Reelin is a positional signal for the lamination of dentate granule cells

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Summary

Reelin is required for the proper positioning of neurons in the cerebral cortex. In the *reeler* mutant lacking reelin, the granule cells of the dentate gyrus fail to form a regular, densely packed cell layer. Recent evidence suggests that this defect is due to the malformation of radial glial processes required for granule cell migration. Here, we show that recombinant reelin in the medium significantly increases the length of GFAP-positive radial glial fibers in slice cultures of *reeler* hippocampus, but does not rescue either radial glial fiber orientation or granule cell lamination. However, rescue of radial glial fiber orientation and

granule cell lamination was achieved when reelin was present in the normotopic position provided by wild-type co-culture, an effect that is blocked by the CR-50 antibody against reelin. These results indicate a dual function of reelin in the dentate gyrus, as a differentiation factor for radial glial cells and as a positional cue for radial fiber orientation and granule cell migration.

Key words: Neuronal migration, Layer formation, Radial glia, Reelin, *reeler* mouse, Dentate gyrus

Introduction

The extracellular matrix protein reelin controls neuronal migration by binding to different reelin receptors (Senzaki et al., 1999; Trommsdorff et al., 1999; Hiesberger et al., 1999; Dulabon et al., 2000). In the mouse mutant *reeler*, migration of neurons in the neocortex, cerebellum and hippocampus is severely altered. The mechanism of reelin action, however, has remained unclear (Tissir and Goffinet, 2003). Reelin is synthesized and secreted by Cajal-Retzius (CR) cells located in the marginal zone of the cerebral cortex (D'Arcangelo et al., 1995; D'Arcangelo et al., 1997; del Rio et al., 1997; Frotscher, 1998). As the marginal zone is almost cell-free in wild-type animals but is invaded by numerous neurons in the *reeler* mutant, reelin has been proposed to act as a stop signal for migrating neurons (Curran and D'Arcangelo, 1998; Frotscher, 1998).

An alternative function of reelin was recently suggested by Förster et al. (Förster et al., 2002), who showed that Disabled1 (Dab1), a molecule of the reelin signaling cascade (Howell et al., 1997; Sheldon et al., 1997; Ware et al., 1997), is expressed by glial fibrillary acidic protein (GFAP)-positive radial glial cells in the dentate gyrus. They also found a malformation of radial glial processes in the *reeler* dentate gyrus and concluded that reelin controls the migration of dentate granule cells by acting on the radial glial scaffold required for migration (Förster et al., 2002; Frotscher et al., 2003; Weiss et al., 2003).

Reelin, which is secreted by CR cells in the marginal zone, forms a component of the local extracellular matrix. It has remained open to question whether this specific distribution of reelin is crucial for its function. Previous studies that addressed this issue (Magdaleno et al., 2002) showed that heterotopic

reelin expression partially rescued the migration defects in the *reeler* mutant, suggesting that reelin does not function simply as a positional signal. In line with this study, incubation of slices from embryonic *reeler* neocortex in the presence of recombinant reelin partially rescued the *reeler* phenotype (Jossin et al., 2004).

Here, we have used slice cultures of postnatal *reeler* hippocampus and recombinant reelin to study the effects of reelin on neuronal migration in the dentate gyrus, a brain region known for its late, largely postnatal neurogenesis. Our findings suggest that reelin acts as both a differentiation factor for radial glial cells, and a positional signal for radial fiber orientation and granule cell migration in the dentate gyrus.

Materials and methods

Preparation of hippocampal slice cultures

For the preparation of hippocampal slice cultures, newborn homozygous *reeler* mouse pups (P0) and young postnatal wild-type mice and rats were used. Brains were removed following decapitation under hypothermic anaesthesia. The hippocampi were dissected and sliced (300 μm) perpendicular to their longitudinal axis with a McIlwain tissue chopper. *reeler* mice were identified by their well-known morphological malformations in the cortex and hippocampus. In addition, the genotype of the mutants was confirmed by PCR analysis of genomic DNA, as described (Deller et al., 1999). All experiments were performed in agreement with the institutional guide for animal care.

For the preparation of co-cultures, hippocampal slices of newborn *reeler* mice were positioned in close vicinity to the outer molecular layer of the dentate gyrus of slices from wild-type mice or young Wistar rats (P2-P5), so that the dentate gyrus of the *reeler* slice came

in direct contact with the reelin-containing marginal zone of the wild-type slice (see Fig. 3A). Slices were placed onto Millipore membranes and transferred to a six-well plate with 1 ml/well nutrition medium (25% heat-inactivated horse serum, 25% Hank's balanced salt solution, 50% minimal essential medium, 2 mM glutamine, pH 7.2). Slices were incubated as static cultures in 5% CO_2 at 37°C for 7 to 10 days (Stoppini et al., 1991). The medium was changed every 2 days.

For the preparation of co-cultures from *reeler* hippocampus and wild-type olfactory bulb, the olfactory bulb of P2-P5 wild-type animals was coronally sectioned with a tissue chopper (300 µm). Then, the lamina glomerulosa of the olfactory slices was removed with a scalpel to expose the reelin-synthesizing mitral cells that are not superficially located like CR cells in the marginal zone of neocortex and hippocampus. Sections of *reeler* dentate gyrus were then positioned in close vicinity to the mitral cell layer of the olfactory bulb.

Transfection of 293 cells with the full-length reelin cDNA and preparation of reelin-containing and control supernatants

293 cells were transfected with the full-length reelin clone pCrl, a generous gift of Dr T. Curran (D'Arcangelo et al., 1997), as described elsewhere (Förster et al., 2002). In brief, full-length reelin-synthesizing clones were identified by RT-PCR, using various primer pairs spanning different parts of the reelin cDNA, and by immunocytochemistry and western blotting of cell supernatants employing the G10 antibody against reelin, kindly provided by Dr A. Goffinet (de Bergeyck et al., 1998). Reelin-enriched supernatants and control supernatants were obtained from serum-free incubation medium of reelin-transfected 293 cells and green fluorescent protein-transfected control cells, respectively. Reelin content was confirmed by western blotting using the G10 antibody (Förster et al., 2002; Frotscher et al., 2003).

