

Lamina-specific cell adhesion on living slices of hippocampus

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SUMMARY

Laminar distribution of fiber systems is a characteristic feature of hippocampal organization. Ingrowing afferents, e.g. the fibers from the entorhinal cortex, terminate in specific layers, which implies the existence of laminar recognition cues. To identify cues that are involved in the laminar segregation of fiber systems in the hippocampus, we used an *in vitro* assay to study the adhesion of dissociated entorhinal cells on living hippocampal slices. Here we demonstrate that dissociated entorhinal cells adhere to living hippocampal slices with a lamina-specific distribution that reflects the innervation pattern of the entorhino-hippocampal projection. In contrast, laminae which are not invaded by entorhinal fibers are a poor substrate for cell adhesion. Lamina-specific cell adhesion does not require the neural cell adhesion molecule or the extracellular matrix glycoprotein *reelin*, as revealed in studies with mutants. However, the pattern of adhesive cues

in the *reeler* mouse hippocampus mimics characteristic alterations of the entorhinal projection in this mutant, suggesting a role of layer-specific adhesive cues in the pathfinding of entorhinal fibers. Lamina-specific cell adhesion is independent of divalent cations, is abolished after cryofixation or paraformaldehyde fixation and is recognized across species. By using a novel membrane adhesion assay, we show that lamina-specific cell adhesion can be mimicked by membrane-coated fluorescent microspheres. Recognition of the adhesive properties of different hippocampal laminae by growing axons, as either a growth permissive or a non-permissive substrate, may provide a developmental mechanism underlying the segregation of lamina-specific fiber projections.

Key words: Cell adhesion, Hippocampus, Entorhinal cortex, Slice cultures, Axon pathfinding, Reeler mouse

INTRODUCTION

Growing axons are guided to their correct targets by molecular cues in their environment (Tessier-Lavigne and Goodman, 1996). They require a substrate that is both adhesive and permissive for axonal growth (Letourneau, 1975). Contact-mediated growth promoting mechanisms as well as contact repulsion play a role in axon guidance and may involve non-diffusible cell surface molecules and extracellular matrix (ECM) molecules (Keynes and Cook, 1995; Kolodkin, 1996). Patterned neuronal outgrowth may result from juxtaposition of two contrasting substrates that differentially influence axonal growth (Keynes and Stern, 1984; Oakley and Tosney, 1993).

In the neocortex and hippocampus, ingrowing afferents terminate in specific layers which implies the existence of laminar recognition cues. Various experimental *in vitro* approaches have been used to study the development of a laminated fiber segregation in the neocortex and hippocampus (Bolz et al., 1990; Yamamoto et al., 1992; Frotscher and Heimrich, 1993; Emerling and Lander, 1994, 1996; Li et al., 1993, 1994, 1995, 1996; Del Rio et al., 1997). Recently we have shown that Cajal-Retzius (CR) cells in the hippocampus provide positional cues for the termination of the entorhinal

projection in distinct hippocampal layers (Del Rio et al., 1996, 1997) and identified the ECM glycoprotein *reelin*, expressed by CR cells, as a laminar cue for entorhinal fibers (Del Rio et al., 1997). Since *reelin* has been shown to be involved in both cell adhesion and axonal growth (Hoffarth et al., 1995; Ogawa et al., 1995; Del Rio et al., 1997; Frotscher, 1997), we used an *in vitro* adhesion assay to study adhesion of dissociated entorhinal cells on living slices of hippocampus. The possibility that cell adhesion could reveal the location of positional cues for growing axons has been previously suggested by investigators studying the developing retinotectal system (Barbera et al., 1973; Gottlieb et al., 1976) and the thalamocortical projection (Emerling and Lander, 1994, 1996). *In vitro*, alternating patterns of adhesive and non-adhesive substrates were found to influence both attachment and neurite outgrowth of mammalian central neurons (Letourneau, 1975; Kleinfeld et al., 1988; Corey et al., 1991; Matsuzawa et al., 1996). We concentrated on examining the adhesive properties of hippocampal slices at postnatal stages because both entorhinal fibers and commissural fibers innervate slices of postnatal hippocampus with correct laminar specificity *in vitro* (Frotscher and Heimrich, 1993, 1995; Frotscher et al., 1997; Li et al., 1993, 1994, 1995, 1996; Del Rio et al., 1997).

Here we show that dissociated cells from the entorhinal area specifically adhere to the same laminae in which the projecting axons of entorhinal neurons are found. This lamina-specific cell adhesion is independent of divalent cations and is mimicked by membrane preparations. In the *reeler*^{-/-} mutant mouse characteristic alterations of the entorhinal projection are associated with corresponding changes in adhesiveness. Neither reelin nor the neural cell adhesion molecule (NCAM) are required for this lamina-specific adhesion. Both cell adhesion and lamina-specific fiber outgrowth on hippocampal slices are abolished after fixation of the slices. We hypothesize that the lamina-specific adhesive cues play a role as positional information for the segregation of developing fiber systems in the hippocampus.

