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Impaired postnatal development of hippocampal neurons and axon projections in the Emx2–/– mutants

Nicolai E. Savaskan,*,† Gonzalo Alvarez-Bolado,‡,[1] Robert Glumm,* Robert Nitsch,* Thomas Skutella§,[2] and Bernd Heimrich*,[2]

*Department of Cell and Neurobiology, Institute of Anatomy, Berlin, Germany

†Department of Neurology, Humboldt University Medical School Charité, Berlin, Germany

‡Department of Molecular Cell Biology, Max Planck Institute of Biophysical Chemistry, Göttingen, Germany

§Neuroscience Research Center, Humboldt University Medical School Charité, Berlin, Germany

Abstract

The specification and innervation of cerebral subregions is a complex layer-specific process, primed by region-specific transcription factor expression and axonal guidance cues. In Emx2–/– mice, the hippocampus fails to form a normal dentate gyrus as well as the normal layering of principal neurons in the hippocampus proper. Here, we analyzed the late embryonic and postnatal development of the hippocampal formation and its axonal projections in mice lacking Emx2 expression *in vitro*. As these mutants die perinatally, we used slice cultures of Emx2 mutant hippocampus to circumvent this problem. In late embryonic Emx2–/– cultivated hippocampi, both the perforant path as well as the distribution of calretinin-positive cells are affected. Traced entorhinal afferents in co-cultures with hippocampus from embryonic Emx2–/– mice terminate diffusely in the prospective dentate gyrus in contrast to the layer-specific termination of co-cultures from wild-type littermates. In addition, in brain slice cultures from null mutants the presumptive dentate gyrus failed to develop its normal cytoarchitecture and mature dentate granule cells, including the lack of their mossy fiber projection. Our data indicate that Emx2 is essential for the terminal differentiation of granular cells and the correct formation of extrinsic and intrinsic hippocampal connections.

Keywords: axon guidance, cortical maturation, dentate gyrus, homeobox gene, perforant path, slice cultures.

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The hippocampus, with its intrinsic and extrinsic projections, is a current model system to study axonal pathfinding and layer-specificity. The *Emx2* gene, which is a vertebrate homeobox gene related to the *Drosophila* empty spiracles (*ems*) gene (Dalton *et al*. 1989), is expressed in the anterior CNS of the developing mouse embryo, including the hippocampal primordium (Simeone *et al*. 1992; Gulisano *et al*. 1996; Mallamaci *et al*. 1998). The role of Emx2 in forebrain development can be inferred from studies carried out in the cortex and olfactory bulb of *Emx2* mutants. In the isocortex, Emx2 is essential not only for correct area determination, but also for proper thalamo-cortical connectivity (Mallamaci *et al*. 2000a). Failure in axonal connectivity has also been reported in the olfactory bulb of *Emx2* mutants (Yoshida *et al*. 1997). In both isocortex and olfactory bulb, Emx2 deficiency is accompanied by histological disorganization, probably caused by improper neuronal migration due to impaired reelin signaling (Cecchi and Boncinelli 2000; Mallamaci *et al*. 2000b).

Because *Emx2* homozygous embryos die by the day of birth, however, knowledge of the involvement of Emx2 in hippocampal development (many of those key processes take place after birth) had lagged behind. In Emx2–/– embryos, the medial limbic cortex and the hippocampus show a reduction in size and a missing dentate gyrus (Pellegrini *et al*. 1996; Yoshida *et al*. 1997), although region-specific

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Address correspondence and reprint requests to Dr Nicolai E. Savaskan, Institute of Anatomy, Department Molecular Cell and Neurobiology, Oskar-Hertwig House, Humboldt University Hospital Charité, Philippstr. 12, D-10115 Berlin, Germany.

E-mail: nicolai.savaskan@charite.de

[1]Present address: Department of Neuroembryology, Max Planck Institute for Molecular Endocrinology, Hannover, Germany.

[2]These investigators are joint senior authors.

Abbreviations used: DIV, days *in vitro*; NGS, normal goat serum; PB, phosphate buffer.

molecular markers still show correctly positioned hippocampal area identity (Tole *et al*. 2000). The main hippocampal connections, however, are formed perinatally, and cannot be studied in these mutants because they die by the day of birth (Pellegrini *et al*. 1996).

The developmental processes in the hippocampus are quite similar to those in the neocortex, i.e. they have an 'inside-out' gradient (Caviness 1973; Caviness and Rakic 1978; Marin-Padilla 1978). The hippocampal pyramidal cells, which later on form the cornu ammonis (CA1–CA3), migrate along radial glia and terminate in the hippocampal plate in an inside-out manner (Angevine 1965). During neurogenesis of granule cells in the dentate gyrus, however, cells are laid down in an 'outside-in' gradient (Stanfield and Cowan 1979; Cowan *et al*. 1980). There, the dentate gyrus is the central afferent target and thus comprises all sensory input to the hippocampus. Major sources of principal extrinsic projections to the hippocampus arise from ipsi- and contralateral entorhinal cortex and hippocampus (Blackstad *et al*. 1967; Raisman *et al*. 1966). Entorhinal and commissural axons terminate in the dentate gyrus in a segregated non-overlapping fashion. Interestingly, the layer-specific termination of entorhino-hippocampal afferents is independent of their final target neurons, i.e. dentate granule cells. Entorhinal axons still find their termination zone after elimination of granule cells by neonatal X-irradiation (Frotscher *et al*. 2001), after altered dentate granule cell migration in the mutant shaking rat Kawasaki (Woodhams and Terashima 1999), and after depletion of reelin in the reeler mutant (Deller *et al*. 1999) or during altered granule cell differentiation in NeuroD mutants (Schwab *et al*. 2000).

The lack of available information on postnatal CNS development in Emx2–/– mice is the result of their prenatal death mainly due to stunted kidney development. To overcome these difficulties we performed organotypic cultures of hippocampi from Emx2–/– embryos. Our findings include a reduced number of reelin immunoreactive Cajal–Retzius cells in the marginal zones of the hippocampus, an altered pattern in the neocortex, and a more diffusely developed entorhinal projection in *Emx2* mutants at late embryonic stage. In addition, the entorhinal projection is partly misrouted and dentate granule cells fail to differentiate dendritic as well as axonal processes in Emx2–/– slice cultures.

Materials and methods

Animals

Emx2 mutant mice were generated and genotyped as previously described (Pellegrini *et al*. 1996). The embryonic day 18.5 was selected as the first developmental stage of analysis (Pellegrini *et al*. 1996; Yoshida *et al*. 1997). At that time point, the hippocampus is developed in wild-type animals. The major hippocampal fields are readily identifiable by either cytoarchitecture or molecular markers at E18.5. A morphological dentate gyrus is evident in wild-type mice by E16.5, and a range of molecular markers distinguish different hippocampal fields and subregions by E15.5 (Caviness 1973; Stanfield and Cowan 1979; Tole *et al*. 2000).

Histology and immunohistochemistry

Pregnant mice were killed by delivering an overdose of pentobarbital at gestation day E 18.5. The embryos were quickly removed by cesarean surgery and decapitated. Brains were removed and immersed in fixative [4% paraformaldehyde (PFA), 0.1 M phosphate buffer (PB)] overnight. Some of the brains were transferred into 30% sucrose for cryoprotection for 24 h, frozen on dry ice and stored at −20°C. Horizontal cryostat sections (30 μm) were cut and sections containing the hippocampal formation were selected for Nissl stain.