Treatment of *reeler* cultures with recombinant reelin

Supernatant (200 µl) from reelin-synthesizing 293 cells was added to each well containing 1 ml normal nutrition medium. In addition, 1 µl of this supernatant was directly applied to each individual *reeler* hippocampal culture 3 times a day from day in vitro (DIV) 0 to DIV 7 (n=16). As a control, 200 µl of the supernatant from 293 cells transfected with a plasmid encoding for green fluorescent protein (GFP) were added to the medium of each well containing 1 ml normal nutrition medium, and 1 µl of this supernatant was applied to each *reeler* culture (n=12).

Incubation of hippocampal co-cultures with the CR-50 antibody against reelin

During the first 3 days after preparation, 1 µl of CR-50 [100 µg/ml, diluted in sterile saline; kindly provided by Dr M. Ogawa (see Ogawa et al., 1995)] was added to each hippocampal co-culture 3 times a day (n=9).

Biocytin labeling

After 9 DIV, a crystal of biocytin (Sigma, Munich, Germany) was placed onto the rescued cell layer in slices from the *reeler* dentate gyrus, or onto the hilar region of the wild-type mouse, or rat co-culture, in order to label *reeler* granule cells and 'commissural' projections to the *reeler* culture, respectively. After 10 DIV, the cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, re-sliced on a Vibratome (50 µm), and incubated with avidin/biotin peroxidase complex (Vector Laboratories, Burlingame, CA). Sections were developed with diaminobenzidine/nickel (DAB-Ni) and counterstained with Cresyl Violet.

Immunocytochemistry

After DIV 7 to 10, the cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 2 hours. Then the cultures were re-sliced on a Vibratome (50 µm). Sections were pre-incubated for 30 minutes with blocking solution (5% normal goat serum, 0.2% Triton-X 100 in 0.1 M PB) at room temperature. After rinsing in 0.1 M PB, sections were incubated with the following primary antibodies overnight at 4°C: mouse anti-reelin G10 (1:1000, a generous gift of Dr A. Goffinet), rabbit anti-GFAP (1:500, DAKO, Denmark), mouse anti-NeuN (Neuron-specific Nuclear Protein; 1:1000, Chemicon, Hofheim, Germany), mouse anti-calbindin (1:500, Chemicon, Hofheim, Germany), rabbit anti-calretinin (1:3000, SWant, Bellinzona, Switzerland), goat anti-calretinin (1:2000, Chemicon, Hofheim, Germany) and rabbit anti-apolipoprotein E receptor 2 (ApoER2; 1:1000, Santa Cruz Biotechnology, Heidelberg, Germany). After washing in 0.1 M PB, sections were incubated in secondary antibodies, Alexa 488 and/or Alexa 568 (1:600, Molecular Probes, Göttingen, Germany), overnight at 4°C. Some sections were incubated with Neurotrace (1:1000; Molecular Probes, Göttingen, Germany) for 30 minutes at room temperature. After rinsing in 0.1 M PB for 2 hours, sections were mounted in Mowiol.

Quantitative analysis of GFAP-positive fibers

GFAP-stained sections were photographed using a confocal microscope (LSM 510, Carl Zeiss, Germany). The length of GFAP-positive fibers was measured by employing analySIS software (Soft Imaging System GmbH, Münster, Germany). To estimate the density of GFAP-positive fibers, 3D reconstructions were made (section thickness, 10 µm; interval, 1 µm). Then the number of cross-sectioned GFAP-positive fibers per area was determined. The length density (Lv) was calculated using the formula: $Lv=2\times N/A$ (N, number of cross-sectioned profiles; A, area). Differences were tested for significance (Student's *t*-test; $P<0.01$).

Western blot analysis for reelin

Western blot analysis included lysates of hippocampal tissue from P5 rats and of slice cultures from rat and *reeler* hippocampus, and supernatants of the different incubation conditions.

Brains of P5 rats were removed following decapitation under hypothermic anaesthesia. The hippocampi were prepared and collected in 1.5 ml tubes on ice. The samples were weighed and immediately frozen in liquid nitrogen. Wild-type and *reeler* hippocampal slices treated with recombinant reelin were incubated for 7 days and then collected in 1.5 ml tubes on ice. The samples were weighed and immediately frozen in liquid nitrogen. Six volumes (v/w) of hypotonic lysis buffer [50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% protease inhibitor cocktail (Sigma, Munich, Germany), pH 7.6] were added to each sample and the tissue lysed by repeated thawing at 37°C and freezing in liquid nitrogen (five times). After sonication for 5 minutes and trituration with a pipette tip to homogenize larger tissue pieces, the suspension was centrifuged at 20,000 *g* for 20 minutes (0°C). The resulting crude supernatants were stored at –80°C.

The incubation medium of slice cultures was collected at different time points and stored at –80°C. One microliter recombinant reelin supernatant, 1 µl control supernatant, 1 µl each of the different media, and 6 µl of the supernatants from freshly prepared tissue and slice cultures (6 µl in this case because reelin in tissue and slice cultures was diluted 1:6 during the extraction) were diluted with sample buffer (Invitrogen, Karlsruhe, Germany) and boiled for 5 minutes. Proteins were separated by 3-8% gradient Tris-Acetate gel electrophoresis (SDS-PAGE, Invitrogen, Karlsruhe, Germany) and transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes. A monoclonal antibody against reelin (G10, provided by Dr A. Goffinet) was used as primary antibody at a dilution of 1:3000, followed by an alkaline phosphatase-conjugated secondary antibody (1:10000, Invitrogen, Karlsruhe, Germany). The immunoreaction was visualized by a chemiluminescence reaction (Invitrogen, Karlsruhe, Germany).

Results

The *reeler* phenotype is preserved in slice cultures of hippocampus

In slice cultures of newborn wild-type hippocampus the characteristic cell layers of the hippocampus proper and dentate gyrus are retained as visualized by staining for NeuN (Neuna60 – Mouse Genome Informatics), a neuronal marker (Fig. 1A). By contrast, slice cultures of *reeler* hippocampus show the characteristic migration defects of pyramidal neurons and granule cells (Fig. 1B). Pyramidal cells in CA1 of the *reeler* hippocampus form two layers, and the granule cells, normally arranged in a densely packed layer, are scattered all over the dentate gyrus. Staining of wild-type cultures for GFAP reveals long immunoreactive processes that traverse the granular layer perpendicularly (Fig. 1C), as is characteristic for radial glial fibers (Förster et al., 2002; Weiss et al., 2003). Long radially oriented fibers are not observed in slice cultures from *reeler* hippocampus. Rather, GFAP-positive cells give rise to short processes, thus resembling astrocytes (Fig. 1D). These results show that the characteristic neuronal and glial phenotype of the *reeler* hippocampus (Stanfield and Cowan, 1979; Drakew et al., 2002; Weiss et al., 2003) is preserved in slice culture.