MATERIALS AND METHODS

Preparation of hippocampal slices

For the present experiments Sprague-Dawley rats, aged 4-21 days, and adult *NCAM*^{-/-}, *NCAM*^{+/-} mutant mice (Cremer et al., 1994) or Jackson *reeler*^{-/-} mutant mice were used. Genotyping of NCAM mutant mice was performed by PCR analysis of genomic DNA (Cremer et al., 1994). Adult *reeler*^{-/-} mice were identified by their phenotype, i.e., characteristic deficits in motor coordination and well-known morphological malformations in the cortex and hippocampus. Brains were removed from rat pups decapitated under hypothermic anaesthesia. Adult rats or mice were decapitated under deep pentobarbitone anaesthesia (50 mg Nembutal/kg bodyweight), and the brains were removed. All experiments were performed in agreement with the institutional guide for animal care. The hippocampi were dissected using fine spatulas. Hippocampi were sliced perpendicular to their longitudinal axis with a McIlwain tissue chopper. Section thickness was 200-400 µm. Slices were placed in a drop of 50-100 µl incubation medium (50% (v/v) Minimal Essential Medium (MEM), 25% (v/v) Hank's balanced salt solution (HBSS), and 25% (v/v) heat inactivated horse serum, 2 mM glutamine and 0.044% sodium bicarbonate (final concentration), adjusted to pH 7.3) or HBSS in a sterile plastic dish. For the adhesion assay in the absence of divalent cations, HBSS was replaced by Ca²⁺/Mg²⁺-free HBSS + 1 mM EDTA. Until seeding of labeled dissociated cells, slices were incubated at 37°C in a humidified 5% CO₂ atmosphere.

Dissociation and labeling of cells

For dissociation of embryonic cells, embryos were removed by cesarean section from time-pregnant Sprague Dawley rats (plug date = E0), anaesthetized by an overdose of pentobarbitone. The brain was removed under sterile conditions in incubation medium. For dissociation of cortical cells from postnatal stages, brains were removed from rat pups decapitated under hypothermic anaesthesia. Adult rats or mice were decapitated under deep pentobarbitone anaesthesia and brains were removed. The brain regions of interest (neocortex, entorhinal cortex, hippocampus) were dissected using fine spatulas. Until dissociation, the tissue was incubated in HBSS at 4°C. For the adhesion assay in the absence of divalent cations, HBSS was replaced by Ca²⁺/Mg²⁺-free HBSS + 1 mM EDTA.

Neural tissue was dissociated essentially as described by Emerling and Lander (1994). In short, neural tissue was incubated in phosphate-buffered saline (PBS) with 0.05% trypsin (Boehringer Mannheim) and 0.1 mg/ml DNase (Boehringer Mannheim) in a 15 ml polypropylene tube for 10 minutes at 37°C. The tissue was then carefully triturated with a flame-polished Pasteur pipette and incubated for another 5 minutes at 37°C. The solution was brought to a final volume of 10 ml with HBSS; undissociated tissue was allowed to settle for approximately 10 minutes and the supernatant was transferred to a

new tube. The cell suspension was centrifuged at 100 g for 10 minutes and the pellet gently resuspended in labeling medium. Labeling medium consisted of either a 2.5% Fluorogold solution (Fluorochrome, Inc.) diluted in serum-free incubation medium or of fluorescent cell tracker dye (Molecular probes, C-2925) which was diluted to a final concentration of 10 µM in serum-free incubation medium. Cells were resuspended in the staining solution and incubated for 15-30 minutes at 37°C. The staining solution was subsequently removed by three washes with fresh medium. Further incubation of cells labeled with cell tracker dye (37°C, 60 minutes) allowed the intracellular enzymatic cleavage of the fluorescent dye. Different fluorescent markers were used to avoid possible effects on adhesion due to the fluorescent marker. Cells were incubated at 37°C for 30-60 minutes and then centrifuged at 100 g for 10 minutes. The pellet was resuspended in MEM and cells were checked for viability using Trypan Blue. Usually more than 90% of cells were alive after staining with Trypan Blue (developmental stages P4 and younger). The cell suspension was diluted with MEM to approximately 3×10⁶ cells/ml. Cell concentrations and volumes in the assay were varied to detect possible effects due to differing concentrations or volumes. For some experiments, tissue was dissociated by mechanical force. Brain tissue was cut into small pieces with a razor blade and then carefully triturated with a flame-polished Pasteur pipette. Primary cultures of cerebellar granule cells were prepared as described (Kaltschmidt et al., 1995), 3T3 cells were harvested by trypsinization; dissociated cells were labeled with a fluorescent marker and further treated as described above.

Preparation of membranes and coating of microspheres

Membranes were prepared as described by Cox et al. (1990). Cortices from P0 rats were removed under sterile conditions and transferred to homogenization buffer containing PBS (pH 7.4), urea (4 M), and spermidine (10 mM). Immediately before homogenization, a cocktail of protease inhibitors was added. Cortices were homogenized with 20 strokes of a dounce homogenizer. For control experiments, neural tissue was first dissociated by trypsinization and trituration as described above and then again incubated in PBS with 0.5% trypsin, 0.1 mg/ml DNase and 1 mM EDTA for 30 minutes at 37°C. Cell nuclei and unlabeled cells were pelleted in a swing-out rotor (16000 g, 30 minutes at 4°C). The supernatant was centrifuged again in a fixed-angle rotor (125000 g, 60 minutes at 4°C), and cell membranes were pelleted. The supernatant was discarded and the pellet was washed three times in PBS by resuspension and centrifugation with a fixed-angle rotor (57000 g, 10 minutes) at 4°C in order to completely remove urea. Membranes were resuspended in PBS. Protein concentration was adjusted to 2 mg/ml. Membrane fractions were coated onto fluorescent microspheres (Boehringer Mannheim, 0.1 µm diameter; Molecular Probes, 4 µm diameter) prior to use, according to the manufacturer's instructions. As controls, BSA or the membranes prepared from thoroughly trypsinized cortical cells were coated onto the microspheres. Labeled membrane-coated microspheres were resuspended in Ca²⁺/Mg²⁺-free HBSS + 1 mM EDTA.

