Laminating the hippocampus

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Abstract | Lamination of neurons and fibre projections is a fundamental organizational principle of the mammalian cerebral cortex. A laminated organization is likely to be essential for cortical function, as studies in mutant mice have revealed causal relationships between lamination defects and functional deficits. Unveiling the determinants of the laminated cortical architecture will contribute to our understanding of how cortical functions have evolved in phylogenetic and ontogenetic development. Recently, the hippocampus, with its clearly segregated cell and fibre layers, has become a major subject of studies on cortical lamination.

Principal neurons
A term that describes glutamatergic hippocampal pyramidal neurons and dentate granule cells that outnumber GABAergic interneurons.

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In mammals, cortical layers form according to common rules[1], and functional principles inherent to the cerebral cortex are similar, suggesting that a laminar organization is associated with the specific functions of the mammalian cortex. What could be the role of lamination in cortical function? And what functional properties are achieved by packing neurons and fibres into layers?

One way to approach these questions is by studying the signals that govern the development of the laminated cortex. In this case, the hippocampal formation comes to mind. When compared with the neocortex, the architecture of the phylogenetically old hippocampus (allocortex) is relatively simple, making this cortical region an excellent model to investigate the development of lamination. The hippocampus proper and the dentate gyrus each consists of only one layer of principal neurons — the pyramidal cells and the granule cells, respectively. This contrasts with the six-layered architecture of the neocortex. Afferent fibre projections, arising from distant sources, terminate in distinct hippocampal layers in a non-overlapping fashion. They impinge on defined segments of the principal neuron dendrites that traverse the fibre layers perpendicularly. This highly ordered organization is particularly evident in the dentate gyrus (BOX 1; FIG. 1).

As most dentate granule cells are born in the early postnatal period[2–6], the developmental processes that contribute to lamination, such as neurogenesis, neuronal migration and axonal pathfinding, are easier to study in the dentate gyrus than in the prenatally generated neocortex. Moreover, as a region with persisting postnatal neurogenesis, the dentate gyrus provides a unique model for understanding how newly born neurons become integrated into a differentiated, laminated network. Here, we discuss recent studies that shed light on the molecular determinants that govern the formation of cell and fibre layers in the dentate gyrus, and consider the functional significance of hippocampal lamination.

Laminating afferent projections

During development, afferent fibres to the hippocampus grow for long distances to reach the hippocampus proper and dentate gyrus where they terminate in distinct layers in a non-overlapping fashion and establish synapses with their target cells. This developmental process can be specified further as axonal pathfinding, target layer recognition and synapse formation. Several studies have addressed the roles of different axon guidance molecules, such as the semaphorins, netrins, the Slit–Robo system, and the ephrin family of tyrosine kinases and their ligands, for the pathfinding of axons destined to the hippocampus[7]. Here, we focus on the factors that guide afferent fibres to and keep them in sharply segregated hippocampal layers, allowing them to establish synaptic contacts with defined dendritic segments of their target neurons.

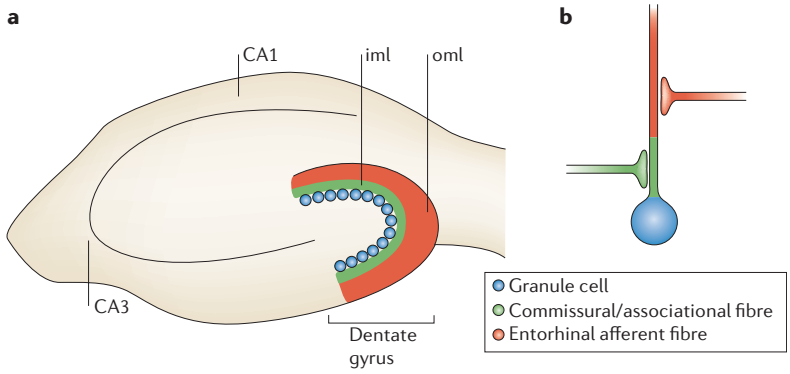
Ruling out temporal factors. How can the formation of the strictly lamina-specific termination of afferent projections to the hippocampus be explained? It has been suggested that the time of arrival of the different afferent fibre projections in the dentate molecular layer might be the key to understanding the development of their lamina-specific termination[6,8,9]. Early, prenatally arriving fibres from the entorhinal cortex terminate in the outer molecular layer on distal dendritic segments of granule cells, whereas late, postnatally arriving contralateral and ipsilateral hippocampal fibres (commissural/associational afferents) terminate in the inner molecular layer on proximal dendrites (BOX 1). The temporal hypothesis has been addressed by grafting experiments *in vivo*, and

Box 1 | **Lamination of the dentate gyrus**

In the dentate gyrus, a region with a particularly clear lamination (FIG. 1), the commissural/associational fibres, which originate from the mossy cells in the contralateral and ipsilateral hilar region, terminate in the inner molecular layer (iml), whereas entorhinal fibres, which originate from projection neurons in the superficial layers of the entorhinal cortex, terminate in the outer molecular layer (oml)[99,100]. This lamina-specific termination of fibre projections results in a subcellular specificity of afferent input (panel **a**). Therefore, commissural/associational fibres contact only proximal dendritic segments, whereas entorhinal fibres make synaptic contacts with only the distal dendritic segments of the same target cells (panel **b**). Axonal projections from deeper layers of the entorhinal cortex are an exception — they do not follow this rule, and innervate proximal dendritic portions and cell bodies of granule cells[101].