Recombinant reelin increases radial glial fiber length and density

When recombinant reelin was added to slice cultures of *reeler*

Fig. 1. The *reeler* phenotype is preserved in slice cultures of hippocampus. (A) Slice culture of wild-type hippocampus, prepared on P0 and incubated for 7 days in vitro (DIV). Staining for NeuN reveals dense packing of pyramidal neurons in CA1 and CA3, and of granule cells in the granular layer (g) of the dentate gyrus (DG). (B) Slice culture of *reeler* hippocampus, prepared on P0 and incubated for 7 DIV. NeuN-stained pyramidal neurons and granule cells show the migration defect characteristic of the *reeler* hippocampus. Pyramidal neurons in CA1 form a double layer (asterisks), and the granule cells are scattered all over the dentate gyrus. (C) Double-labeling for NeuN (red) and GFAP (green) in a slice culture of wild-type dentate gyrus. Long GFAP-positive radial glial fibers run perpendicular to the granular layer. g, granular layer; h, hilus; m, molecular layer. (D) Detail of *reeler* dentate gyrus double-labeled for NeuN and GFAP. Granule cells do not form a circumscribed layer, and GFAP-positive cells have short processes, thus resembling typical astrocytes. Scale bars: 100 μm in A,B; 20 μm in C,D.

hippocampus, we noticed a significant increase in the length and density of GFAP-positive fibers in the dentate gyrus (Fig. 2A-F). However, these longer fibers did not show a preferential orientation as seen in wild-type slices. They rather traversed the dentate gyrus in all directions often crossing each other at right angles (Fig. 2B). Counterstaining for NeuN demonstrated that application of recombinant reelin did not rescue the granule cell migration defect. Neurons were scattered all over the dentate gyrus in these cultures (Fig. 1C,D), which were indistinguishable from untreated *reeler* cultures. These results show that reelin in the culture medium is effective by acting
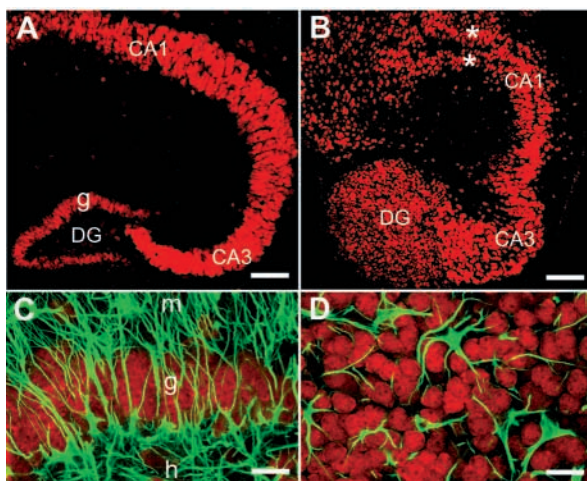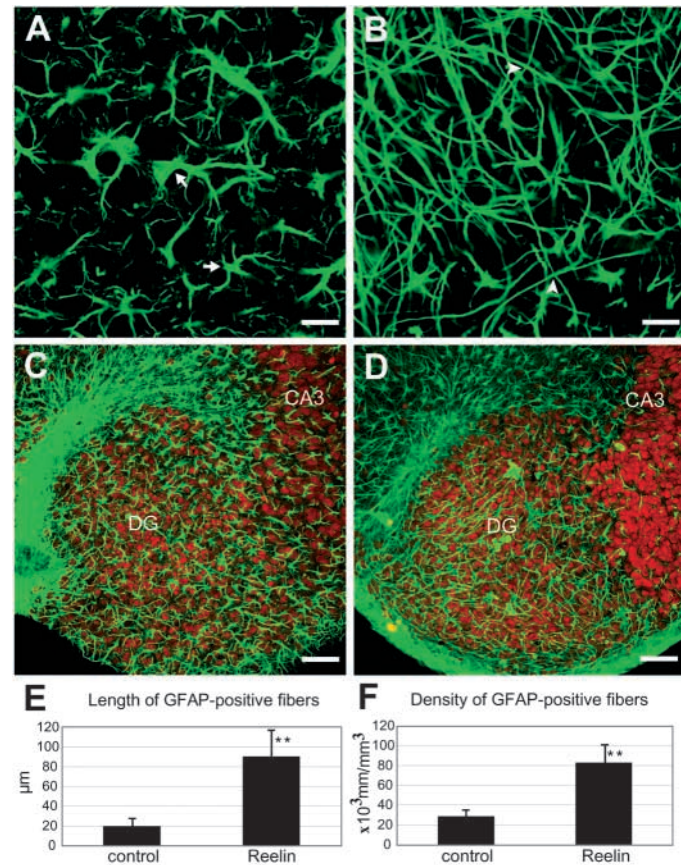