Washing of slices after seeding of dissociated cells or membrane-coated microspheres

Slices with adherent cells or microspheres were carefully transferred to a large volume of incubation medium or, for studies in the absence of divalent cations, to Ca²⁺/Mg²⁺-free HBSS + 1 mM EDTA. The slices were then washed by carefully pipetting them up and down with a 1 ml Eppendorf pipette (pipette tips were cut to a large diameter with a razor blade).

Analysis of adherent cells and membrane-coated microspheres

Freshly cut slices were mounted on microscope slides and were analyzed under a Zeiss fluorescence microscope using a 10×, 20× or

40× objective lens. Slices that were cultured according the method of Stoppini et al. (1991) were left on the millipore membranes and analyzed under the microscope. Hippocampal laminae such as the dentate molecular layer, the granule cell layer or the pyramidal cell layer, were discernable under the microscope. As controls, some slices were counterstained with the fluorescent dye DAPI (Boehringer Mannheim) to confirm the laminar boundaries under UV fluorescence.

Fluorogold-labeled cells were visualized under UV fluorescence. Cell tracker labeled cells were visualized under rhodamine fluorescence, and fluorescent microspheres under rhodamine or UV fluorescence. For statistical analysis, cells or membrane-coated microspheres (Molecular Probes) on different hippocampal laminae were counted in a defined window of the slice that included all hippocampal layers.

As internal control, yellow fluorescent microspheres (Molecular Probes, F8858) were coated either with BSA or membranes from thoroughly trypsinized cortical cells. Red fluorescent microspheres (Molecular Probes, F8859) were coated with untreated membrane preparations. The red and yellow microspheres with different coating were then seeded on DAPI-stained slices in equimolar ratios. After washing of the slices, adherent microspheres with different coating could be distinguished by their different fluorescent color.

Fast Blue labeling of hippocampal commissural neurons

15 Sprague-Dawley rat pups, aged 1-3 days were used in this study. All surgical procedures were performed under ether anaesthesia. For retrograde labeling of commissural neurons 0.1 µl Fast Blue (FB, 3%, Illing, Germany) in 10 mM phosphate buffer (PB, pH 7.8) was injected into the contralateral hippocampus using a 0.5 µl syringe. After 2-5 days, animals were killed by decapitation and the brains were removed. The CA3 region containing the retrogradely labeled commissural neurons was dissected from hippocampal slices and dissociated.

Phaseolus vulgaris-leucoagglutinin tracing and Dil tracing of entorhinal fibers

Six adult (male and female) reeler mutant mice (Jackson Laboratories) and six adult (male and female) control mice housed under standard laboratory conditions were used for *Phaseolus vulgaris*-leucoagglutinin (PHAL) tracing in this study. Homozygous control mice (*reelin*^{+/+}) were obtained from heterozygous (*reelin*^{+/-}) breeding pairs (Jackson Laboratories, USA) using standard breeding and cross-breeding techniques. All surgical procedures were performed under deep anesthesia: midazolam (0.1 mg/kg body weight), fentanyl (3.15 mg/kg b.w.), and fluanison (1 g/kg b.w.).

For anterograde tracing of entorhinal fibers, PHAL (2.5%; Vector Laboratories) in 10 mM PB, pH 7.8) was iontophoretically delivered (Gerfen and Sawchenko, 1984) using a glass micropipette (tip diameter 15-30 µm, 5 µA positive current, on-period and off-period 5 seconds for 20 minutes). The animals received two or three tracer deposits; stereotaxic coordinates from bregma according to Franklin and Paxinos (1997; L -3,5; V -4; AP -4,7; L -3,0; V -4; AP -4,7; L -3; V -4,7; AP -4,5). The animals were allowed to survive for 10 days following the injection of the anterograde tracer. After this time period the mice were deeply anesthetized with nembutal and transcardially perfused with a fixative containing 4% paraformaldehyde, 0.1% glutaraldehyde, and 15% picric acid in 0.1 M PB (pH 7.4). After rinsing in PB, brains were embedded in 5% agar and 50 µm thick frontal sections were cut on a vibratome and immersed in 0.1% H₂O₂ in 0.1 M PB for 15 minutes to inactivate endogenous peroxidase. For PHAL immunocytochemistry, free floating sections were incubated for 2 days at 4°C in biotinylated goat anti-PHAL (1:800), 1% normal horse serum, 0.5% Triton X-100, 0.1% NaN₃ in 0.1 M PB. After rinsing the sections in PB, they were incubated in the avidin-biotin-peroxidase complex (1:50; ABC-Elite, Vector Labs) for 3 hours. Following subsequent washes, the sections were reacted for 5-10 minutes in a nickel/DAB solution (0.05%

3,3'-diaminobenzidine, 0.02% nickel-ammonium chloride, 0.024% cobalt chloride, 0.001% H₂O₂) which resulted in a deep-blue labeling of PHAL containing fibers. Sections were then placed on gelatin-coated slides, dehydrated in ethanol, and mounted in Eukitt.