Although the cell bodies are not arranged in layers like those of hippocampal principal cells, inhibitory GABAergic interneurons are known to give rise to a clearly laminated input to the different compartments of the principal neurons. In the dentate gyrus, different types of basket cell innervate the cell body region[102], axo-axonic (chandelier) cells are specialized for the axon initial segments of the granule cell axons (the mossy fibres)[103], and various types of interneuron project to the molecular layer to innervate granule cell dendrites[104–106].

Subcortical projections to the dentate gyrus, such as the noradrenergic and serotonergic projections from the brainstem, and the cholinergic and GABAergic projections from the septum, are less clearly laminated, terminating 'diffusely' in all hippocampal and dentate layers.



through the use of sequential organotypic slice cultures *in vitro*. Grafting of embryonic tissue to the adult hippocampal region revealed that afferents growing out from the transplanted tissue innervated the adult hippocampus with proper laminar specificity[10]. In reverse, embryonic hippocampal transplants were shown to be properly innervated by host dentate granule cell axons[11]. Therefore, grafting experiments did not support the temporal hypothesis.

In organotypic co-cultures[12–16], the normal arrival of afferent fibre projections to the hippocampus can be reversed, and this has allowed the temporal hypothesis of laminated fibre projections to be tested directly[14]. A hippocampal target slice culture was co-cultured with a second hippocampal slice, thereby allowing for the formation of a 'commissural' projection to the target slice. Next, the same target hippocampal slice, being already innervated by commissural axons, was confronted with entorhinal fibres derived from an entorhinal slice culture. In this way, the normal sequence of arrival of these two different fibre systems was reversed. However, despite the reversal of sequential ingrowth, the entorhinal and commissural fibre systems maintained their correct laminar specificity to the dentate molecular layer[14]. The results of these two types of experiment suggest that the laminar specificity of hippocampal afferent projections does not depend on time of arrival but rather on specific molecular interactions between ingrowing fibre systems and their proper target layers. Therefore, a lamina-specific distribution of cellular targets and/or molecular recognition cues of the extracellular matrix (ECM) might account for the laminar specificity of the different fibre projections.

Pioneer neurons. The concept of transient pioneer neurons providing a scaffold for growing fibre projections in the developing vertebrate cortex was first proposed by McConnell and colleagues[17]. Pioneer neurons serve as temporary targets to direct later differentiating fibre projections to their proper target lamina. In line with this concept, transient Cajal–Retzius cells in the marginal zone, the prospective outer molecular layer of the dentate gyrus, were suggested to serve as intermediate targets for ingrowing entorhinal fibres[18–20] (FIG. 2). Synapse formation between entorhinal afferents and Cajal–Retzius cells in the dentate outer molecular layer was detected before the formation of synapses between entorhinal fibres and their final targets, the granule cell dendrites[20]. In addition, Cajal–Retzius cells in the outer molecular layer were shown to form an early axonal projection to the entorhinal cortex, suggesting a role for these axons as a guidance scaffold for the first ingrowing entorhinal afferents that are seeking their proper target layer[21]. After ablation of Cajal–Retzius cells in the dentate gyrus of organotypic slice cultures, entorhinal fibres no longer innervated the dentate molecular layer[20], thereby confirming the requirement for Cajal–Retzius cells for the ingrowth of entorhinal afferents.

A similar pioneering role has been proposed for GABA (γ-aminobutyric acid)-containing interneurons in the stratum radiatum of the hippocampus proper, whereby they act as guideposts for commissural/associational fibres to the stratum radiatum[22]. However, in mutant mice that are deficient for the transcription factors distal-less homeobox 1 and 2 (DLX1/2) and, as a consequence, lack GABAergic interneurons in the hippocampus, commissural fibres still terminate with proper laminar specificity in the stratum radiatum[23]. In addition, laminar specificity of entorhinal fibres projecting to the adjacent stratum lacunosum-moleculare is retained and does not overlap with that of commissural fibres, precluding the requirement for GABAergic interneurons as pioneer neurons for the laminated termination of commissural/associational fibres[23]. This indicates that pioneer neurons are not required for laminar target specificity in all cases.

Afferent fibres and target cells. Trophic interactions between afferent entorhinal fibres and granule cell dendrites seem to be required for the maturation of distal granule cell dendritic segments that extend into the dentate outer molecular layer, as this is affected in the absence of entorhinal input[24,25]. Moreover, entorhinal afferents are required in adult animals for the maintenance of distal segments of granule cell and basket cell

Organotypic slice culture
A culture system that preserves the environment of the cultured cells, as tissue sections and not dissociated cells are cultivated.

Cajal–Retzius cell
A type of early-generated neuron that populates the marginal zone of the cerebral cortex. Originally described by Gustaf Retzius and Santiago Ramón y Cajal, these cells were recently found to synthesize and secrete the glycoprotein reelin. Reelin is important for the proper migration of cortical neurons.