Fig. 2. Treatment of *reeler* slice cultures with recombinant reelin increases the length and density of GFAP-positive fibers. (A) Portion of the hilus of the dentate gyrus in an untreated control culture of *reeler* hippocampus. GFAP-positive cells have relatively thick, short processes, reminiscent of astrocytes. Two characteristic cells are labeled by arrows. (B) Portion of *reeler* dentate gyrus after incubation with recombinant reelin for 7 days. The length of GFAP-positive fibers has dramatically increased. The fibers run in all directions, often crossing each other at right angles (arrowheads). (C) Same control culture as that shown in A, counterstained for NeuN. As is characteristic for the migration defect in the *reeler* mutant, dentate granule cells are scattered all over the dentate gyrus (DG). (D) Same culture as that shown in B, counterstained for NeuN. Treatment with recombinant reelin, while increasing the length of GFAP-positive fibers, did not rescue the granule cell migration defect characteristic of the *reeler* dentate gyrus. CA3, hippocampal region CA3. Scale bars: 20 μm in A,B; 75 μm in C,D. (E,F) Incubation of the cultures with recombinant reelin significantly (**) increased both the length and the density of GFAP-positive fibers in the hilar region of *reeler* cultures ($n=10$; $P<0.01$).
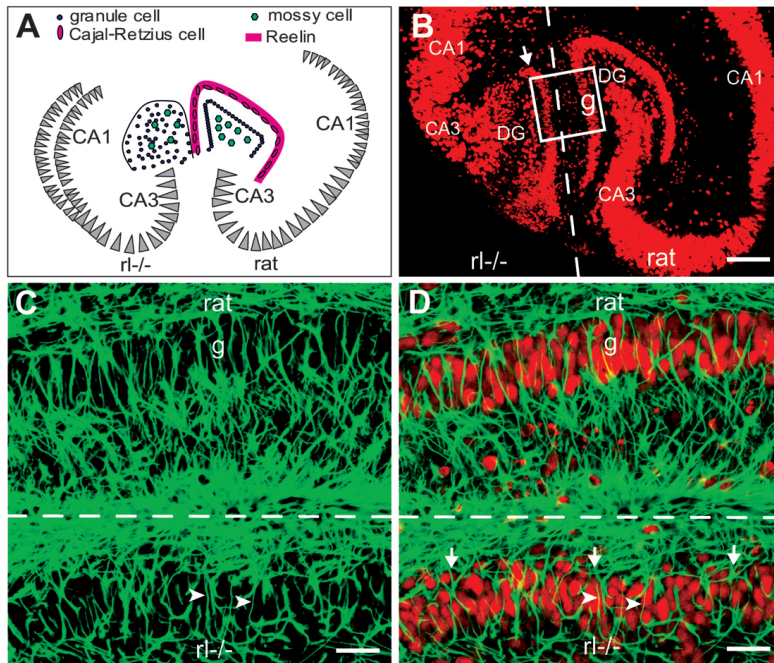
Fig. 3. Co-cultivation of *reeler* (rl–/–) dentate gyrus with wild type rescues radial fiber orientation and neuronal lamination. (A) Schematic diagram illustrating the experimental design. The rat dentate outer molecular layer was co-cultured next to the *reeler* dentate gyrus to provide the *reeler* dentate gyrus with a reelin-containing zone in normotopic position. (B) NeuN staining of a rat hippocampal culture co-cultured with the *reeler* hippocampus, as indicated in A. Note the formation of a compact cell layer in the *reeler* dentate gyrus (arrow) adjacent to the rat outer molecular layer. Dashed line indicates border between the two cultures. The boxed area is shown in C and D at a higher magnification. (C) Boxed area shown in B, immunostained for GFAP. Note the long, vertically oriented radial fibers (arrowheads) in the *reeler* dentate gyrus adjacent to the rat outer molecular layer. The dotted line indicates the border between the cultures. (D) Same detail as is shown in C, counterstained for NeuN. Arrows indicate the compact cell layer formed in the vicinity of the rat outer molecular layer. Scale bars: 150 μm in B; 40 μm in C,D.

on the differentiation of glial processes, but it does not rescue the migration of dentate granule cells.

Rescue of radial fiber orientation and granule cell migration by wild-type co-culture

With the concept that reelin might provide a positional signal for radial glial fiber orientation and granule cell migration, we attempted to add reelin to *reeler* hippocampal cultures in its normal topographical location. We reasoned that co-culturing of the *reeler* dentate gyrus with the dentate gyrus of wild-type mouse or rat such that the two molecular layers were in close apposition, would provide reelin in an almost normal topographical location to a defined portion of the *reeler* dentate gyrus (Fig. 3A). With this approach, we not only observed the formation of a densely packed cell layer in exactly those parts of the *reeler* slices that faced the wild-type mouse or rat slice (Fig. 3B,D), but also noticed a preferential orientation of GFAP-positive fibers towards the wild-type culture (Fig. 3C,D). As seen in the *reeler* cultures treated with recombinant reelin, GFAP-positive fibers adjacent to the wild-type tissue had increased in length. Portions of the *reeler* slice remote from the co-cultured wild-type tissue showed neither a densely packed neuronal cell layer nor long, radially oriented GFAP-positive fibers. Remarkably, a dense cell layer only formed in *reeler* cultures prepared at early postnatal age (P0-P2), when numerous granule cells are being generated, and not in *reeler* cultures from P4, suggesting that only newborn, migrating granule cells were capable of responding to wild-type tissue by layer formation.

A topic effect of wild-type tissue, probably of reelin in the marginal zone of the dentate gyrus, was further confirmed in triplet cultures in which a wild-type culture was placed in between two *reeler* cultures (Fig. 4). One of the *reeler* cultures was placed next to the outer molecular layer of the wild-type culture as described above, whereas the other *reeler* culture was placed next to CA1 in a position remote from the wild-type

marginal zone. Whereas the *reeler* culture next to the wild-type marginal zone formed a compact cell layer, the culture placed next to CA1 retained its loose distribution of neurons all over the dentate area.

Together, these findings point to a topic effect of the co-cultured slice on radial glial fiber length and orientation, and on granule cell lamination in the *reeler* culture, which is likely to be caused by reelin present in the adjacent wild-type marginal zone.

Rescue of radial fiber orientation and granule cell lamination is caused by reelin in the normotopic position

The experiments with wild-type co-cultures suggested an effect of reelin in the marginal zone of the wild-type culture on the
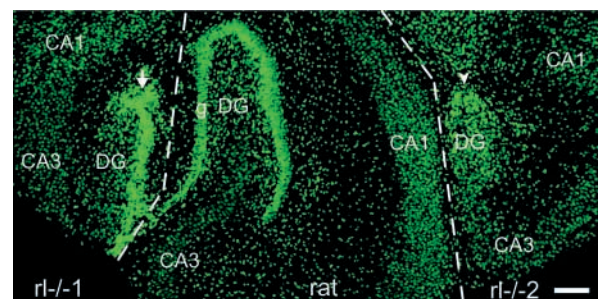