Several brains were used for the combined calretinin immunocytochemistry and Nissl counterstain of the hippocampal formation. After fixation brains were rinsed in 0.1 M PB and cut on a vibratome into 50-μm thick horizontal sections. Prior to incubation with the primary antibody, the endogenous peroxidase was blocked by incubating the slices with 2% H_2O_2 for 30 min. After several rinses, the sections were permeabilized in PB containing 0.2% Triton X-100 for 30 min. This was followed by incubating the sections with 5% normal goat serum (NGS). The primary antibody anti-calretinin (dilution: 1 : 2500, Swant, Bellinzona, Switzerland) was administered at 4°C overnight. As secondary layer biotinylated goat anti-rabbit IgG (dilution 1 : 1000) was used. On the next day the slices were washed and transferred into a ABC solution (ABC-elite, 1 : 50, Vector Laboratories, Burlingame, CA, USA) at room temperature for 2 h and subsequently reacted with 0.07% 3,3′-diaminobenzidine tetrahydrochloride in PB containing 0.02% $(NH_4)_2(NiSO_4)_2$ and 0.024% $CoCl_2$ for intensification. Sections were developed by adding 0.001% H_2O_2. Sections that exhibited immunopositive cells were mounted onto gelatin coated slides, Nissl counterstained, dehydrated, through an ascending series of ethanol, flat-embedded with Hypermount and coverslipped. Sections were digitally photographed (Olympus BX-50).

Some hippocampal and neocortex sections were selected for immunostaining with a mouse monoclonal antibody against the extracellular matrix protein reelin (1 : 1000, G10, generously provided by Dr Goffinet, Namur, Belgium), which is a marker to label layer I Cajal–Retzius cells. Secondary antibody (Cy2-conjugated anti-mouse IgG; 1 : 1000) was administered for 4 h. This was followed by nuclear staining with Hoechst 33528 (1 : 6000) for 20 min. After thorough rinsing, section were mounted onto gelatin-coated slides embedded with Moviol (Hoechst, Frankfurt, Germany), coverslipped, and examined under epifluorescence microscopy.

Tracing of the entorhino-hippocampal pathway *in vitro*

Pregnant mice were killed with an overdose of ether at embryonic stages E18. Embryos were removed and the skull was opened. Brains were removed and immersed in a solution containing 4% paraformaldehyde in 0.1 M PB. A single injection of DiI (Molecular Probes, Eugene, OR, USA) was delivered into the entorhinal cortex under visual control via a glass micropipette. Then, brains were stored in fixative for 2–3 weeks in the dark. Coronal vibratome

sections (80 μm) were mounted on slides and coverslipped with Mowiol Sections displaying entorhinal projection were digitally photographed using a TRITC filter set.

Organotypic slice cultures

Complex slice cultures of entorhinal cortex connected to the adjacent hippocampus were prepared from E18 mice. Slices (350-μm thick) were cultivated on membranes for up to 14 days as described (Stoppini *et al*. 1991; Zafirov *et al*. 1994; Frotscher *et al*. 1995). For anterograde biocytin tracing, slice cultures of at least 10 days *in vitro* (DIV) were used only. One set of experiments was performed to label dentate granule cells with their mossy fiber axons. Under visual control, small crystals of biocytin were placed onto the presumptive dentate gyrus (DG) (from –/– mutant mice) or on the delineated granule cell layer (from wild-type littermates). Cultures were further incubated to allow for anterograde transport of the tracer (36–48 h). They were then fixed for 2 h in a solution containing 4% PFA, 0.1% glutaraldehyde. After several rinses, cultures were resliced on a vibratome (50 μm), and sections were incubated with ABC-elite complex overnight (1 : 50; Vector Laboratories, Burlingame, CA, USA). Subsequent DAB reaction was heavy metal-intensified as described (Schwab *et al*. 2000). Sections were counterstained (cresyl violet), dehydrated, and coverslipped. Some of the co-cultures that had been incubated for 9 days were selected to trace of an entorhino-hippocampal pathway developed *in vitro*. Therefore, crystals of biocytin were placed onto the superficial layers of the entorhinal cortex, the tracer allowed for anterograde transport and the tissue further processed as described above.

For silver impregnation, the method of Golgi–Collonier was performed. For all semiquantitative analysis (reelin staining, Golgi impregnation) neuron counts were performed by two independent observers according to the criteria described by Clark and Oppenheim (1995). Calbindin and calretinin immunofluorescence were used to visualize dentate granule cells and hilar mossy cells, respectively. Briefly, after 10–14 DIV, cultures were fixed in 4% PFA in PB (2 h), vibratome-sectioned (50 μm), incubated with 5% NGS blocking solution (30 min) and permeabilized with 0.1% Triton-X for 30 min before applying the primary antibody (anti-calretinin, 1 : 2500; anti-calbindin, 1 : 6000, Swant, Bellinzona, Switzerland) (4°C, overnight). Secondary antibody (Alexa Fluor 488 anti-rabbit IgG; 1 : 800) was administered for 4 h. This was followed by Hoechst nuclear stain (1 : 10 000) for 20 min. After thoroughly rinsing for 3 h, sections were mounted and embedded on glass slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Cultures showing immunofluorescent neurons were digitally photographed.

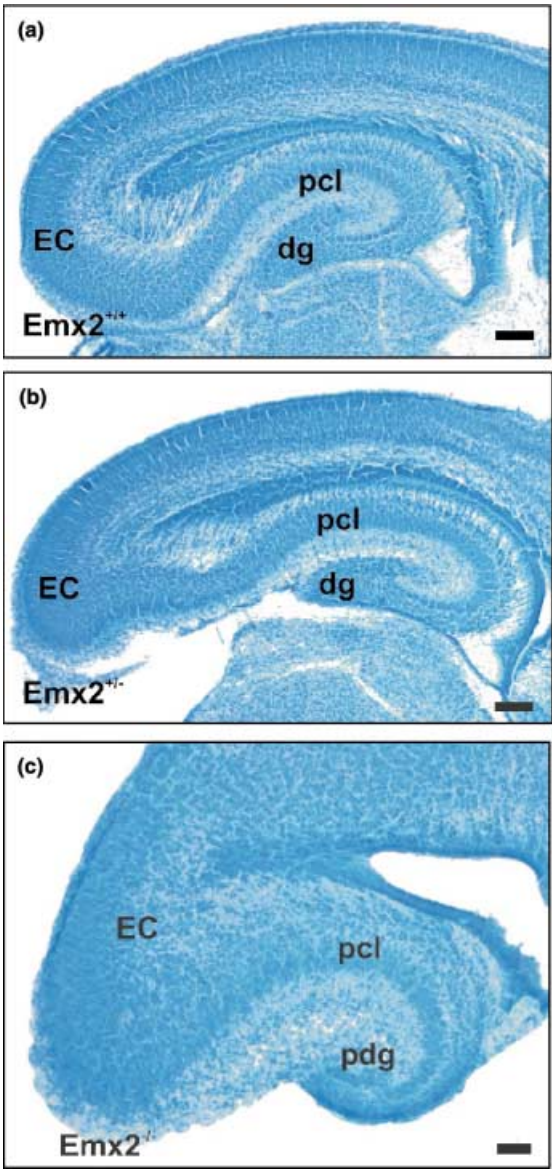

Fig. 1 Cytoarchitectural organization of the embryonic (E 18.5) hippocampal formation. Nissl-stained horizontal sections through the hippocampus of wild-type (a), heterozygous (b), and Emx2–/– homozygous (c) mutant mice. In (a) and (b), the pyramidal cell layer and the granule cell primordium is present. (c) The hippocampus is reduced in size in the Emx2–/–mutant. Somata of the presumed dentate gyrus are Nissl stained, although the typical C-shaped cytoarchitecture is missing. dg, dentate gyrus; pcl, pyramidal cell layer; pdg, presumed dentate gyrus; EC, entorhinal cortex. Scale bars: 170 μm (a), 230 μm (b), 300 μm (c).