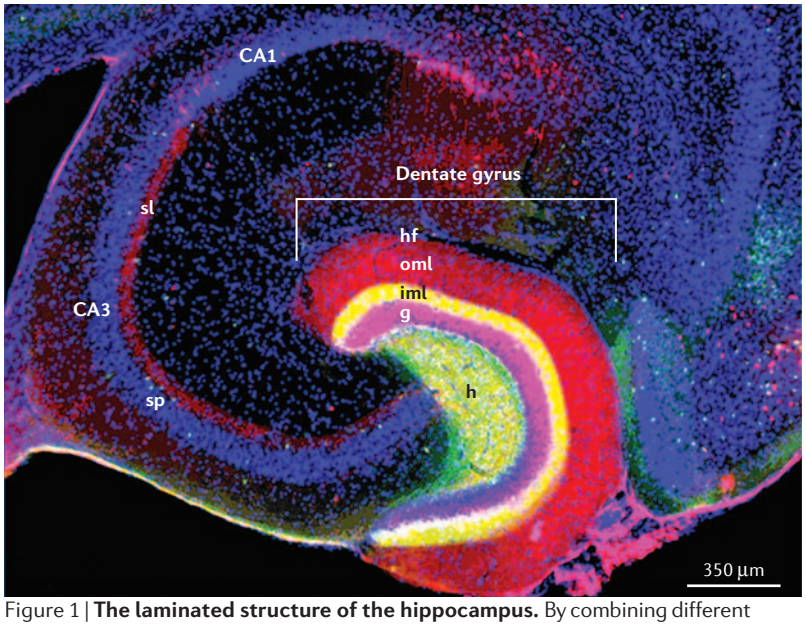


Figure 1 | **The laminated structure of the hippocampus.** By combining different labelling techniques, the arrangement of neurons and fibre projections in layers in the mouse hippocampus can be visualized. A particularly clear-cut lamination is seen in the dentate gyrus. The mossy cells in the hilar region (h) of the hippocampus are immunostained green for the calcium-binding protein calretinin. The axons of these neurons give rise to a laminated projection in the inner molecular layer (iml) on the ipsilateral and contralateral sides (commissural/associational fibres). The outer molecular layer (oml) is the termination zone of fibres from the entorhinal cortex. Neuronal cell bodies are counterstained with DAPI (blue), thereby labelling the pyramidal layer (sp) in areas CA1 and CA3, and the granule cell layer (g) of the dentate gyrus. The granule cells are also labelled for calbindin (red), which heavily stains their dendrites extending towards the hippocampal fissure (hf) and their axons, the mossy fibres, terminating in the stratum lucidum (sl) of CA3.

dendrites[26]. The nature of these interactions between presynaptic entorhinal terminals and postsynaptic target cell dendrites is unknown. Blockade of neuronal activity in entorhinal–hippocampal co-cultures by application of the sodium channel blocker tetrodotoxin did not alter the development of the lamina-specific termination of entorhinal fibres or the differentiation of granule cell dendrites[25]. Therefore, neither laminar ingrowth of entorhinal fibres nor the development of granule cell dendrites depends on electrical activity.

It has been proposed that laminar specificity of afferent projections depends on the presence and correct position of postsynaptic partners. In line with this hypothesis, a number of *in vivo* tracing studies have provided evidence that the correct alignment of the dentate granule cell layer is required for the proper lamina-specific ingrowth of commissural/associational fibres in the inner molecular layer[27,28]. In mice that are deficient in the glycoprotein reelin, malpositioned granule cells are distributed across the entire dentate gyrus[29]. In these mutants, commissural fibres are misrouted and seem to follow their malpositioned target cells[28]. Mutant mice that lack reelin or its receptors, apolipoprotein receptor 2 (APOER2) and the very low density lipoprotein receptor (VLDLR), show graded defects of granule cell malpositioning[30,31].

Anterograde tracing studies have revealed that changes in granule cell distribution are reflected in the aberrant distribution of commissural fibres[32]. These findings are supported by tracing studies in hippocampal slice co-cultures. Zhao and colleagues[16] have anterogradely traced commissural fibres in co-cultures of two hippocampal slices from wild-type mice, and in a wild-type hippocampal slice co-cultured with a hippocampal slice from a reeler mouse. Whereas the commissural fibres projecting to the wild-type target slice terminated with correct laminar specificity in the inner molecular layer of the dentate gyrus, the commissural fibres projecting to the reeler target slice were found to intermingle with the malpositioned granule cells in the hilar region, just as they do in reeler mutants *in vivo*[28,31]. These findings suggest that recognition cues for commissural fibres are expressed by granule cells and exposed on their dendrites (FIG. 2). When commissural fibres from a reeler hippocampal slice were traced to a wild-type slice, the fibres terminated with correct laminar specificity in the inner molecular layer, precluding a cell-autonomous defect in the reeler commissural neurons[16].

In contrast to commissural fibres, entorhinal fibres that grew into the dentate gyrus of reeler slices maintained their laminar specificity to the outer molecular layer despite malpositioned granule cells[16]. These *in vitro* data are in line with *in vivo* tracing studies[28] and demonstrate that lamination of entorhinal fibres is independent of the positioning of their final target cells (see below).

