *et al*., 2002), which precludes migration as an explanation.

An early transient population of Reln*-expressing cells in the wild-type pallium*

Cortical *Reln*-expressing cells are transient and disappear early postnatally (Derer & Derer, 1990). Unexpectedly, we have found in wild-type as well as $Emx2^{-/-}$ brains a population of *Reln*-expressing cells born at E10.5 that seems to disappear within the next 3 days (Fig. 8). Analysis of *Reln*-expressing cells in the $Emx2^{-/-}$ neocortex (Mallamaci *et al*., 2000) detected a discrepancy between actual histological detection of reelin cells in the mutant at E11.5, and BrdU + reelin cell counting at E19.5 showing that cells born at E12.5 were absent. Mallamaci *et al*. (2000) solved this discrepancy by postulating the existence of two different populations of *Reln*-expressing cells: an early transient, not dependent on *Emx2*; and a late population, still detectable around birth, dependent on *Emx2*. In the present study, we have actually injected BrdU much earlier, at E10.5 and at E11.5, and we have in the hippocampus (and in the neocortex) data that permit to confirm this conjecture. Their disappearance cannot be in this case attributed to reduced proliferation, and our data with the TUNEL method preclude cell death as the mechanism. Intriguingly, these early transient *Reln*-expressing cells are able to migrate and invade the pallial marginal zone also in the $Emx2^{-/-}$ brain, suggesting that, unlike the ones born later, they do not depend on *Emx2* for tangential migration. Accordingly, their disappearance could mean that they migrate away from the pallium; alternatively, they could downregulate *Reln* as part of their normal differentiation.

Different development of neocortical vs. hippocampal Reln*-expressing cells*

In $Emx2^{-/-}$ brains, the neocortical marginal zone keeps its normal thickness and neocortical *Reln*-expressing cells transiently accumulate and then completely disappear (Mallamaci *et al*., 2000). Our results, however, indicate that the region around the developing mutant fissure region shows atrophic marginal zones but no abnormal accumulation or complete disappearance of *Reln*-expressing cells. This differential phenotype is consistent with reported differential survival of neocortical and hippocampal *Reln*-expressing cells (Del Rio *et al*., 1996; Drakew *et al*., 1998; Abraham & Meyer, 2003). Here we show that $Pax6^{Sey/Sey}$ has differential effects on neocortical and hippocampal *Reln*-expressing cells of the marginal zone as well (very increased in neocortex vs. slightly reduced in the fissure). Intriguingly, while *Pax6* and *Emx2* are antagonists in neocortical regionalization (Bishop *et al*., 2000, 2002; Shinozaki *et al*., 2002) and have opposite effects on neocortical *Reln*-expressing cells of the marginal zone (Stoykova *et al*., 2003), in the developing hippocampal fissure both mutations translate into a reduced number of *Reln*-expressing cells (although the role of *Pax6* is less dramatic and seems restricted to the OMZ, not DMZ). Hippocampal and neocortical marginal zone *Reln*-expressing cells have differential, specialized roles in the formation of layer-specific hippocampal connections (Del Rio *et al*., 1997) and in neocortical organization (Alcantara *et al*., 2006), respectively. Our results indicate that, in keeping with this functional diversity, the *Reln*-expressing cells found in the neocortex and hippocampus show differential developmental processes.

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Abbreviations

CA, Cornu Ammonis, Ammon's horn; DMZ, dentate marginal zone; GFAP, glial fibrillary acidic protein; IMZ, inner marginal zone; OMZ, outer marginal zone; TA, transcriptional activation.

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