Results

Cajal–Retzius cells are reduced in number but correctly orientated in Emx2 null mutants

At embryonic day E18.5, Nissl staining of the hippocampal formation revealed a timely normal development of the principal cell layers in wild-type and heterozygous mice. In the dentate gyrus (DG), the suprapyramidal blade of the granule cell layer had started to form (Figs 1a and b). A smaller CA1 and CA3 cell layer, but a demarcated hilar region and a granule cell layer were consistently missing in Emx2 deficient mice (Fig. 1c). Instead, a dense band of cells persisted in vicinity to the ventricular zone. The curling of

the developing dentate gyrus over itself to adopt its characteristic convoluted C-shape appeared delayed in Emx2–/– compared to wild-type littermates (Fig. 2). It has been reported that Emx2-deficient isocortex has Cajal–Retzius cell problems which translate into inappropriate neuronal migration and histogenesis (Mallamaci *et al.* 2000b). Calretinin immunocytochemistry was carried out to examine the existence of early generated layer I Cajal–Retzius cells. In all sections of the examined genotypes [wild-type ($n = 3$), heterozygous ($n = 5$) and Emx2–/– ($n = 4$)] calretinin immunopositive neurons were found. Immunolabeled cells were packed densely in the marginal zones of the dentate gyrus and the hippocampal anlage from Emx2+/– mice (Figs 2a and b). Only a few calretinin positive cells were detected in these zones of Emx2–/– mutant embryos with a loosely distribution not only restricted in the marginal zone (Figs 2c and d). We confirmed these results with the use of a monoclonal antibody against the extracellular matrix protein reelin which is mainly contained in Cajal–Retzius cells (Frotscher *et al.* 2001). Reelin immunopositive cells were found near to the hippocampal fissure, regardless of the genotype. The marginal zones of hippocampi from wild-type and heterozygous mice were densely populated by the Cajal–Retzius cells (Figs 3a and b), whereas the number of stained cells in Emx2–/– mice appeared to be reduced (Fig. 3c). This is in line with the pattern of immunoreactivity in layer I of the corresponding entorhinal cortices. A subpial localization of reelin positive Cajal–Retzius cells were observed in the neocortex of *Emx2* wild-type and heterozygous mice. Like in the hippocampal formation, immunoreactivity for reelin was markedly lower in Emx2–/– mutants (Fig. 3f). These results show that Emx2 is involved in the production and differentiation of Cajal–Retzius cells in the hippocampus.

Emx2–/– mice display abnormalities in the entorhino-hippocampal pathway

As our observations on reelin-positive cells in the Emx2 deficient hippocampus match reported observations in the isocortex, we asked if the mutant hippocampus showed also axonal targeting problems similar to those which have been reported for the thalamo-cortical projection (Mallamaci *et al.* 2000a). To test this effect, embryos of the various *Emx2* genotypes were injected with the lipophilic dye DiI. In all genotypes, outgrowth of entorhinal fibers towards the hippocampus could be observed (wild type $n = 4$, heterozygous $n = 5$ and homozygous $n = 5$). The outgrowth pattern in both wild-type and heterozygous embryos was channeled along the alveus and the perforant path (Figs 4a and b). DiI-labeled entorhinal afferents were mainly confined to the outer marginal zone of the hippocampus proper. A few fibers crossed the hippocampal fissure. In contrast, the majority of Emx2 deficient perforant path fibers showed a diffuse distribution in the hippocampus and presumptive

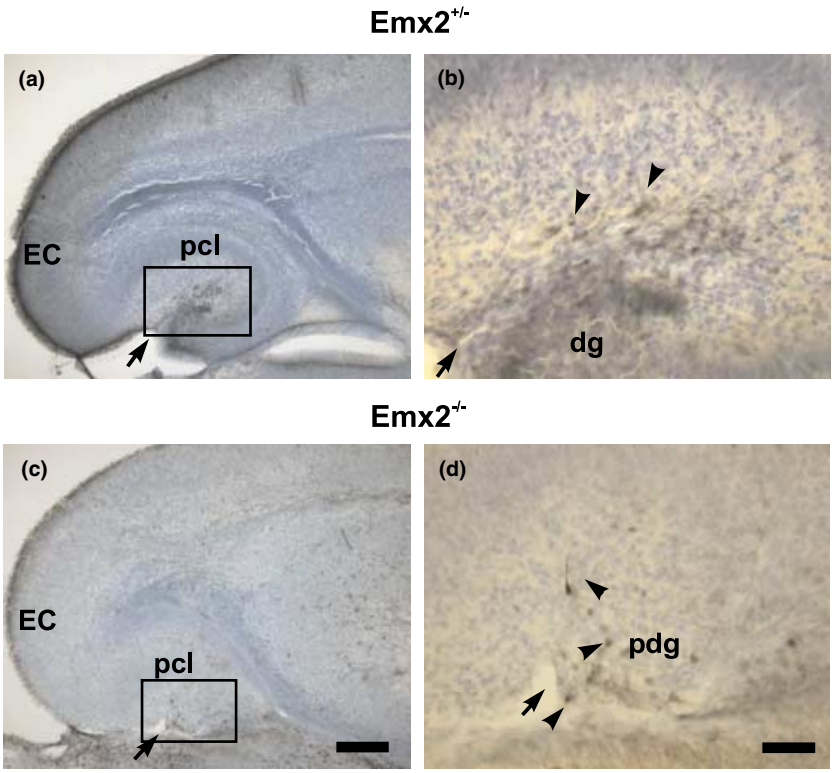

Fig. 2 Calretinin immunoreactivity in the hippocampal formation of heterozygous and Emx2–/–mutant embryos (E 18.5). Calretinin immunopositive cells are observed along the developing hippocampal fissure in the heterozygous (a and b) and in Emx2–/– (c and d) sections. Note that the hippocampal anlage is far beyond the mature state and appears to be delayed in the Emx2–/– mutants. (b) and (d) show examples of calretinin immunopositive cells with no obvious morphological differences. Arrowheads mark immunopositive cells in the marginal zone. The arrow marks the developing hippocampal fissure. EC, entorhinal cortex; pcl, pyramidal cell layer; dg, dentate gyrus; pdg, presumed dentate gyrus. Scale bars 150 μm (a and c), 25 μm (b and d).