Fig. 4. A specific position of the rat hippocampal slice culture is required to induce a compact cell layer in the *reeler* dentate gyrus. Two *reeler* cultures (rl–/–1 and rl–/–2) are co-cultured with a rat hippocampal slice. A compact cell layer (arrow) has only formed in rl–/–1, which was co-cultured next to the outer molecular layer of the rat dentate gyrus. In rl–/–2, which was cultured next to the stratum oriens of CA1, the *reeler*-specific loose distribution of neurons in the dentate gyrus is retained (arrowhead). Dashed lines represent borders between cultures. Scale bar: 200 μm.
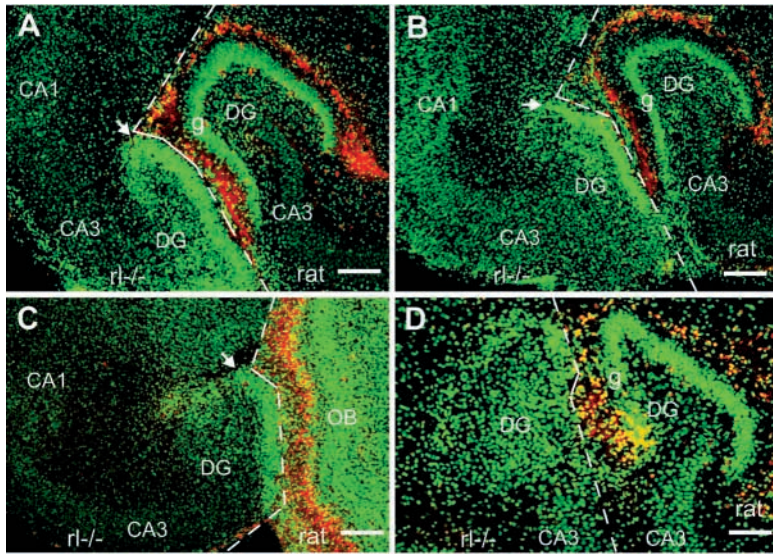
Fig. 5. Rescue of neuronal lamination is induced by reelin. (A,B) *Reeler* hippocampus co-cultured next to rat hippocampus; the dotted line represents the border between the two cultures. Neuronal somata stained for Neurotrace (green); counterstained for reelin (anti-reelin G10; red). A compact neuronal layer (arrows) has only formed in those portions of the *reeler* culture that are juxtapositioned to reelin-synthesizing cells in the outer molecular layer of the rat culture. (C) *Reeler* hippocampal culture co-cultured next to a rat olfactory bulb (OB) culture. The border between the two cultures is marked by a dashed line. A dense neuronal layer (arrow) in the *reeler* culture has formed near the border of the rat olfactory bulb culture, containing numerous reelin-synthesizing mitral cells (red). (D) A compact neuronal layer in the *reeler* dentate gyrus next to reelin-synthesizing neurons in a rat co-culture fails to form when the two cultures were incubated in the presence of the reelin-blocking CR-50 antibody. Scale bars: 175 μm in A,B,C; 150 μm in D.

adjacent *reeler* slice. In order to test this possibility, we stained these co-cultures with an antibody against reelin (G10, kindly provided by Dr A. Goffinet, Bruxelles). In the wild-type culture, reelin-immunoreactive cells were located in the outer portion of the molecular layer surrounded the band of granule cells (Fig. 5A). In the *reeler* culture, a dense cell layer had only formed in those portions of the *reeler* dentate gyrus that were adjacent to the reelin-positive cells of the wild-type culture, strongly suggesting that this layer formation in the *reeler* culture was caused by the juxtapositioned reelin in the wild-type tissue (Fig. 5A,B).

In order to substantiate an effect of wild type-derived reelin (and not of some other factors of the co-cultured wild-type hippocampus), we next co-cultured *reeler* slices with slices of the olfactory bulb known to contain numerous reelin-synthesizing mitral cells throughout postnatal life (Drakew et al., 1998; Hack et al., 2002). Like with the co-cultures of wild-type hippocampus, *reeler* dentate gyrus neurons formed a compact cell layer in portions of the slice adjacent to the co-cultured mitral cells of the olfactory slice (Fig. 5C). Finally, we aimed at neutralizing the reelin effects of co-cultured wild-type slices by incubating co-cultures of wild-type and *reeler* hippocampus in the presence of CR-50, an antibody known to block reelin effects (Ogawa et al., 1995) (kindly provided by Dr M. Ogawa). Incubating the co-cultures with CR-50 abolished the rescue effect of the wild-type culture (Fig. 5D), strongly indicating that reelin, provided by wild-type tissue in specific topographical arrangement, had induced the formation of a compact cell layer in the *reeler* culture. As expected, co-culturing of two *reeler* hippocampal slices did not induce the formation of a compact cell layer (data not shown).

An involvement of reelin in the various experimental approaches described here was confirmed by western blot analysis (Fig. 6). This figure not only shows that reelin is present in fresh hippocampal tissue and in slice cultures from young postnatal rats used for the present rescue experiments (lanes 5, 6), but also demonstrates the presence of reelin in the supernatant of reelin-transfected cells and in *reeler* slice cultures incubated with this supernatant (lanes 1, 7). Interestingly enough, the concentration of reelin and its

fragments appeared higher in the tissue of *reeler* cultures treated with recombinant reelin than in wild-type cultures (lanes 6, 7). This finding makes it unlikely that the lack of a rescue of granule cell lamination in slice cultures treated with recombinant reelin is due to low reelin concentration. We conclude that reelin is required in its normal topographical position to exert its effects on granule cell lamination.

The rescued cell layer contains granule cells developing their normal dendritic and axonal orientation

The dentate gyrus of normal rodents is characterized by co-aligned granule cell somata, displaying a characteristic bipolar morphology with their dendrites extending into the molecular layer, and the axons, the mossy fibers, invading the hilar region. The band of granule cells is clearly segregated from hilar mossy cells. In the mouse hippocampus, both cell types can be differentiated by their different content of calcium-binding proteins. Whereas the granule cells are known to
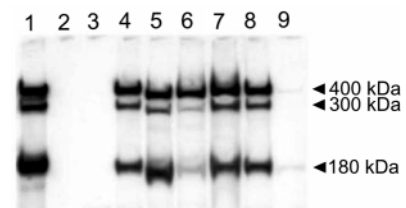

Fig. 6. Western blots for reelin under the different experimental conditions. Lane 1, supernatant of reelin-transfected 293 cells. Arrowheads indicate the full-length protein and its characteristic fragments. Lane 2, supernatant of control 293 cells. Lane 3, freshly prepared culture incubation medium. Lane 4, freshly prepared supernatant (200 μl) from reelin-transfected 293 cells, added to 800 μl incubation medium (as was used in the experiments with *reeler* cultures). Lane 5, lysate of rat hippocampus (P5). Lane 6, lysate of slice cultures of P5 rat hippocampus incubated in vitro for 7 days. Lane 7, lysate of slice cultures of P0 *reeler* hippocampus incubated in the presence of reelin (DIV 7). Lane 8, same incubation medium as in lane 4, after 2 days of incubation. Lane 9, incubation medium from wild-type slice cultures after 2 days of incubation.