For Dil tracing, brains from transcardially perfused animals were removed and small Dil crystals were placed on the entorhinal cortex with a thin metal needle, under inspection through a dissection microscope. After injection, brains were stored in 4% paraformaldehyde, 0.1 M PB at room temperature for up to 3 weeks. Brains were then coronally sectioned at 80 µm with a vibratome. Sections were mounted on slides, coverslipped and analyzed under the fluorescence microscope as described by Supèr and Soriano (1994).

Cryocultures and biocytin tracing of fibers

Freshly cut hippocampal slices were immersion-fixed with 1.5% paraformaldehyde (PFA) for 30 minutes at 4°C; animals were perfusion-fixed with 1.5% PFA. Perfused brains were removed and then frozen and cut on a cryotome. These cryosections were placed on millipore membranes in PBS. After a short incubation time, PBS was replaced by culture medium. Immersion-fixed slices from freshly cut hippocampus were washed several times with PBS and then transferred to culture medium. Next, freshly prepared slice cultures of hippocampus and entorhinal cortex were placed either on the cryosections from perfusion-fixed brains or on the immersion-fixed slices and cultivated for various periods. For the tracing of outgrowing fibers, small crystals of biocytin (Sigma) were placed on the slices using a glass micropipette. Next, cultures were incubated for another 48 hours at 37°C. Slices were then rinsed with HBSS and fixed by using a mixture of 4% paraformaldehyde, 0.08% glutaraldehyde and 15% picric acid in 0.1 M PB (pH 7.3). After 2 hours of fixation, the cultures were rinsed in PB. Biocytin was visualized using an ABC procedure (Hsu et al., 1981) and a diaminobenzidine (DAB)-nickel/cobalt intensification (Adams, 1981).

RESULTS

Lamina-specific adhesion of entorhinal cells on living hippocampal slices

To examine the adhesion of dissociated entorhinal cells on living slices of hippocampus, tissue of the entorhinal area from P4 rat pups was dissociated, labeled with Fluorogold or fluorescent cell tracker dye and allowed to settle onto freshly cut slices from rat hippocampus (see Methods). After 2 hours of incubation at 37°C, the cells were first visualized by fluorescence microscopy and showed a uniform distribution over the slice surface. Then, the slices were gently rinsed to remove unattached cells. Adhesion of fluorescent cells was now restricted to defined laminae of the hippocampus (Fig. 1A-D). Fluorescent cells adhered to the stratum oriens, the stratum lacunosum moleculare, the outer molecular layer of the fascia dentata and to the hilar region. In contrast, the pyramidal cell layer, the granule cell layer, the stratum radiatum and the innermost portion of the dentate molecular layer were devoid of adherent cells (qualitatively similar results: *n*=50). Thus, the distribution of the adherent cells corresponded to the distribution of fibers projecting from the entorhinal cortex to the hippocampus in vivo (Blackstad, 1958; Supèr and Soriano, 1994; Deller et al., 1996; Fig. 1B) and in vitro (Frotscher and Heimrich, 1993; Li et al., 1993).

We also analyzed cell adhesion on the slices after incubation times between 30 minutes and 6 hours. The number of adherent cells on the outer molecular layers, stratum lacunosum-

moleculare and stratum oriens, however, did not change with incubation time but rather reflected the cell density initially seeded on the slice. The stratum pyramidale, granule cell layer and inner molecular layer remained poor substrates for dissociated cells after longer adhesion times. On the hilar region where cells adhered even after short incubation times, the number of adherent cells increased with incubation time. However, the principal division of the hippocampal slice surface in areas which were either permissive or non-permissive for cell adhesion remained unchanged independent of the incubation time ($n > 50$).

Some hippocampal slices were cultured for up to 5 days *in vitro* prior to the seeding of dissociated cells. Also on these slices laminar specificity of cell adhesion was apparent. However, the lamination was less distinct, most likely because the slice cultures flatten after several days *in vitro* resulting in a less pronounced lamination of the structures (qualitatively similar results: $n = 8$, data not shown). Thus, lamina-specific cell adhesion appeared to be independent of the incubation time of hippocampal slices prior to seeding of cells. This finding is important with respect to the lamina specificity of ingrowing entorhinal or hippocampal afferents, which is maintained in slice cultures (Frotscher and Heimrich, 1993, 1995; Frotscher et al., 1997, Li et al., 1993, 1994, 1995, 1996; Del Rio et al., 1997).

Adhesion of other cell types on living hippocampal slices

To find out whether lamina-specific cell adhesion is a cell type independent phenomenon, we labeled 3T3 cells and cerebellar granule cells with fluorescent cell tracker dye and performed the adhesion assay with these cells. Both 3T3 cells and cerebellar granule cells demonstrated a different adhesive behavior to dissociated entorhinal cells. 3T3 cells also adhered to the pyramidal cell layer and the granule cell layer (Fig. 1E). Also, when in contact with the pial surface, 3T3 cells extended long processes running in parallel with the pial surface (Fig. 1F) within less than 60 minutes after seeding. This behaviour was exclusively observed for 3T3 cells. In contrast, 3T3 cells which did not have contact to the pia maintained their spherical shape. Cerebellar granule cells adhered to all hippocampal layers at a similar density except for the pyramidal cell layer, where the cells adhered at a lower density (Fig. 2A,B).