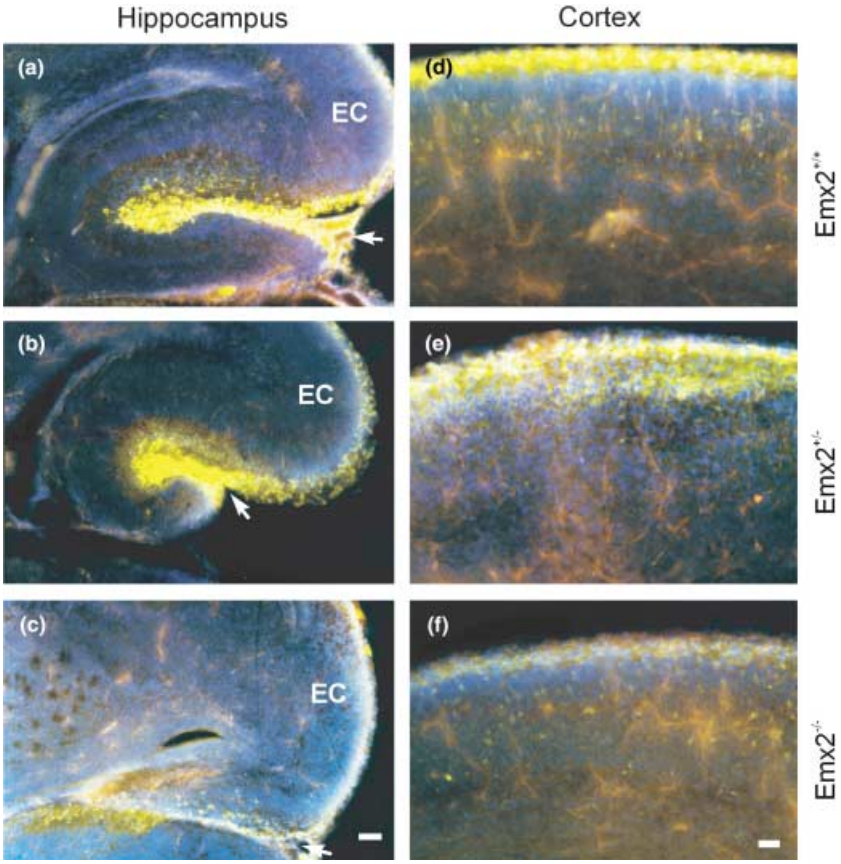

Fig. 3 Distribution of reelin-containing Cajal–Retzius cells during mouse hippocampal and cortical development of *Emx2* wild-type, heterozygous, and homozygous mice at E 18.5. In section of the hippocampal formation from wild-type (a) and heterozygous (b) mice intense reelin immunoreactivity shows the typical subpial distribution along the hippocampal fissure and in the marginal zone of the entorhinal cortex. (c) A section from an Emx2–/– mouse which shows a similar pattern but reduced number of reelin immunoreactive cells in the marginal zones of the hippocampus and entorhinal cortex. (d) This section from neocortex of an *Emx2* wild-type mouse shows the reelin containing Cajal–Retzius cells strictly confined to layer I. (e) Reelin immunopositive Cajal–Retzius cells show similar subpial distribution in this section from a heterozygous mouse, but also appeared to be slightly broadened and distributed with the highest concentration in layer I. (f) In the section of an Emx2–/– neocortex the immunoreactivity is markedly reduced in the correct layer. Arrow points to the hippocampal fissure. EC, entorhinal cortex. Scale bars for (a), (b) and (c) represent 400 μm, for (d), (e) and (f) is 75 μm.

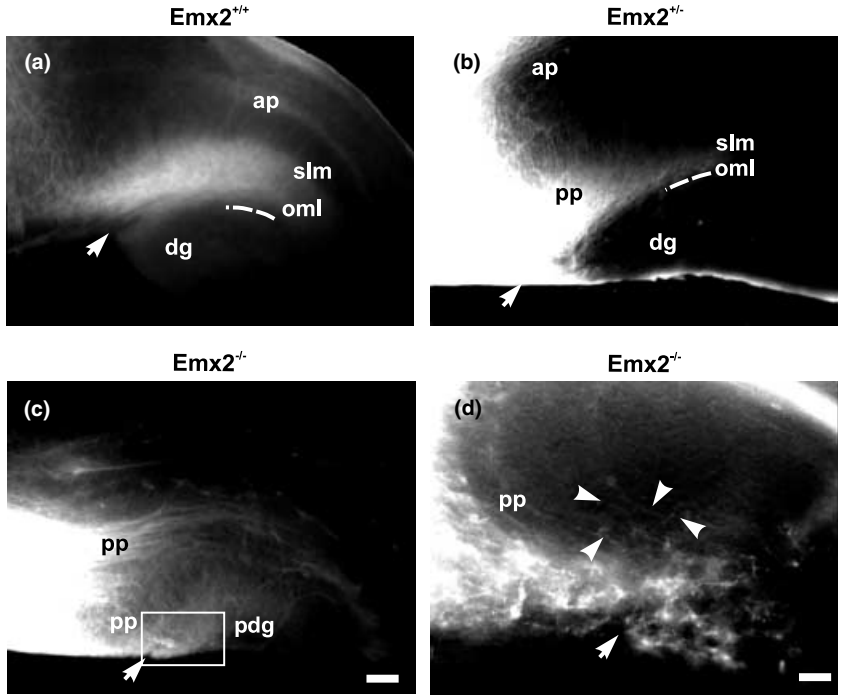

Fig. 4 Anterograde DiI tracing of the developing entorhino-hippocampal pathway in wild-type, heterozygous and homozygous *Emx2* mice at E 18.5. In all three genotypes, the alvear and perforant pathway are initiated at this stage of development. In the wild-type (a) and heterozygous (b) animals prominent fiber projection is visible in distinguishable marginal zones of hippocampus and dentate gyrus. (c and d) In this section of an Emx2–/– mouse DiI labeled entorhinal fibers are loosely distributed irrespectively of normal termination borders (d). The arrow and the dashed lines point to the hippocampal fissure in (a), (b), (c), and (d). Arrowheads in (d) show loosely distributed axonal terminals in the presumed dentate gyrus. ap, alvear path; oml, outer molecular layer; pp, perforant path; dg, dentate gyrus; pdg, presumed dentate gyrus; slm, stratum lacunosum moleculare. Scale bar in (c) is 200 μm and in (d) is 15 μm.

dentate gyrus disregarding laminar boundaries (Figs 4c and d). Our results show that, at least at this early phase of the invasion of the hippocampus by perforant path axons, Emx2 is essential for the segregation of the incoming axons to their corresponding target layers.