contain calbindin, hilar mossy cells can be stained by applying antibodies to calretinin. In *reeler* mice, calbindin-positive granule cells and calretinin-positive mossy cells are intermingled (Drakew et al., 2002). When we immunostained our co-cultures of wild-type and *reeler* hippocampus for these calcium-binding proteins, we noticed that only calbindin-positive granule cells established a compact cell layer, whereas calretinin-positive mossy cells were scattered underneath in the hilar region (Fig. 7A-D). These findings suggest a cell-specific response of the granule cells to reelin, probably because only late-generated granule cells, and not early-generated mossy cells, were able to migrate in the present postnatal cultures. Alternatively, the mossy cells could be located too far away from the reelin source, when compared with granule cells, or could lack reelin receptors or other molecules of the reelin signaling cascade. The former

Fig. 7. Rescued neurons in the compact cell layer are calbindin-positive granule cells. (A,C) Low-power micrographs of *reeler* hippocampal cultures co-cultured next to the rat hippocampus to promote the formation of a dense neuronal layer (arrows) in the *reeler* cultures (staining for Neurotrace). (B) Boxed area in A counterstained for calbindin (red). Note that the neurons in the compact cell layer of the *reeler* culture are calbindin-positive, like the granule cells in the rat dentate gyrus. The dotted line represents the border between cultures. (D) Boxed area in C immunostained for calretinin (red). Only hilar neurons in the *reeler* mouse dentate gyrus are immunoreactive, indicating that these neurons do not participate in the formation of the densely packed cell layer (as a species difference, hilar mossy cells in the rat are not calretinin-positive). (E) Calretinin-immunopositive mossy cells (red) intermingle with granule cells in a slice culture of *reeler* hippocampus (culture from P0 mouse, DIV7; counterstained with Neurotrace, green). (F) High-power magnification of a calretinin-immunoreactive mossy cell (red) in the dentate area of a *reeler* culture (P0; DIV7). The cell was double-labeled for ApoER2 (green puncta). Scale bars: 200 μm in A,C; 85 μm in B; 55 μm in D; 80 μm in E; 4 μm in F.

assumption could be discarded, as mossy cells and granule cells intermingle in the *reeler* dentate gyrus (Fig. 7E) (Drakew et al., 2002); the latter hypothesis was tested using double-labeling experiments. Fig. 7F shows a mossy cell, identified by immunostaining for calretinin, which co-localizes with punctate labeling for ApoER2, one of the lipoprotein receptors known to bind reelin. These findings are in line with our previous observation that virtually all neurons in the hilar region, including mossy cells, express the adapter protein Dab1 (Förster et al., 2002). We conclude that the mossy cells can sense reelin but are unable to migrate under the present experimental conditions because they are postmigratory, differentiated neurons. By specifically influencing the migration of late born granule cells, reelin contributes to the segregation of these two neuronal types.

As revealed by Golgi impregnation, many granule cells in the dentate gyrus of the *reeler* hippocampus have lost their characteristic bipolar morphology and orientation (Stanfield and Cowan, 1979; Drakew et al., 2002). Similarly, when we labeled granule cells in slice cultures of *reeler* hippocampus by extracellular application of biocytin, we found that they extended their dendrites in all directions (Fig. 8A,B). We next wanted to know whether granule cells in the rescued granular layer of *reeler* cultures co-cultured with wild type had developed their characteristic dendritic and axonal orientation. In the newly formed dense band of the *reeler* culture, we observed that the majority of the labeled neurons gave rise to dendrites directed towards the adjacent wild-type culture, whereas the granule cell axons invaded the hilar region (Fig. 8C,D). These results provide evidence that reelin may not only have an effect on the directional growth of GFAP-positive radial fibers, but on the granule cell dendrites as well.