Instruction by the extracellular matrix. The idea that adhesion of dissociated projection neurons on living brain slices might reveal position-specific information for the growing axons of these neurons has been tested by researchers studying different brain regions, such as the retinotectal projection[33,34] and the thalamocortical projection[35]. In an adhesion assay using living hippocampal slices, dissociated entorhinal neurons were found to adhere with laminar specificity to the dentate outer molecular layer, suggesting a role for these laminar adhesive cues as mediators of the laminar specificity in this case[36]. Lamina-specific adhesion can be disrupted by treating the hippocampal slices with hyaluronidase, an enzyme that digests hyaluronan, which is a glycosaminoglycan of the ECM of many tissues. In line with the adhesion assays, laminar specificity of entorhinal fibre growth to the outer molecular layer was disrupted by hyaluronidase treatment[16,37]. By contrast, hyaluronidase treatment did not affect the pathfinding of entorhinal fibres to the dentate molecular layer or the laminar specificity of other fibre projections, such as the commissural/associational projection to the dentate inner molecular layer or the mossy fibre projection[16]. What could the role of hyaluronan be? Chondroitinsulphate proteoglycans (CSPGs), which are ECM components that are highly expressed in the developing dentate outer molecular layer (as shown with an antibody against chondroitinsulphate[16]), are associated with hyaluronan through linker proteins. Together, these data suggest that laminar specificity of entorhinal fibres that project to the outer molecular layer requires

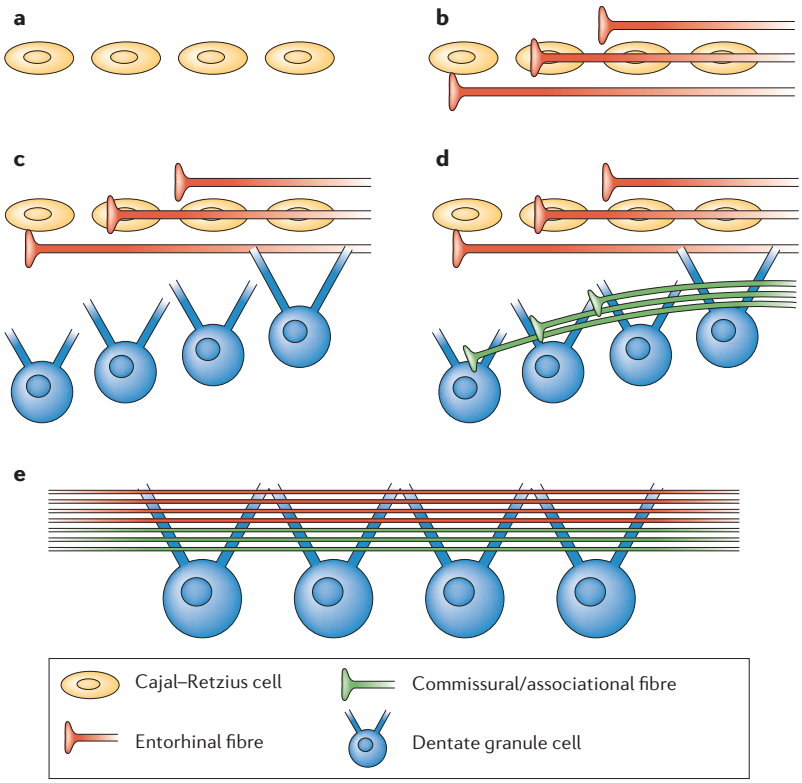


Figure 2 | **Different signals control the laminated termination of entorhinal and commissural/associational fibres to the dentate gyrus. a** | Tangentially migrating Cajal–Retzius cells populate the marginal zone of the dentate gyrus, the outer molecular layer, early in development. **b** | Cajal–Retzius cells serve as early targets of entorhinal fibres in the outer molecular layer. **c** | Dentate granule cells are born in the hilar region of the dentate gyrus and migrate radially towards the marginal zone, a process that is controlled by reelin-secreting Cajal–Retzius cells. **d** | Commissural/associational fibres, arriving in the molecular layer after the entorhinal fibres, grow towards their definitive targets, the granule cells. **e** | The characteristic laminated structure of the dentate gyrus has formed: granule cells have reached their final positions, with their cell bodies forming the granule cell layer. Commissural fibres, having 'travelled' together with the migrating granule cells, occupy the space between the outer molecular layer and the granule cell bodies — the inner molecular layer. Entorhinal fibres occupy the outer molecular layer. Granule cell dendrites now extend throughout the molecular layer. Commissural/associational fibres in the inner molecular layer innervate proximal granule cell dendrites, whereas entorhinal fibres in the outer molecular layer contact distal granule cell dendritic segments, which replace Cajal–Retzius cells as targets.

Radial glia
A type of glial cell that gives rise to long, radially oriented processes. These processes provide a scaffold for radially migrating neurons. Recent studies have shown that radial glial cells are neuronal precursors.

Suprapyramidal blade
Describes the part of the granule cell layer that is close to hippocampal area CA1, and is located above the pyramidal cell layer in CA3.

the presence of hyaluronan-associated ECM molecules. However, laminar specificity of commissural fibre and mossy fibre projections to the hippocampus is determined by factors other than hyaluronan and its associated molecules.

The ECM molecule reelin is expressed and secreted by Cajal–Retzius cells in the marginal zone of the hippocampus. Besides its role in neuronal migration, reelin has been shown to influence the branching pattern of entorhinal fibres projecting to the outer molecular layer. In the reeler mouse, entorhinal fibres are less branched than they are in wild-type mice, which suggests that reelin acts on entorhinal axons and influences their branching pattern[20]. However, reelin is not required for the laminar specificity of entorhinal fibres, because in reeler mutants entorhinal fibres still innervate the outer molecular layer[28]. When an entorhinal slice and a reeler hippocampal slice are co-cultured, entorhinal fibres still innervate the reeler dentate gyrus with proper laminar specificity[16].