Alterations in *Emx2* null mutants in long-term organotypic slice cultures

We wanted to know if the developmental alterations in Emx2 deficient hippocampus was a transient defect (compensated for later by other mechanisms) or perhaps only in axonal targeting due to a possible general delay in the maturation of the hippocampus in these mutants. To overcome the early lethality of renal malformation in Emx2 mice we performed entorhino-hippocampal organotypic slice cultures. In this culture system, hippocampal CA1 pyramidal cell layers maintained their typical cellular organization in all slices prepared from mice independent of their genotype, i.e. wild-type, heterozygous, and homozygous *Emx2* mutants (Table 1). In contrast, a clearly distinguishable CA3 cell layer and a dentate granule cell layer developed only in cultures from wild-type and heterozygous mice, but was never detected in the absence of Emx2, even after extended incubation periods (Fig. 5). In Emx2+/– slice cultures, a C-shaped granule layer developed during incubation and the calretinin immunoreactive Cajal–Retzius cells exhibited their normal topographical distribution in the outer molecular layer above the densely packed dentate granule cells (Fig. 5c). Hilar mossy cells, which also stain for calretinin, were present in the examined cultures of heterozygous and mutant cultures (Figs 5f and g). A characteristic hilar mossy cell axonal projection into the inner molecular layer of the dentate gyrus was only observed in slice co-cultures from Emx2+/– (Fig. 5g, arrows). Calbindin immunolabeling was used as a marker for differentiated dentate granule cells as well as hippocampal interneurons. Calbindin immunolabeled cells could be found in their normotypical distribution in the granule cell layer (Fig. 5e). A similar cellular arrangement of calbindin immunoreactive neurons was never observed in slice cultures from Emx2–/– mutants ($n = 8$). In these cultures, calbindin-containing neurons showed a dispersed distribution (Fig. 5d). Therefore, the histogenetic alterations of Emx2 deficient hippocampus are present also in long-term cultured slices of E18.5 mutant brain, whose development corresponds in theory to several postnatal days.

Table 1 Altered neuronal maturation in long-term slice cultures of Emx2–/– mutants: comparative analysis of Golgi-impregnated organotypic slice cultures

Genotype	Cell type	Abundance	*n*
Wild type	Dentate granule cells	+ + +	14
	Hilar neurons	+ + +	
	Pyramidal neurons	+ + +	
Emx2+/–	Dentate granule cells	+ +	19
	Hilar neurons	+ + +	
	Pyramidal neurons	+ + +	
Emx2–/–	Dentate granule cells	–	20
	Hilar neurons	+ +	
	Pyramidal neurons	+ + +	

Following 14 days *in vitro*, slice cultures from all three genotypes were processed for Golgi impregnation. Regions from the dentate gyrus, hilar area, and the CA 1 layer were analyzed. Semiquantitative data are scored as: + + +, highly abundant; + +, abundant; +, found in some cases; –, not found in any cases. n = number of analyzed sections.

Biocytin tracing of extrinsic and intrinsic afferents in culture

Anterograde tracing of entorhinal fibers with biocytin both in wild-type (data not shown), Emx2+/–, and Emx2–/– slice cultures revealed a normotypic orientated perforant pathway. Entorhinal fibers developed their layer specific termination only in slice cultures of heterozygous littermates (Figs 6a and b). The entorhino-hippocampal projection was partly altered in slice cultures deficient of this gene (Figs 6c and d). Although the projection to the stratum lacunosum-moleculare of the hippocampus proper has developed the layer-specific termination, fibers which normally terminate in the outer molecular layer of the dentate gyrus did not establish this normotypic pattern. These fibers spread throughout the presumed dentate gyrus (Figs 6c and d). From these results, we conclude that the improper segregation of entorhinal afferents in Emx2 deficient hippocampus is not a transient phenomenon but a final defect showing that Emx2 is essential for proper targeting of perforant afferents to the molecular layer.

Emx2 is essential for the generation and/or differentiation of hippocampal granule cells

In addition, tracer application of biocytin into the developing dentate gyrus/hippocampus revealed numerous labeled neurons (Fig. 7a). However, while in slice cultures from heterozygous mice we could observe Schaffer collaterals as well as en passant boutons in the CA3 region (Figs 7b and c; characteristic of differentiated granule cell axons), these axons were not observed in slices from mutant mice (data not shown). The Schaffer collaterals were also maintained in slice cultures from Emx2+/– mice just above a well-developed pyramidal cell layer (Fig. 7c).

The absence of the typical granule cell projections in mutant hippocampus led us to examine the neuronal differentiation on a more detailed structural level. To this purpose we performed Golgi-impregnation in slice cultures from heterozygous and mutant mice 14 DIV. We did not detect any cell resembling a mature granule cell in any of the Emx2–/– slices (Fig. 8, Table 1). However, spiny hilar