Rescue of the commissural projection to the *reeler* dentate gyrus

In addition to the dense packing of granule cells, the normal dentate gyrus is characterized by a laminated termination of afferent fiber systems (Blackstad, 1956; Blackstad, 1958). Thus, commissural/associational fibers originating from contralateral and ipsilateral hilar mossy cells give rise to a sharply delineated projection to the inner molecular layer, impinging on proximal granule cell dendrites. Fibers from the entorhinal cortex, by contrast, are known to form a dense projection to the outer molecular layer. In the *reeler* dentate gyrus, the characteristic layer-specific termination of the commissural fibers, but not that of entorhinal axons, is lost. We were recently able to show that this loss of laminar specificity of commissural fibers is not due to a cell-autonomous effect of the *reeler* mutation on the projecting neurons, but mimics the scattered distribution of the target granule cells (Gebhardt et al., 2002; Zhao et al., 2003). Here, we wondered whether the establishment of a granule cell layer in *reeler* cultures co-cultured next to wild type would also rescue the lamina-specific termination of commissural axons. For this purpose, we co-cultured P0 *reeler* slices with wild-type cultures for 9 days to allow fiber projections between the two cultures to develop. As a control, wild-type sections were co-cultured with slices from P4 *reeler* mice, which do not respond to wild type by forming a compact cell layer (see above). Then, mossy cells in the wild-type culture, known to give rise to a 'commissural'
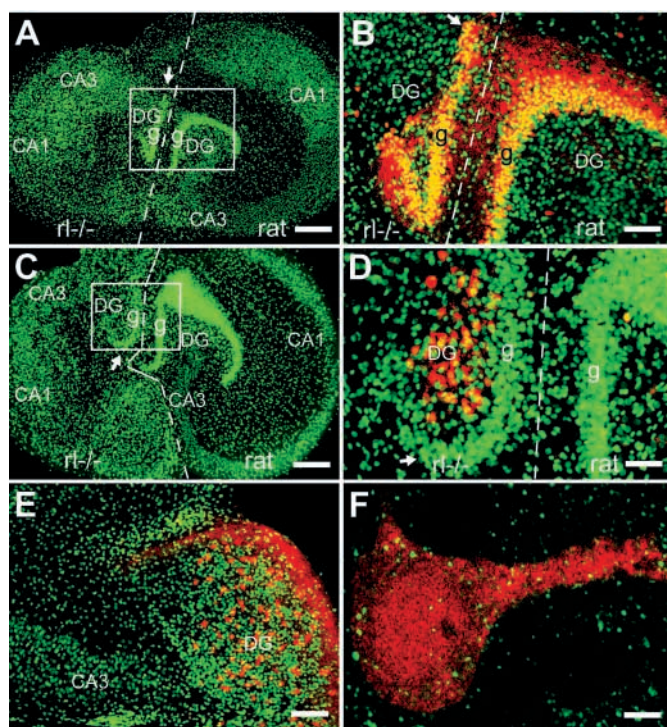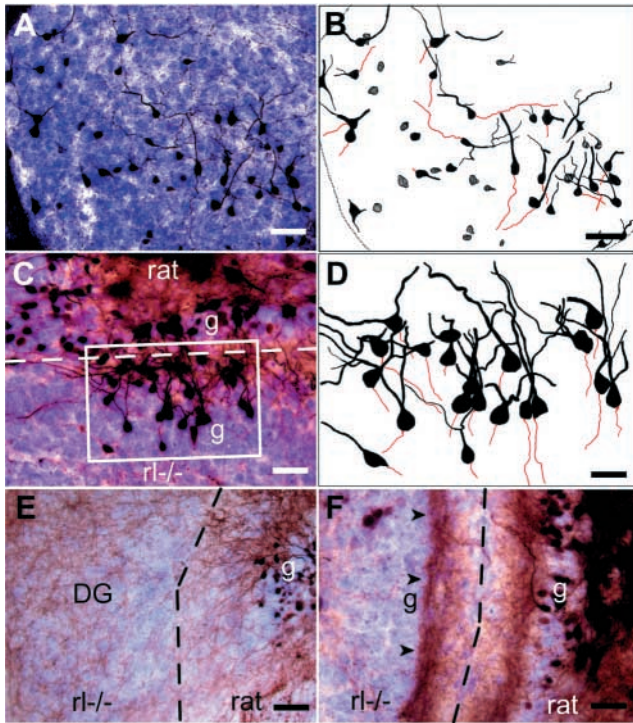
Fig. 8. Rescue of granule cell orientation and layer-specific commissural input. (A,B) Scattered distribution of biocytin-labeled granule cells in the dentate gyrus of a *reeler* hippocampal slice culture. Granule cell dendrites and axons (red in B) extend in all directions. In A, neuronal somata were counterstained for Cresyl Violet to show the loose distribution of granule cells. (C,D) Many biocytin-labeled granule cells in the rescued granular layer of a *reeler* slice culture show normal dendritic and axonal orientation. Neuronal somata are counterstained for Cresyl Violet (C) to illustrate the formation of a granular layer in the *reeler* culture. Dashed line represents border between cultures; the boxed area is depicted in D. (E,F) The laminated projection of commissural fibers to the *reeler* dentate gyrus is only rescued when a compact granule cell layer has formed. (E) Co-culture of rat dentate gyrus and P4 *reeler* dentate gyrus. 'Commissural' fibers from the rat dentate gyrus are scattered all over the *reeler* dentate gyrus, like their target granule cells, counterstained for Cresyl Violet. (F) By contrast, when a rat dentate gyrus is co-cultured with P0 *reeler* dentate gyrus, a granular layer and a compact 'commissural' projection (arrowheads) have formed. Dashed lines represent borders between cultures. Scale bars: 45 μm in A,B; 50 μm in C; 30 μm in D; 70 μm in E; 50 μm in F.

projection to the adjacent *reeler* culture (Zhao et al., 2003), were labeled by the extracellular application of anterogradely transported biocytin. Whereas a diffuse, broad commissural projection had developed in wild-type/P4 *reeler* co-cultures, wild-type/P0 *reeler* cultures with a re-established dense granular layer showed a normal, compact layer-specific termination of 'commissural' axons in the inner molecular layer (Fig. 8E,F). These results demonstrate that correcting granule cell migration is associated with the formation of a layer-specific commissural projection, suggesting that the target granule cells carry positional information for these axons. The findings indicate that the loss of laminar specificity of commissural/associational axons is secondary to the migration defect of the granule cells.

Discussion

Our results provide evidence for two distinct functions of reelin in the dentate gyrus. When added to the culture medium, recombinant reelin significantly increased the length of GFAP-positive processes in hippocampal sections from *reeler* mutants. By contrast, recombinant reelin in the medium did not rescue the formation of a granular layer. Our results with wild-type co-cultures suggest that reelin needs to be in normotopic position in order to govern the formation of a granule cell layer. These findings provide new insights into the mechanisms underlying layer formation in the dentate gyrus.

Methodological considerations

The late generation of the granule cells allowed us to use postnatal hippocampal slices to study layer formation in the dentate gyrus. Many granule cells are still forming when other types of hippocampal neuron have already completed their migration and are differentiating their processes and synaptic connections. Likewise, GFAP-positive cells in the dentate gyrus still show the characteristics of radial glia, contrasting with the GFAP staining of astrocytes in the hippocampus proper. Reelin, either added to the medium or provided by wild-type co-culture, may selectively act on the migration of granule cells in these postnatal cultures. In fact, we did not find an effect on early generated pyramidal neurons and mossy cells, and observed a rescue of granule cell lamination only in *reeler* slices from P0-P2, and not in slices from later stages. These specific experimental conditions do not allow us to generalize our results, and it remains open as to what extent the present findings can be extrapolated to other brain regions and to other developmental stages. Unlike the present results, incubation of slices from embryonic *reeler* neocortex in the presence of recombinant reelin partially rescued the *reeler* phenotype (Jossin et al., 2004), as did heterotopic reelin expression (Magdaleno et al., 2002).

Reelin acts on GFAP-positive radial glial fibers in the dentate gyrus

We recently demonstrated that Dab1, a cytoplasmic adapter protein of the reelin signaling cascade, is expressed by GFAP-positive radial glial cells in the dentate gyrus (Förster et al., 2002). Moreover, we were able to show that GFAP-positive radial glial cells responded to reelin in the stripe choice assay (Förster et al., 2002; Frotscher et al., 2003). In *reeler* mice, as well as in mutants lacking the reelin receptors apolipoprotein E receptor 2 (ApoER2) and/or very low density lipoprotein receptor (VLDLR), and in *scrambler* mice lacking Dab1, there were severe malformations of the radial glial scaffold in the dentate gyrus (Weiss et al., 2003). Although together these findings suggested an effect of reelin on GFAP-positive radial glial fibers in the dentate gyrus, which is mediated via lipoprotein receptors and Dab1, the nature of this effect remained unclear.