Laminating neuronal cell bodies

The strict lamination of neuronal cell bodies is as characteristic of the hippocampus and dentate gyrus as is the segregated termination of afferent projections. As for neurons in the neocortex, two main modes of migration guide hippocampal neurons to their final destination. Whereas excitatory principal neurons — that is, pyramidal cell and granule cell precursors — migrate radially from the neuroepithelium in the ventricular zone towards their final destination near the pial surface, inhibitory interneurons migrate tangentially from the basal telencephalon towards their final destination in the hippocampus. Here, we focus on the radially migrating granule cells, which, unlike hippocampal interneurons, form a compact cell layer.

Radial migration of granule cells. Dentate granule cell precursors originate in the neuroepithelium near the cortical hem[38], which is the medial margin of the dorsal telencephalon. Precursor cells then migrate along radial glial fibres from the ventricular zone towards the anlage of the dentate gyrus near the pial surface[39]. Tracing studies have shown that these early migrating cells consist of both postmitotic cells and precursor cells that maintain their capacity for cell division[40–42]. These observations were later confirmed by retrovirus injections into the ventricles to identify labelled postmigratory cells in the hippocampus[43]. Granule cells in the suprapyramidal blade of the dentate gyrus originate from early cell divisions, whereas granule cells in the infrapyramidal blade originate from the subsequent division of precursor cells in the hilar region[40,41].

In principle, there might be two different modes of radial migration, as previously described for neocortical neurons: neuronal migration, guided by radial glial fibres, and nuclear translocation[44]. The exact mode of migration of granule cells emerging from the secondary proliferation zone in the hilus remains to be determined. Notably, most studies of granule cell migration have not taken into account the fact that cortical radial glial cells are precursors of radially migrating neurons[45–48].

As for radially migrating neurons in the neocortex[49], reelin has a pivotal role in controlling the proper positioning of radially migrating hippocampal neurons. In the absence of reelin, the normal sequence of neuronal layer formation in the neocortex — with early generated neurons forming deep layers and late generated neurons forming superficial layers (inside-out sequence) — is reversed. In the dentate gyrus of reeler mutants, granule cells are not arranged in a compact layer, but are loosely distributed across the entire dentate gyrus[29,50], and malpositioning of hippocampal pyramidal neurons results in a characteristic duplication of the pyramidal cell layer in CA1 (REF. 29).

Box 2 | **Crosstalk between signalling cascades**

In addition to reelin, which is known to be a key player in neuronal lamination, certain components of other signalling cascades have been shown to interfere with neuronal migration in the hippocampus. Cyclin-dependent kinase 5 (CDK5) is a member of the serine/threonine family of kinases, and is regulated by two proteins, p35 and p39 (REF. 107). In CDK5-deficient mice, neuronal migration is disrupted, producing a phenotype similar to that of the reeler mouse, including malpositioning of neurons in the hippocampus[108]. Simultaneous inactivation of p35 and p39 induces defects that are indistinguishable from CDK5 deficiency[109]. Interaction of CDK5 and reelin signalling in controlling hippocampal cell migration has been shown by combining genetic and biochemical approaches[107]. The migration of pyramidal cells in double mutants lacking both p35 and the reelin receptor APOER2 is more disturbed than that in mutants lacking only one of these molecules[107], which suggests the presence of parallel, partially interacting signalling pathways.

Doublecortin (DCX) is a microtubule-associated protein that is encoded by an X-chromosome-linked gene and is required for proper neocortical and hippocampal layer formation[110–113]. Male mice that are hemizygous and female mice that are heterozygous for doublecortin show hippocampal lamination defects. These migration defects are most prominent in the CA3 region of the hippocampus, where the pyramidal layer is split into two. More subtle migration defects have been described in CA1, and no migration defects were found in the dentate gyrus[112]. Severe defects in humans and minor defects in mice have been shown to result from mutation of the gene that encodes lissencephaly 1 (LIS1), another microtubule regulatory protein[114], pointing to closely related roles of these proteins. The different migration defects in different hippocampal subregions suggest that these proteins have regionally specified functions in the control of neuronal migration and lamination, respectively.

The chemokine stromal cell-derived factor 1 (SDF1) and its receptor CXCR4 have been shown to have a role in the migration of dentate granule cells from the ventricular zone to the dentate gyrus[43]. Chemokines belong to a family of peptide ligands that act via G-protein-coupled seven-transmembrane spanning receptors[115]. SDF1 seems to function as a chemoattractive factor for dentate granule cells, as misexpression of SDF1 by electroporation of slice cultures resulted in aberrant granule cell positioning[43].

As in the neocortex, reeler-like migration defects are also seen in the dentate gyrus in mutants with defects in other molecules of the reelin signalling cascade. Therefore, mutants lacking the intracellular adaptor protein disabled 1 (DAB1) or the reelin receptors VLDLR and APOER2 show migration defects similar to those seen in the reeler mutant[30,51]. Granule cell migration defects in mutant mice lacking only one of VLDLR or APOER2 are less severe and slightly different, which implies that the two receptors have different roles in granule cell positioning[31]. The exact roles of the receptors and of the signalling cascades involved in radial migration remain to be characterized (BOX 2).