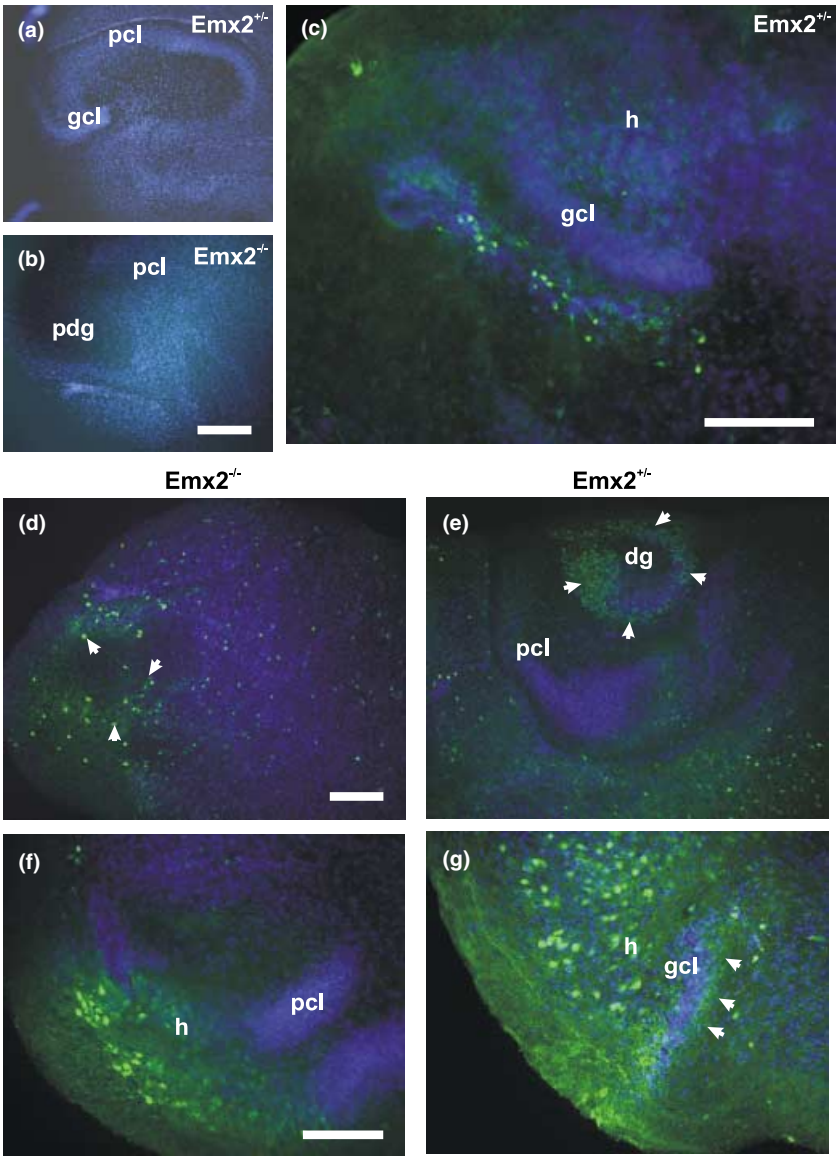

Fig. 5 Development of slice co-cultures of entorhinal cortex and hippocampus from heterozygous and Emx2–/– mice at 14 days *in vitro*. (a and b) Surveys of Hoechst dye stained cellular organization in Emx2+/– (a) and Emx2–/– (b) slice cultures (blue stained nuclei). The pyramidal cell layers developed in slice cultures from heterozygous mice (a) and from homozygous mice (b) whereas a granule cell layer is detected only in slice cultures from heterozygous mice. (c) In Emx2+/– slice cultures, calretinin immunoreactive Cajal–Retzius (green) cells are arranged along the hippocampal fissure. (d and e) Calbindin immunofluorescence staining reveals a typical C-shaped clustering of differentiated granule cells in a slice culture of Emx2+/– superimposed to the Hoechst stained (blue) granule cell layer (e, marked by arrows). This pattern of calbindin positive granule cell is not found in Emx2–/– slice cultures (d). In this mutant slice culture, labeled calbindin-positive cells are scattered and no sign of the C-shaped HOECHST stained dentate granule cell layer is visible. (f and g) Hilar mossy cells are stained with anti-calretinin in slice cultures of both genotypes. Only in slice cultures of Emx2+/– mice is the typical distribution of calretinin immunoreactive mossy cell axons (arrows) above the granule cell layer (gcl) observed (g). In Emx2–/– cultures calretinin immunoreactive cells are found to be clustered but no hilar mossy cell projection above granule cells is developed (F). pcl, pyramidal cell layer; dg, dentate gyrus; h, hilus; pdg, presumed dentate gyrus. Scale bars represent 800 μm (b), 150 μm (c) and 300 μm (d and f).

neurons could be found in Emx2–/– slices, which indicates that these mutants do not have a general neuronal differentiation defect (Fig. 8). In the analyzed cultures, we found entorhinal fibers invading the stratum lacunosum-moleculare which appeared bundle-like organized (Fig. 8a, Table 1). Dentate granule cells could, however, be easily identified in slice cultures from Emx2+/– (Figs 8c and d). These neurons developed many of their structural characteristics during the incubation period. They possessed one or two stem dendrites which arborized. The dendrites were densely covered with spines of different shape, which indicated differentiation and maturation of these neurons *in vitro*. Therefore, we conclude that Emx2 is essential for the generation and/or differentiation of hippocampal granule cells.

Discussion

In summary, our data show that Emx2–/– mutants bear a hitherto undescribed defect in the formation of extrinsic and intrinsic connectivity and neuronal differentiation in the hippocampus (summarized in Fig. 9).

Emx2 is required for postnatal dentate granule cell maturation and hilar neuronal organization

Emx2–/– mice show arrest of hilus and dentate gyrus development. At E 18.5 the curling of the hippocampus is delayed making it comparable to 15-day-old wild-type embryos. Because *Emx2* gene expression defines the most medial areas of the embryonic cerebral cortex (Yoshida *et al.* 1997; Mallamaci *et al.* 1998), it is reasonable to assume that

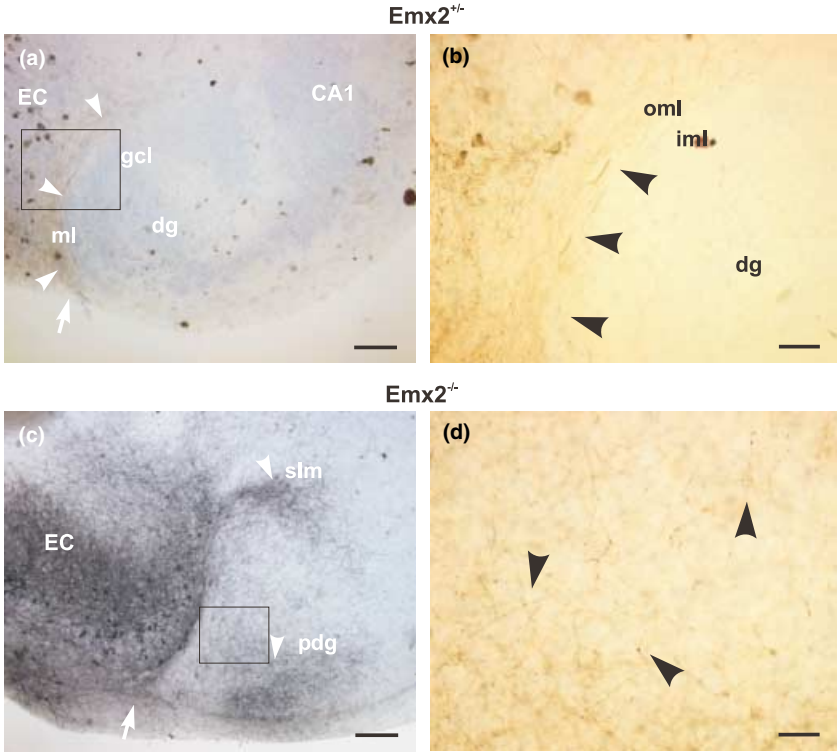

Fig. 6 Biocytin-traced entorhinal projection in heterozygous and knockout co-cultures after 12 days *in vitro*. In heterozygous entorhino-hippocampal slice cultures axonal projections (arrowheads) are confined to the appropriate termination zone, the outer molecular layer of the dentate gyrus (dg) (a; b is a higher magnification of boxed area in a). Cresyl violet staining revealed that both the supra- and infra-pyramidal blade of the dentate granular cell layer had developed. In a slice culture from Emx2**–/–**, an entorhinal projection is also formed (c and d is a higher magnification of boxed area in c). The majority of traced entorhinal afferents is distributed throughout the presumed dentate gyrus (pdg) and the layer specificity is only retained for those fibers which entered the stratum lacunosum-moleculare (slm). The arrow indicates the hippocampal fissure. Arrowheads mark axonal profiles in the presumed dentate gyrus, and stratum lacunosum moleculare. gcl, granule cell layer, ml, molecular layer, EC, entorhinal cortex; CA1, regio superior. Scale bar represents for 300 μm (a) and (c); 20 μm in (b); and 15 μm in (d).

Emx2 specifies areal identities in part at least of the dentate gyrus, i.e. the polymorph hilar region and the principal layer with its granule cells. Based on the role of Emx2 in area specification Pellegrini *et al*. (1996) suggested a lack of area specification in affected subregions of the hippocampus. A recent study by Tole *et al*. (2000) using molecular region markers showed that each hippocampal subfield is specified with its typical neuronal messenger RNA. Furthermore, each cell population develops in its correct position relative to each other and expression analysis of *Wnt5a* gene indicates the presence of dentate granule cell precursors. However, most of the dentate granule cells are known to be generated and migrate to their final position during the first few postnatal weeks (Angevine 1965). In consequence, granule cell maturation first takes place during postnatal stages.