Our present results clearly show that reelin increases the length of GFAP-positive fibers in the dentate gyrus, an effect that was also observed in neocortical radial glial cells, but not radial glial cells from the basal ganglia (Hartfuss et al., 2003). In *reeler* mutants, GFAP-positive cells show morphological characteristics of astrocytes, suggesting a premature transformation of radial glial cells. Reelin added to the medium seems to prevent this premature astroglial differentiation. By

increasing the length of GFAP-positive fibers, reelin may maintain a radial glial scaffold in the postnatal dentate gyrus, thereby supporting the migration of postnatally generated granule cells. Lack of reelin in the *reeler* mutant would then result in a premature astrocytic differentiation and an altered granule cell migration, with many granule cells remaining near their site of generation in the hilus.

Reelin is required in a normotopic position to exert its function on granule cell migration

It is a major finding of the present study that reelin in the medium does not rescue layer formation in the *reeler* dentate gyrus. Granule cells in *reeler* cultures treated with recombinant reelin were scattered all over the dentate area. Our results also show that ubiquitous reelin, while increasing the length of glial processes, does not result in the formation of a regular radial glial scaffold. In wild type, radial glial fibers extend from the subgranular zone to the pial surface, thereby traversing the granule cell layer perpendicularly. Such a regular radial glial orientation was only achieved in the present experiments when a *reeler* slice was co-cultured with a wild-type slice with the wild-type marginal zone in close apposition. Moreover, only under these conditions did we observe the formation of a granule cell layer. Our findings suggest that reelin is required in a normotopic position for the directed growth of radial glial fibers, which form a regular scaffold suitable for granule cell migration and lamination (Fig. 9).

The observed effect of reelin on the radial glial scaffold does not allow us to exclude direct effects of reelin on neurons. In situ hybridization for *Dab1* mRNA revealed strong labeling of the granule cells in addition to radial glial cells in the dentate gyrus (Förster et al., 2002). The presence of molecules of the reelin pathway in both cell types is not too surprising in view of recent studies showing that radial glial cells are neuronal precursors (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001).

Previous studies suggested that reelin acts on neurons directly, by functioning as a stop signal (Curran and D'Arcangelo, 1998; Frotscher, 1998). Forming a component of the extracellular matrix in the marginal zone (the future layer I of the cortex), reelin seems to stop neurons in their migration, resulting in a cell-poor layer I in wild-type animals. Recent studies indicate that this effect may be brought about by the phosphorylation of Dab1 at Tyr220 and Tyr232, which appears to be important for the detachment of the neuron from the radial glial fiber (Sanada et al., 2004). In *reeler* mice and *Dab1* mutants, layer I is densely filled with neurons, and these recent findings suggest that the detachment from the radial glial fiber is severely altered in these animals. Like layer I of the neocortex, the molecular layer of the dentate gyrus, the marginal zone of this brain region, is a cell-poor layer with the granule cells accumulating underneath. Like in the marginal zone of the neocortex, reelin in the outer molecular layer of the dentate gyrus may directly act on granule cells by stopping their migration and initiating their detachment from the radial fiber by the phosphorylation of Dab1 (Sanada et al., 2004). In fact, in our rescue experiments with wild-type co-cultures, we regularly observed a zone that was almost free of granule cells in the *reeler* tissue directly attached to the wild-type marginal zone (e.g. Fig. 3D, Fig. 4), suggesting that reelin does function as a stop signal for the migrating granule cells in the *reeler*
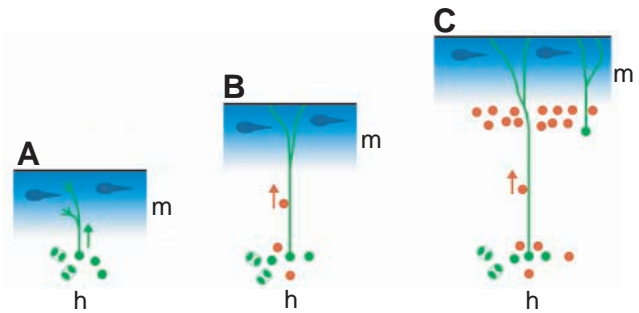

Fig. 9. Schematic diagram hypothesizing a dual function of reelin in the dentate gyrus. (A) Reelin (blue), synthesized by Cajal-Retzius cells (dark blue) in the marginal zone (m) provides a positional signal for radial glial fibers (green, arrow). The glial cell bodies are located in the secondary proliferation zone, the future hilus (h). (B) Glial cell processes have reached the pial surface, providing a scaffold for the migration of neurons (red). (C) Reelin provides a stop signal for migrating granule cells. Following migration, granule cells accumulate in a layer directly underneath the marginal zone. Alternatively, some radial glial cells may retract their long hilar process (soma translocation), thereby accumulating directly underneath the granular layer (right radial glial cell). Here they may divide and become neurons, inheriting the radial glial apical processes (Miyata et al., 2001).

tissue under these experimental conditions. Arrest of migration eventually leads to the accumulation of granule cells in a densely packed granular layer (Fig. 9). Alternatively, granule cell precursors, i.e. radial glial cells, may migrate by soma translocation and retract their basal (hilar) processes (Miyata et al., 2001). In this scenario, we would have to assume that reelin stops soma translocation (Fig. 9). Further studies are required to clarify precisely how granule cells migrate from their site of origin in the secondary proliferation zone of the hilar region to the granule cell layer.

Rescue of granule cell polarity and orientation

The uniform bipolar morphology and parallel alignment of granule cells in the wild-type dentate gyrus is largely lost in *reeler* mutants, and in mutants lacking VLDLR and ApoER2 (Stanfield and Cowan, 1979; Drakew et al., 2002; Gebhardt et al., 2002). We noticed a remarkable rescue of dendritic orientation in *reeler* sections co-cultured with wild type. Rescue of granule cell lamination and dendritic orientation were paralleled by a normalization of the laminated termination of commissural axons, indicating the presence of positional cues for these fibers on granule cell dendrites (Zhao et al., 2003). It remains to be analyzed in detail whether or not this re-direction of granule cell dendrites towards the pial surface is a direct effect of reelin on dendritic growth, similar to its effect on radial glia fiber orientation. Assuming that radial glial cells are precursors of neurons (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001), the radial fiber may be inherited by the neuron and become its apical dendrite. If this scenario holds true for dentate granule cells, then the normalized dendritic orientation would simply result from the rescue of radial fiber orientation. Alternatively, reelin may act on dendritic orientation and branching directly (Niu et al., 2004), similar to its effects on radial fibers and axon terminals (del Rio et al., 1997). With the different effects of reelin

described in the present study, and the recently discovered effects of reelin on chain migration (Hack et al., 2002) and synaptic plasticity (Weeber et al., 2002), we are beginning to unveil the diverse functions of this molecule.

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