We also wanted to know whether lamina-specific adhesion in our assay is a unique property of dissociated cells derived from the entorhinal area. Therefore, we dissociated cells from entire rat neocortex (E18 to P4), labeled the cells with fluorescent cell tracker dye and performed the adhesion assay as described above. We found that also these cells, taken from different developmental stages, adhered with a similar

distribution as cells from the entorhinal area (qualitatively similar results: $n > 50$).

For quantification, the densities of adherent cortical cells (Fig. 3A) and of cerebellar granule cells (Fig. 3B) on different laminae were determined for 10 slices after 2 hours. The highest density of adherent cortical cells was counted on the stratum lacunosum moleculare (slm) and the outer molecular layer (oml) with 1400–2400 cells/mm², followed by a density of up to 1000 adherent cells/mm² on the stratum oriens. On the hilar region up to 400 adherent cortical cells/mm² were

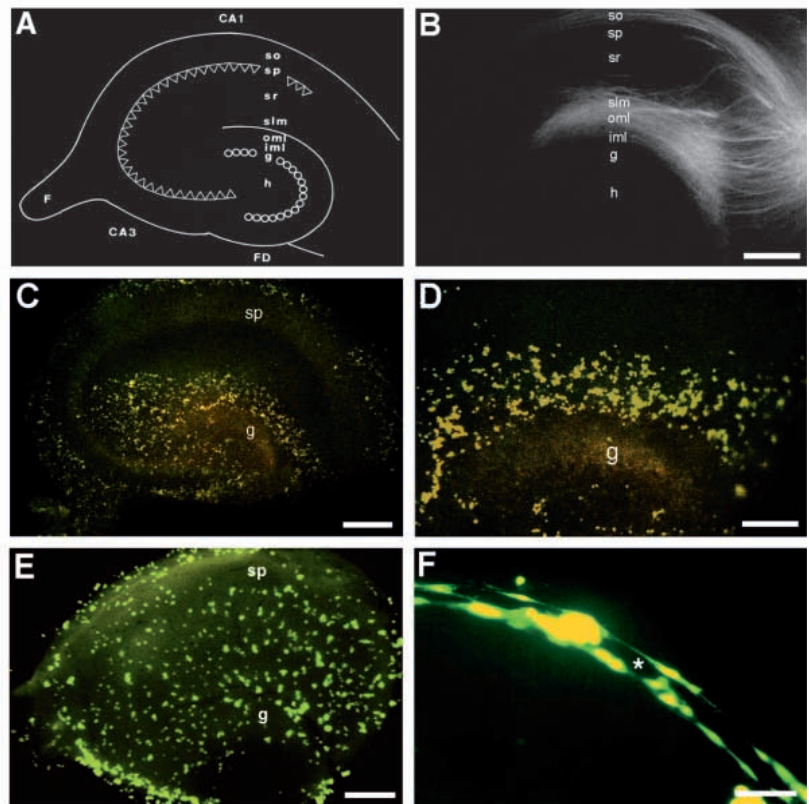


Fig. 1. Schematic illustration of the laminar hippocampal organization (A), lamina-specific projection of entorhinal axons (B), and lamina-specific adhesion of dissociated cells on living slices of rat hippocampus (C–F). (A) Schematic illustration of hippocampal lamination. so, stratum oriens; sp, pyramidal cell layer; sr, stratum radiatum; slm, stratum lacunosum-moleculare; ml, dentate molecular layer (oml, outer ml; iml, inner ml); g, granular cell layer; h, hilus; F, fimbria. (B) Lamina specificity of entorhinal fibers in the hippocampus (P1) as visualized by tracing with the fluorescent dye DiI. Entorhinal fibers are found in the stratum lacunosum moleculare, and in the outer molecular layer of the fascia dentata. Bar, 200 μ m. (C) Entorhinal cells (P4) were dissociated, labeled with the fluorescent dye Fluorogold, and seeded on hippocampal slices (P4). After 2 hours of incubation, non-adherent cells were removed. An example of a hippocampal slice with adherent dissociated entorhinal cells is shown. Cells adhere only to the stratum oriens, the stratum lacunosum moleculare, the outer molecular layer and the hilus region. No cells adhere to the pyramidal cell layer, the granule cell layer or the stratum radiatum (see A for orientation about layers). Bar, 200 μ m. (D) Adhesion of dissociated entorhinal cells to the stratum lacunosum-moleculare and outer molecular layer of the fascia dentata at higher magnification. No cells adhere to the granule cell layer or the inner molecular layer. Bar, 100 μ m. (E) Adhesion of dissociated 3T3 cells to a hippocampal slice after 2 hours incubation time. 3T3 cells did not show lamina-specific adhesion. Note that 3T3 cells also adhered to each other and formed small clusters Bar, 200 μ m. (F) 3T3 cells extend processes (above asterisk) along the pial surface when in contact with the pia. Bar, 25 μ m.