Another function of Emx2 has to be suggested when considering the results from the slice culture experiments in the present study. Here, our immunocytochemistry data show that calbindin-positive cells and hilar mossy cells are present in Emx2–/– mutants. However, the well-defined and characteristic C-shaped layering of dentate granule cells has been found neither in Emx2–/– slice cultures nor the in the morphology of labeled cells resembling differentiated granule cells, even after extended incubation periods. This indicates an arrest of normal migration and differentiation of this cell type. These developmental processes were not impaired in cultures from wild-type and heterozygous animals that showed a normal distribution of typical granule cells. Moreover, the arrangement of hilar mossy cells identified by their immunoreactivity for the calcium binding protein calretinin was not as compact. As a result of this cellular disorganization, they did not develop their normal axonal projection to the proximity of granule cell dendrites in Emx2–/– mutants. Lack of Golgi impregnated neurons with morphological characteristics of dentate granule cells further show that Emx2 is essential in dentate granule cell maturation. Data from the Golgi technique have to take with caution since the nature of the Golgi impregnation is still poorly understood. However, spiny hilar neurons have been found in the same slices which lack identifiable granule cells.

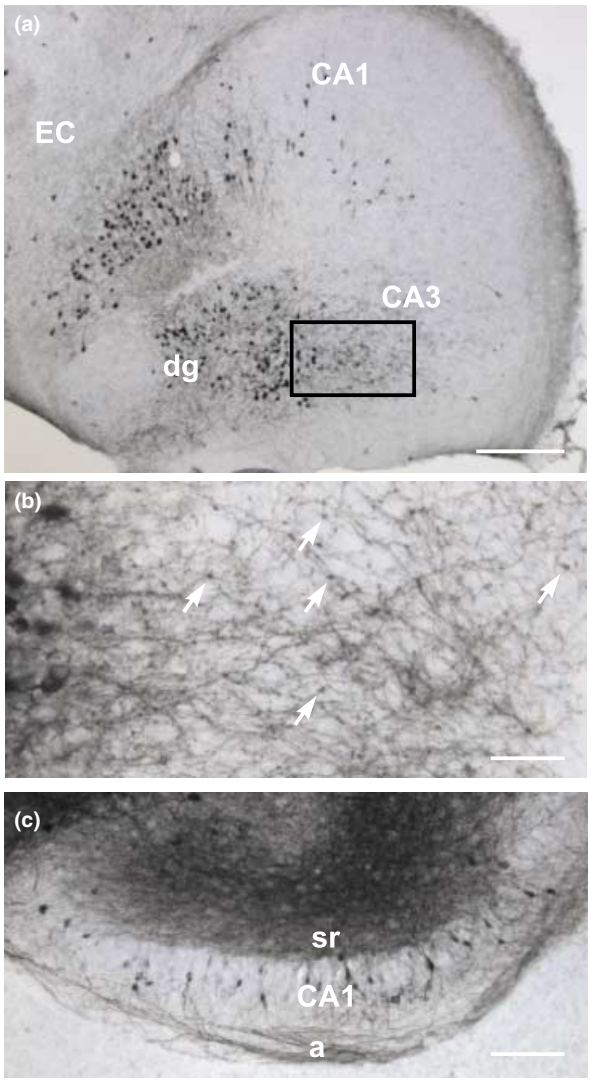

Fig. 7 Biocytin tracing of heterozygous entorhino-hippocampal co-cultures at 12 days *in vitro*. Overview of a co-culture after tracer application into the dentate gyrus/subiculum (a). Numerous black stained cells have taken up the tracer. The boxed area is shown in (b). In the CA3 region a plexus of anterogradely labeled fibers is visible. Many of the stained axons protrude large en passant mossy fiber boutons (white arrows) indicating that the dentate granule cell projection has developed. (c) This micrograph shows numerous biocytin labeled neurons in the CA1 region. The neurons express the morphology of pyramidal cells, extend their apical dendrites into the stratum radiatum (sr), and are aligned in a layer. In the stratum radiatum a heavily stained Schaffer collateral projection arising from CA3 pyramidal cells is observed. Labeled fiber bundles are found to run along the alveus. EC, entorhinal cortex; dg, dentate gyrus; CA3, regio inferior; CA1, regio superior; sr, stratum radiatum; a, alveus. Scale bars are 150 μm (a), 40 μm (b), and 200 μm (c).

Therefore, Emx2 deficiency does not generally affect neuronal positioning and differentiation, but specifically affects the maturation of dentate granule cells.

Emx2–/– mutants fail to form intrinsic hippocampal projections

Signs for the lack of neuronal differentiation in Emx2–/– mutants also came from the analyses of the development of intrinsic connections *in vitro*. In wild-type cultures, dentate granule cells were sending their axons via the mossy fiber tract to the regio inferior of the cornu ammonis (CA3) in a region-specific fashion as has already been seen in *in vivo* (Blackstad *et al*. 1967) and in *in vitro* studies (Schwegler *et al*. 1988; Frotscher *et al*. 1995). In addition, the granule axons developed their characteristic giant en passant boutons, which indicated normal synaptogenesis. Emx2–/– mutants failed to develop these features of dentate granule cells even after long-term incubation. In the case of hilar mossy cells, which are present in Emx2–/– slices, they never formed their normal efferent projections. As hippocampal area identities are generated in Emx2–/– mutants, failure of intrahippocampal connectivity can be directly attributed to the lack of Emx2 expression subsequently due to the lack of signals presented by their targets, the mature dentate granule cells.

Emx2 is required for extrinsic projections to the dentate gyrus

Entorhinal fibers were correctly directed towards the adjacent hippocampus in Emx2–/– mutants. This indicates that the general ability towards axon growth appeared to be unaffected. However, entorhinal fibers that have crossed the presumptive hippocampal fissure showed a random distribution in the presumptive dentate gyrus and had obviously lost their laminar specificity. Axons that ran the alvear path and along the stratum lacunosum-moleculare respected the normal lamination. This is in line with experiments in which the final targets, the dentate granule cells, have been ablated or dislocated but in which entorhinal axons still innervate and terminate accurately, indicating that the dentate granule cells are not required for target recognition (Deller *et al*. 1999; Schwab *et al*. 2000; Frotscher *et al*. 2001). It is known from earlier studies that entorhinal pathfinding is determined by Cajal–Retzius cells (Ceranik *et al*. 1999; Del Rio *et al*. 1997; Schwab *et al*. 2000). In fact, already at a late embryonic stage of mutant brain, reelin-expressing Cajal–Retzius cells appeared to be strongly reduced in number but showed typical morphology, orientation, and correct positioning in the marginal zones of the hippocampal formation. In addition, subpial localization of reelin containing Cajal–Retzius cells was altered in mutant neocortex supporting the role of the *Emx2* gene on neuronal migration (Mallamaci *et al*. 2000a). The layer-specific termination of entorhinal afferents in the stratum lacunosum-moleculare of the CA1 region and on the other hand the perturbed termination pattern of these fibers in the presumptive dentate gyrus coincides with the cytoarchitectural organization of the corresponding hippocampal subfields. The observed misrouting