Lack of granule cell lamination in mutants with defects in the reelin signalling cascade is, at least in part, due to radial glial defects[52–54]. In parallel with the granule cell migration defects described above, radial glial alterations in mice lacking only one of the reelin receptors, VLDLR and APOER2, are mild and different in nature[54]. How does reelin act on radial glial cells? Using hippocampal slice cultures as a model, this question could be addressed by investigating whether reelin needs to be secreted in the marginal zone to exert its effect on granule cell positioning. Zhao and colleagues[50] added recombinant reelin to the incubation medium of reeler hippocampal slices not expressing reelin. In these reelin-treated cultures, the length of glial fibrillary acidic protein (GFAP)-expressing glial fibres was significantly increased compared with untreated control slices. However, the elongated GFAP-positive glial fibres did not form the characteristic radially oriented glial scaffold of the dentate gyrus. In line with this, recombinant reelin in the medium did not rescue the formation of a compact dentate granule cell layer, and granule cells remained scattered across the dentate gyrus[50].

The outcome of the experiment was different when a reeler hippocampal slice was co-cultured with a wild-type hippocampal slice, such that the reelin-containing dentate marginal zone was closely apposed to the reeler slice (FIG. 3). In this co-culture, reelin secreted by the wild-type slice induced growth of radial glial fibres in the reeler dentate gyrus that was oriented towards the source of reelin, namely the wild-type marginal zone. In accordance with this, it was found that radial glial cells express molecules of the reelin signalling pathway and show a preference for a reelin-coated substrate in a stripe choice assay[52,53]. Moreover, in parallel with the rescued radial glial scaffold in reeler slices co-cultured to wild type, the formation of a dense granule cell layer was seen in the reeler slice[50]. These findings suggest that reelin needs to be present in a specific topographic position — that is, the marginal zone — to exert its effect on granule cell positioning. Remarkably, rescue of granule cell lamination in reeler mice by wild-type reelin expression also led to the rescue of laminar specificity of commissural fibres. This confirms the hypothesis that granule cells carry positional cues for the layer-specific termination of commissural fibres[50].

Transcription factors. Various studies have investigated the transcription factors that control the early migration from the neuroepithelium towards the dentate anlage, as well as the formation of the dentate granule cell layer. Loss-of-function mutations in genes known to be expressed in the ventricular zone of the hippocampal anlage were shown to regulate the formation of glutamatergic pyramidal neurons and dentate granule cells. For example, mutant mice with loss-of-function mutations in the high mobility group (HMG)-box transcription factor LEF1, or in the basic helix–loop–helix transcription factor neurogenic differentiation 1 (NeuroD, also known as BETA2), show defects in granule cell generation[55,56], but still have interneurons in this region[56,57]. Interestingly, formation of heterotopic granule cell clusters in these mutants has been reported to be associated with epilepsy[57]. Characteristic features of laminar commissural fibre termination are present in the absence of NeuroD despite the absence of most granule cells, which suggests that a few granule cells provide sufficient instructive information for lamina-specific fibre termination[58].

WNT signalling mutants show decreased dentate granule cell production and radial glial defects[59]. The entire hippocampal region is eliminated by inhibition of β-catenin-mediated WNT signalling in WNT3A mutants[60], or by a mutant allele of LEF1 (REF. 56).

Mutations in Lim homeobox 2 (*Lhx2*), *Lhx5* and empty spiracles homologue 2 (*Emx2*) disrupt the development of hippocampal glutamatergic neurons to varying degrees[61–66]. Although studies of these mutations have provided important information about the genes

Infrapyramidal blade
Describes the part of the granule cell layer that is furthest from CA1, and is located underneath the pyramidal cell layer in CA3.

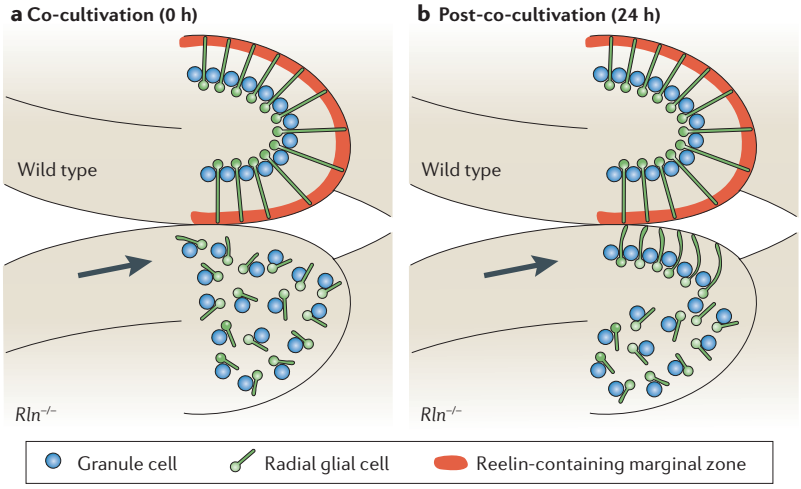


Figure 3 | **Rescue of granule cell lamination in the reeler dentate gyrus. a** | A wild-type hippocampal slice, placed next to a hippocampal slice from a reelin mutant (reeler) mouse (*Rln*$^{-/-}$), provides the reeler slice with a reelin-containing marginal zone. Note the different organization of the dentate gyrus in the two slices. In the wild-type slice, granule cells form a densely packed cell layer. Processes of radial glial cells traverse the granular layer and project towards the reelin-containing marginal zone. In the reeler mutant, granule cells are scattered across the dentate gyrus and the characteristic radial orientation of radial glial fibres is lost (arrow). **b** | As early as 24 h post-co-cultivation, radial glial fibres in the reeler dentate gyrus project radially towards the reelin-containing marginal zone of the wild-type slice, and nearby granule cells have formed a compact granule cell layer (arrow). The results point to a dual function for reelin — that is, in the formation of a regular radial glial scaffold and in the migration of granule cells[50].

involved in the formation of hippocampal and dentate neurons, they have been less conclusive regarding the determinants of hippocampal lamination.