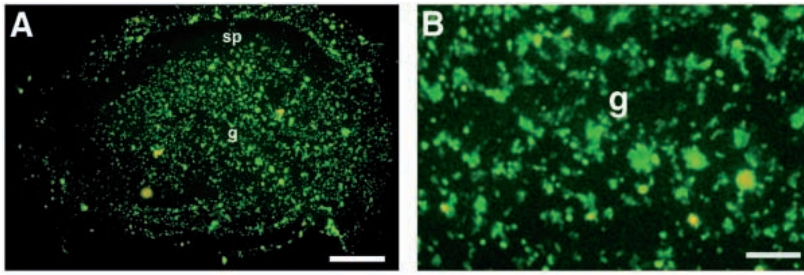


Fig. 2. (A) Adhesion of dissociated cerebellar granule cells (P6) to a hippocampal slice (P4) after 2 hours incubation time. Cerebellar granule cells adhered to all layers, however with significantly lower affinity to the pyramidal layer (sp). Bar, 200 μm . (B) Higher magnification of 2 A. Note that dissociated granule cells also adhere to the granule cell layer and iml, in contrast to dissociated entorhinal cells (see Fig. 1D). Bar, 100 μm .

counted. The lowest densities of adherent cortical cells were observed on the stratum radiatum (0-200 cells/ mm^2), the granule cell layer (g)/inner molecular layer (iml; 0-100 cells/ mm^2), and the pyramidal cell layer (0-100 cells/ mm^2). Note that for quantification slm/oml and iml/g were grouped together, since the densities of adherent cells were very similar on these laminae.

In contrast, dissociated cerebellar granule cells adhered at high density to all hippocampal laminae (1700-3400 cells/ mm^2) except for the pyramidal cell layer, where the number of adherent cells was significantly lower (300-1400 cells/ mm^2).

Finally, we tested adhesion of dissociated hippocampal commissural neurons. These neurons were first prelabeled by injection of the retrograde tracer Fast Blue (FB) into the contralateral hippocampus of rat pups (P1-P3) 2-5 days prior to the experiment. The CA3 pyramidal cells including the prelabeled cells were then dissected from hippocampal slices and dissociated. Unlike the entorhinal fibers, commissural fibers are known to project in the iml. In these experiments, however, we could not detect cell adhesion to the iml. Adherent labeled cells were detected on the stratum oriens, stratum radiatum, stratum lacunosum moleculare, outer molecular layer and the hilar region. These results, taken together with the results of the experiments with dissociated neocortical cells, indicate the presence of repellent cues in the iml.

Lamina-specific cell adhesion to hippocampal slices in the absence of divalent cations

In order to distinguish between Ca^{2+} -dependent and Ca^{2+} -independent adhesion molecules, which both might be involved in mediating lamina-specific adhesion (Hynes and Lander, 1992), the assay was performed in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS+EDTA (see Methods). Both, hippocampal slices and dissociated cortical cells (E18) were first separately incubated in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS+EDTA before the dissociated cells were seeded on the slices. After 2 hours incubation the slices were gently rinsed to remove unattached cells. We found that lamina-specific cell adhesion is also maintained under divalent cation-free conditions (qualitatively similar results: $n > 50$, not shown). These results indicate that a Ca^{2+} -independent cell adhesion mechanism is sufficient to mediate the lamina-specific cell adhesion.

Lamina-specific adhesion of membrane preparations to hippocampal slices

We wanted to test whether adhesion in our assay requires living cells. Therefore, we prepared membranes from whole rat cortices (P4) and coated these membranes onto fluorescent microspheres in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS+EDTA (see Methods).

The membrane-coated microspheres were allowed to settle on hippocampal slices in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS+EDTA for 30 minutes to 6 hours. Slices were then gently rinsed and the distribution of fluorescent microspheres was monitored under the fluorescence microscope. The distribution of adherent membrane-coated microspheres was similar to that of dissociated cells (Fig. 4A, qualitatively similar results: $n > 50$).

Positional cues for laminated fiber termination should be present in the adult hippocampus, since in heterochronic cocultures with entorhinal slices from young postnatal rats and hippocampal slices from P15, entorhinal fibers correctly project to the dentate outer molecular layer (Li et al., 1995). Furthermore, embryonic transplants from entorhinal cortex were shown to innervate the adult hippocampus with correct laminar specificity (Zhou et al., 1989), and sprouting processes in the hippocampus after lesion of the entorhinal cortex are lamina-specific (Frotscher et al., 1997). Therefore, we also studied adhesion of membrane-coated microspheres on adult hippocampal slices. Also in these experiments, the characteristic, laminar distribution of adherent membrane-coated microspheres appeared with striking precision (Fig. 4B, qualitatively similar results $n = 20$).

As a control, we coated fluorescent microspheres with BSA and incubated the slices for several hours. After washing, almost all BSA-coated microspheres were removed from the hippocampal slice (Fig. 4C).

For quantification of membrane adhesion, we used microspheres with a larger diameter (4 μm) for coating with membrane preparations (see Methods). These microspheres could be counted under the fluorescence microscope (Fig. 4D). The quantification of adherent microspheres reflected the pattern of adherent and repellent laminae (Fig. 5), very similar to the dissociated adherent cells on the slices. Note that on some slices the number of adherent microspheres/ mm^2 was higher than the number of adherent cells/ mm^2 because the concentration of microspheres was higher. As an internal control, we also coated differently colored microspheres with BSA or with membranes from cortical cells that had been thoroughly protease treated before preparation of membranes (see Methods). Adhesion of BSA-coated microspheres (Fig. 4D) and of microspheres coated with pretreated membranes (not shown) was markedly reduced when compared to the different coloured microspheres coated with untreated membranes (Fig. 4D). The few adherent BSA-coated microspheres (Fig. 4D) were again found in the adhesive laminae. The observation that the control microspheres also showed laminar specificity may indicate that repellent cues also play a role in the formation of the adhesion pattern on hippocampal slices.