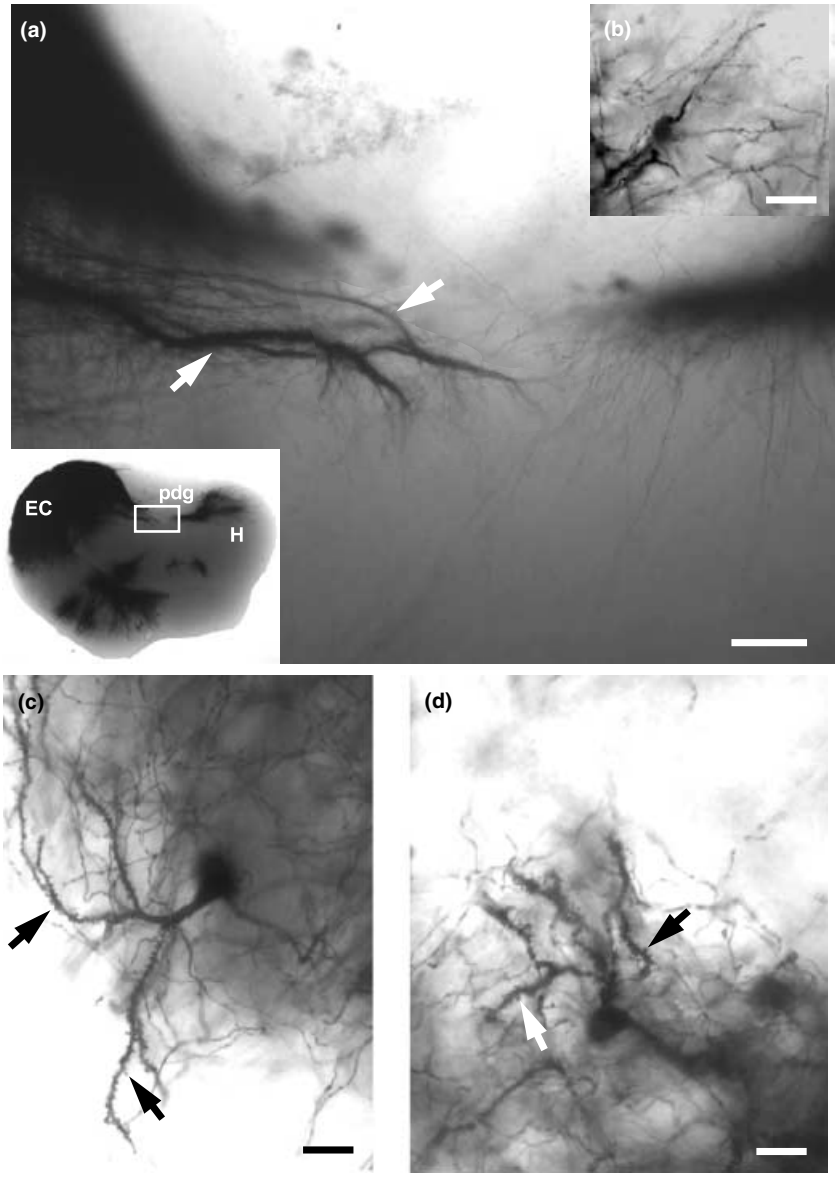

Fig. 8 Golgi impregnated long-term entorhino-hippocampal co-cultures. (a) In this Emx2**–/–** slice culture, Golgi-impregnated entorhinal axons are shown in the stratum lacunosum-moleculare of the hippocampus proper. Axons tend to fasciculate (arrows) in an unusual manner. The bottom left insert shows the complex slice culture with a pronounced Golgi-impregnation in the entorhinal cortex (EC). Note the lack of cellular impregnation in the presumed dentate gyrus (pdg). The boxed area in bottom left insert is enlarged in (a) (H, hippocampus). In (b) a spiny hilar neuron from an Emx2**–/–** slice culture is shown. (c and d) Two examples of identified Golgi-impregnated dentate granule cells in co-cultures from heterozygous mice. The granule cells emerge a branched dendritic arbor and the dendrites are densely covered with spines (arrows). Scale bars are as follows: 50 μm (a), 15 μm (b), 10 μm (c), and 8 μm (d).

of entorhinal fibers in the presumptive dentate gyrus may be accordingly regarded as a secondary effect due to the Emx2 related changes in reelin signaling. In a recent study, it has been shown that during the development of the perforant path Cajal–Retzius cells form a pioneer projection from the hippocampus to the entorhinal cortex and provide a template for later outgrowing entorhinal axons (Ceranik *et al*. 1999). However, most Cajal Retzius cells are confined to their normal position, the marginal zones of the hippocampus in *Emx2* heterozygous and null mutants. Thus, it cannot be excluded that the observed misrouted entorhinal projection is caused by the slightly altered Cajal–Retzius cell patterning. In this line, so far, unknown guidance cues, which are probably regulated by Emx2, may underlie the defects in target recognition seen in *Emx2* mutants.

To overcome the neonatal lethality of Emx2 deficiencies further studies should generate neuron-specific Emx2–/– mutants which would offer the possibilities to analyze the downstream targets of Emx2 regulated genes responsible for the anatomical phenotype.

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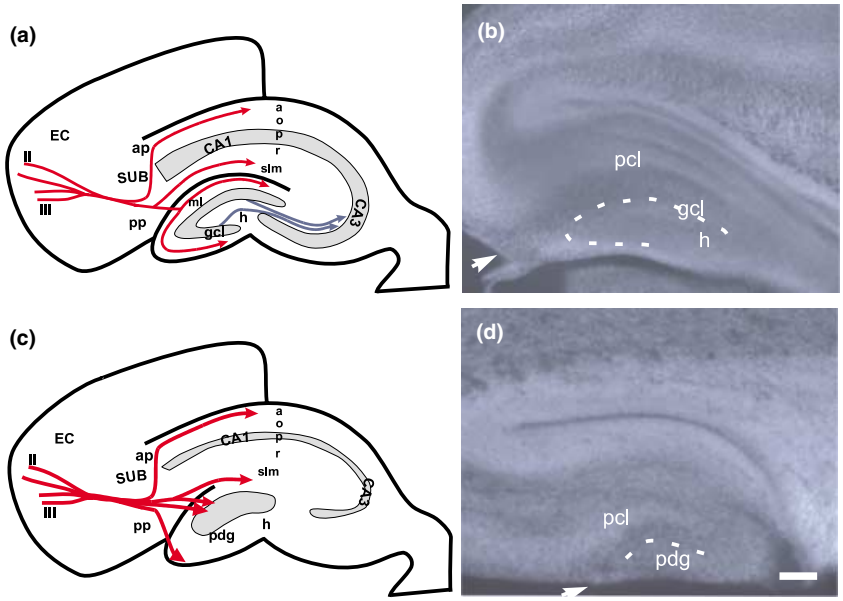

Fig. 9 Summary of the development of hippocampal subfields and extrinsic and intrinsic hippocampal connections in Emx2–/– *in vitro* and *in vivo*. (a, c) In heterozygous mice, the cellular organization and the connectivity pattern is maintained similar to the normal development in wild-type animals. Entorhinal afferents, the intrinsic hippocampal mossy fiber projection and the hilar mossy cell axons occupy their specific termination zones. (b and d) In the Emx2–/– mice, the CA1 and CA3 region is size-reduced and the hilar region is disturbed. A dentate granule cell layer is missing and, instead, only a mass of cells is located in the presumed dentate region (pdg). Entorhinal axons are fasciculated and their layer-specific termination is impaired with the exception of the stratum lacunosum-moleculare. EC, entorhinal cortex (layer II and III), ap, alvear path, sub, subiculum, pp, perforant path, CA1, regio superior, CA3, regio inferior, ml, molecular layer, slm, stratum lacunosum-moleculare, gcl, granule cell layer; h, hilus; a, alveus; o, stratum oriens; p, stratum pyramidale; r, stratum radiatum; pcl, pyramidal cell layer. Scale bar = 150 μm.

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