GABAergic interneurons in the hippocampus and dentate gyrus are not arranged in layers, but their axons show a laminated termination (BOX 1). Recently, it has been shown that most GABAergic hippocampal interneurons are generated in the basal telencephalon, similar to neocortical interneurons. Mice with mutations in both *Dlx1* and *Dlx2* homeobox genes have defects in the development of the lateral and medial ganglionic eminences (LGE and MGE, respectively)[23,67,68]. In wild-type mice, immature GABAergic interneurons migrate tangentially from the basal telencephalon across the developing cortical plate to their final destination in the hippocampus. Disruption of DLX1/2 homeobox function blocked the migration of almost all newly generated interneurons originating from progenitors in the MGE and LGE[23]. However, in these mutants, no major effects on the development of glutamatergic hippocampal projection neurons were observed[23]. Mutant mice that lack the homeodomain transcription factor *Nkx2.1* show a malformation of the MGE and have a 60–70% reduction in hippocampal interneurons, which suggests that this percentage of hippocampal interneurons is derived from the MGE.

Generation and migration of Cajal–Retzius cells. Early-generated Cajal–Retzius cells populate the marginal zones of the hippocampus proper and dentate gyrus, thereby participating in hippocampal lamination (see above). Moreover, reelin-expressing Cajal–Retzius cells are required for the proper lamination of principal neurons, as in the neocortex. The origin of Cajal–Retzius cells has been discussed extensively but remains controversial. It was first proposed that they are generated in the ventricular zone[69–71]. Next, it was suggested that they originate in the MGE and migrate tangentially to their destination in the marginal zone[72]. Later on, evidence for several other possible sources of Cajal–Retzius cells was provided, including the cortical hem[71,73–75], and recently it has been confirmed that Cajal–Retzius cells have many origins[76]. In this last study, the septum was proposed to be an additional source of calretinin-negative Cajal–Retzius cells destined to populate the marginal zone of the hippocampus.

Role of meningeal cells. An intact pial surface seems to be required for the proper formation of the dentate radial glial scaffold and for proper positioning of dentate granule cells in the infrapyramidal blade. Disruption of the pial surface of the infrapyramidal blade by treatment with 6-hydroxydopamine at early postnatal stages induced the disruption of the dentate radial glial scaffold and mal-positioning of dentate granule cells[77]. As a consequence, glial cells and granule cells were redirected towards the suprapyramidal blade, where the granule cells were then innervated as normal by commissural/associational fibres and entorhinal fibres[78]. In contrast to the infrapyramidal blade, the suprapyramidal blade of the dentate gyrus is demarcated by the hippocampal fissure, not by meninges. This indicates that there are differences between the formation of the supra- and infrapyramidal blades of the dentate gyrus[79].

Migration of cortical neurons is also disrupted by targeted deletion of pial basement membrane proteins, such as the laminin γ1 chain, or of receptors for basement membrane proteins, such as integrins[80,81]. Similarly, presenilin-deficient mice have alterations in both radial glia morphology and pial basement membrane organization[82]. Integrin-linked kinase (ILK), a serine/threonine protein kinase, is an important effector of integrin function. Recently, ILK-deficient mutant mice were shown to have cortical lamination defects reminiscent of the defects seen in integrin-deficient mutant mice, including malpositioning of dentate granule cells in the hippocampal region[83]. An abnormal interaction between the pial basement membrane and radial glial fibres is thought to result in radial glial defects, which, in turn, might underlie the defects in neuronal positioning.

In mutant mice that lack the protein p73, the pial surface of the dentate anlage does not invaginate and the hippocampal fissure does not form. As a consequence, Cajal–Retzius cells cannot enter the dentate gyrus, suggesting that the proper formation of the hippocampal fissure is required for the migration of hippocampal Cajal–Retzius cells to the hippocampal marginal zones[84]. The chemokine receptor CXCR4, which is expressed by Cajal–Retzius cells, and its ligand SDF1, which is expressed by meningeal cells, are candidates for a role in controlling the migration of Cajal–Retzius cells[85,86].

Ganglionic eminence
A ventral portion of the telencephalic vesicle. It is a source of GABAergic interneurons destined for the neocortex and hippocampus, and is the anlage of the future striatum.

Functions of hippocampal lamination

That there are common rules governing the development of laminar organization in mammals suggests that proper lamination is a prerequisite for normal function. As discussed above, it is remarkable that proper hippocampal lamination follows a developmental programme that does not depend on neuronal activity. This is in line with the finding that Munc18-1-deficient mice develop normally layered structures, fibre projections and synaptic connections despite the lack of neurotransmitter secretion from synaptic vesicles[87]. The relatively sparse, early generated GABAergic interneurons are first excitatory and then inhibitory during the development of the hippocampus, and have an important role in the functional maturation of the hippocampal network[88].