Lamina-specific adhesion of dissociated cortical cells from NCAM^{-/-} mutant mice on hippocampal slices

Cell adhesion that is mediated by cell adhesion molecules (CAMs) of the immunoglobulin (Ig) superfamily including NCAM, is independent of divalent cations (Hynes and Lander, 1992). NCAM is widely expressed in the developing central nervous system (Edelman, 1986) including the hippocampus (Miller et al., 1993). IgCAMs including NCAM have been shown to be involved in neurite outgrowth and synaptic plasticity (Doherty et al., 1994; Bixby, 1994). Thus, NCAM is a candidate adhesion molecule that could be involved in the lamina-specific cell adhesion on hippocampal slices.

To test the possible involvement of NCAM in lamina-specific cell adhesion, dissociated cortical cells from NCAM^{-/-} or NCAM^{+/-} mutant mice (Cremer et al., 1994) were plated on rat hippocampal slices. Interestingly, also these cells, which do not express NCAM, adhered with the described characteristic laminar distribution on hippocampal slices from wild-type mouse (Fig. 6A,B; qualitatively similar results $n=10$). Therefore, a homophilic cell adhesion mechanism mediated by NCAM does not seem to be required for lamina-specific cell adhesion in the hippocampus.

Lamina-specific adhesion of dissociated cells and membrane preparations to hippocampal slices of *reeler* mice

The extracellular matrix glycoprotein reelin plays a role in the branching pattern of entorhinal fibers to the hippocampus (Del Rio et al., 1997). To examine whether the lamina-specific adhesion is dependent on reelin or is affected in the *reeler* mouse hippocampus, dissociated cells from rat neocortex or membrane-coated microspheres were plated on slices from adult *reeler* mouse hippocampus (Fig. 7A,C,E). The lamina-specific distribution of adhesive cues was also apparent on these slices (qualitatively similar results: $n=20$).

It is of special interest in the context of this study that the entorhinal fiber termination zone is displaced in the temporal hippocampus of *reeler* mice (Bliss and Chung, 1974; Stirling and Bliss, 1978; Stanfield et al., 1979). Indeed, we detected corresponding differences in cell adhesion between slices from the septal (Fig. 7A) and temporal (Fig. 7C,E) hippocampus of

reeler mice. No such differences were found between septal and temporal sections from wild-type animals. Tracing of the entorhinal projection in *reeler* mice with PHAL revealed the normal distribution of entorhinal fibers to the fascia dentata in septal sections only (Fig. 7B), whereas in temporal sections entorhinal fibers invaded the hilar region (Fig. 7D). This particular pattern was similar to the adhesion of membrane-coated microspheres, which adhered to the hilar region in temporal sections of the hippocampus (Fig. 7C). Similarly, in the CA1 region of *reeler* mice there is an abnormal entorhinal