A functional role of hippocampal lamination in the adult is supported by the description of granule cell migration defects in the hippocampus of patients with mesial temporal lobe epilepsy (TLE)[89–91], which points to a causal relationship between lamination defects and epilepsy. Haas and colleagues[91] have shown that granule cell migration defects in the hippocampus of individuals with TLE are inversely correlated with the number of reelin-expressing Cajal–Retzius cells, which suggests that there is a link between reelin secretion and the extent of granule cell dispersion in the hippocampus of patients with epilepsy. Moreover, these studies in tissue samples from human patients suggest that reelin not only controls the formation of granule cell lamination during development but keeps granule cells in register throughout postnatal life, a possibility that can be tested in animal models of epilepsy associated with granule cell dispersion.

In a properly laminated dentate gyrus, afferent input to the granule cells is strictly separated from the output of these neurons. Therefore, afferent fibres project to the molecular layer and terminate on granule cell dendrites, whereas the output fibres — that is, the mossy fibres — are separated from the input by the granule cell bodies. What could be the reason for such a strictly laminated hippocampal organization, and how can this question be addressed experimentally? Malpositioned granule cells disrupt the strict lamination rule and, therefore, the separation of input and output sites. In epilepsy associated with granule cell dispersion, mossy fibre collaterals establish aberrant synapses with granule cell dendrites. It has been proposed that these aberrant projections alter the balance between inhibitory and excitatory input to the dentate gyrus[92,93]. The p35-deficient mouse, which has been used to test this hypothesis, shows granule cell migration defects and spontaneous seizures[94,95]. Electrophysiological studies on this mutant suggest that recurrent axon collaterals of malpositioned granule cells contribute to seizure activity in these mice. However, no such spontaneous seizure activity has been described in the reeler mutant, which also has granule cell migration defects. It is hoped that further electrophysiological and morphological characterization of displaced granule cells in slices from p35-deficient mice and reeler mutants will contribute to our understanding of the functional significance of hippocampal lamination.

Conclusions and future directions

Many details of the development of hippocampal lamination remain to be addressed in future experiments. The molecular cues required to keep commissural fibres in the inner molecular layer of the dentate gyrus remain to be identified. As recent experiments provided evidence that these molecules are associated with granule cell dendrites[16], isolation of membranes from granule cells might be a first step towards characterization of the relevant molecules. Growth of commissural fibres on isolated membrane fractions *in vitro* might be tested using patterned substrates, such as the stripe choice assay developed by Walter *et al.*[96].

The mechanism of reelin function in granule cell lamination is still poorly understood. What is the role of radial glial cells in granule cell migration? Do granule cells migrate along radial glial fibres or do they move by soma translocation? And what are the differences in signalling mediated by the different reelin receptors? An experimental challenge is to dissect the action of reelin on radial glial cells and migrating neurons. Future studies might address these questions by, for example, monitoring migration of single green-fluorescent protein-transfected granule cells in wild-type and mutant hippocampal slices using real-time microscopy of living cells.

In the dentate gyrus, new granule cells are generated throughout life. Newly generated granule cells are functionally integrated in the dentate granule cell layer. What are the mechanisms that ensure proper migration and functional integration of the continuously generated granule cells into the pre-existing and already 'wired' granule cell layer? Recent evidence suggests that GABAergic innervation of proliferating progenitors promotes their neuronal differentiation[97], and we are beginning to unveil the different stages of their functional integration[98].

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Competing interests statement
The authors declare no competing financial interests.

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Summary

- A characteristic feature of the hippocampus is its lamination of neuronal cell bodies and afferent fibre projections. This review summarizes recent studies on the molecular determinants that govern the formation of hippocampal cell and fibre layers.
- Initial experiments using sequential slice co-cultures ruled out temporal factors in the layer-specific termination of afferent projections to the dentate gyrus.
- Afferent fibres from the entorhinal cortex are guided to the dentate gyrus by axons of pioneer neurons (Cajal–Retzius cells). By contrast, commissural/associational fibres to the dentate gyrus do not require pioneer neurons. They are guided to the inner molecular layer by positional cues on proximal segments of granule cell dendrites.
- The layer-specific termination of entorhinal axons in the outer molecular layer of the dentate gyrus is controlled by the extracellular matrix molecule hyaluronan. The lamination of granule cells is under the control of the extracellular matrix protein reelin.
- In the marginal zone of the dentate gyrus, reelin is a positional signal for the extension and orientation of radial glial fibres. A regular radial glial scaffold is necessary for the directed migration of granule cells. Reelin also acts as a stop signal for migrating granule cells, preventing them from invading the molecular layer.
- A laminated dentate gyrus is required for the proper function of the hippocampus. Temporal lobe epilepsy is associated with a loss of granule cell lamination (granule cell dispersion) and decreased reelin

expression. Decreased reelin expression associated with granule cell dispersion in epilepsy suggests that reelin controls granule cell lamination not only during development, but throughout postnatal life.

TOC blurb

The relatively simple architecture of the hippocampus makes it an ideal model to study cortical lamination. Förster *et al.* review recent work on the molecular mechanisms that guide the formation of cell and fibre layers in the hippocampus.