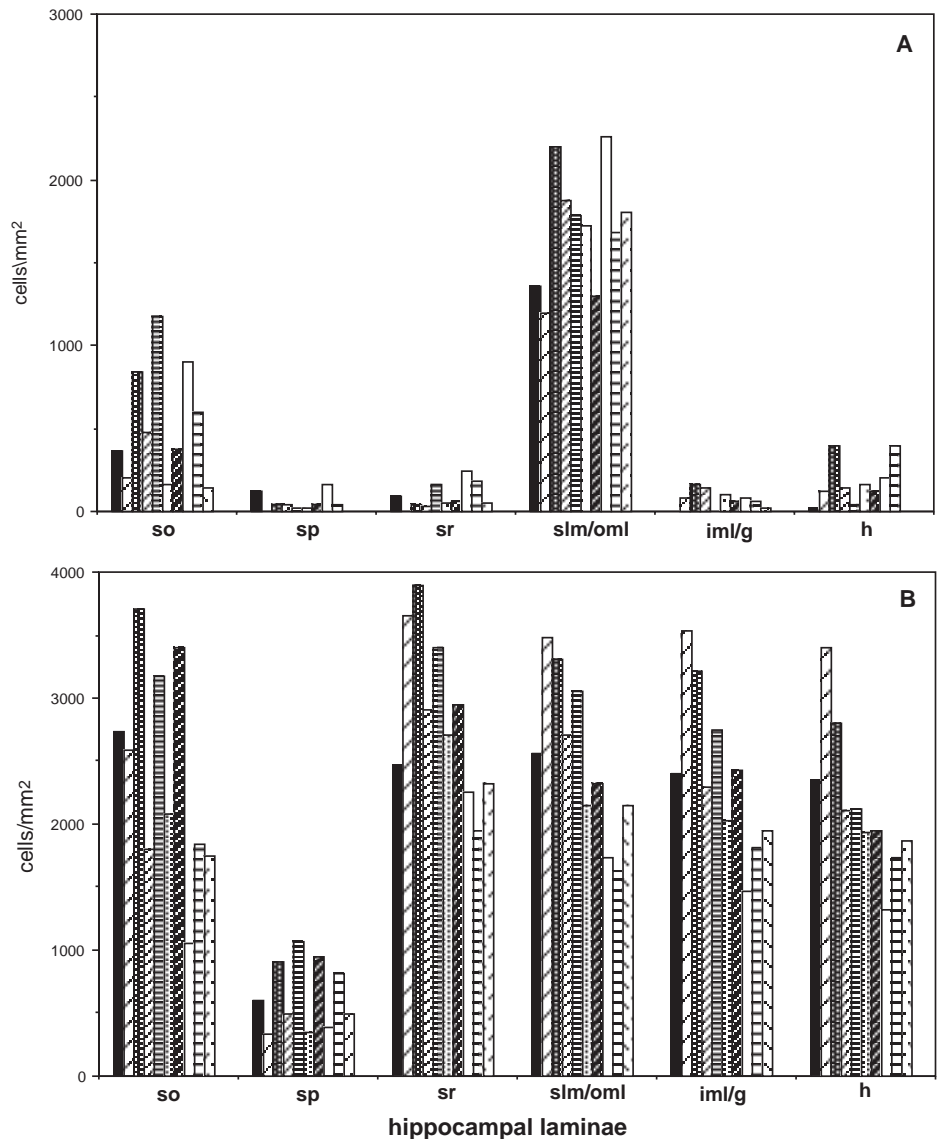


Fig. 3. Densities of adherent cells on different hippocampal laminae. (A) Densities of adherent cortical cells on different hippocampal laminae after 2 hours of incubation. Densities on the different hippocampal laminae, stratum oriens (so), stratum pyramidale (sp), stratum radiatum (sr), stratum lacunosum-moleculare + dentate outer molecular layer (slm/oml), inner molecular layer + granule cell layer (iml/g), hilus (h), were determined for 10 hippocampal slices (individual columns). Cell densities were significantly different between adjacent layers (Wilcoxon Rank Sum Test, two-tailed: $P<0.05$). (B) Densities of adherent cerebellar granule cells after 2 hours of incubation. Densities on the different hippocampal laminae were determined for 10 hippocampal slices as described in 3A. Only on the pyramidal cell layer (sp) the cell density differed significantly from all other layers (Wilcoxon Rank Sum Test, two-tailed: $P<0.05$).

projection to a layer sandwiched between the two pyramidal layers in this mutant (Fig. 7F). In the adhesion assay, membrane-coated microspheres were found to adhere to this zone in the mutant (Fig. 7E). These observations suggest a link between the adhesive cues and the layer-specific projection of entorhinal fibers.

Lamina specificity of cell adhesion and fiber outgrowth is abolished on cryoslices of hippocampus

It has been reported by several authors that region-specific adhesion or neurite outgrowth can be maintained on cryosections of brain, nerve or muscle (Carbonetto et al., 1987; Sandrock and Matthew, 1987; Covault et al., 1987; Watanabe and Murakami, 1989; Savio and Schwab, 1989; Crutcher, 1989; Geisert, 1991; Tuttle and Matthew, 1991; Stier and Schlosshauer, 1995). Other investigators have found that frozen sections could not substitute for living slices in revealing lamina-specific attachment (Emerling and Lander, 1994). To study cell adhesion on fixed and frozen hippocampal slices, we analyzed cell adhesion on paraformaldehyde (PFA)-fixed ($n=20$) or unfixed ($n=20$) cryoslices and on freshly cut immersion-fixed slices ($n=30$) of hippocampus (see Methods). Dissociated cells were seeded on these slices and incubated in vitro for up to 6 hours. After gentle washing, freshly cut immersion-fixed slices or perfusion-fixed cryoslices were not found to support cell adhesion. On cryoslices from unfixed tissue, few dissociated cells adhered, however, without lamina specificity (data not shown).

To analyze fiber growth on cryoslices, living slices of hippocampus including the entorhinal area or of hippocampus alone (P4) were confronted with fixed or unfixed cryoslices of hippocampus (P4 or adult, $n=40$) and incubated together for up to 14 days in vitro (Fig. 8A). As visualized by tracing with biocytin, a large number of neurites had grown out from neurons in the living slices (Fig. 8B). However, no lamina-specific orientation or preferential direction of neurite growth was observed on the cryoslices (Fig. 8C,D). This undirected fiber growth is very much in contrast to the observations made in living slice co-cultures of hippocampus and entorhinal cortex or hippocampus and hippocampus where projecting entorhinal and commissural fibers, respectively, precisely innervate their correct target layers (Li et al., 1993, 1994, 1995, 1996; Frotscher and Heimrich, 1993, 1995; Frotscher et al., 1997; Del Rio et al., 1997). The loss of lamina-specific neurite outgrowth correlates with the loss of lamina-specific cell adhesion on cryoslices. These results support the interpretation that the adhesive cues may provide positional information for the segregation of different fiber systems in the hippocampus.

DISCUSSION

Our results demonstrate that dissociated cells from the entorhinal area adhere to living slices of hippocampus with a lamina distribution that

mimics the innervation pattern of entorhinal projection neurons. However, the lamina-specific cues in the termination zone of entorhinal fibers are also recognized by cells from other cortical regions. Lamina-specific cell adhesion is independent of divalent cations, does not require reelin or NCAM and can be mimicked by membrane-coated microspheres. The distribution of the adhesive cues in the hippocampus of the *reeler* mutant mouse reflects characteristic alterations of the entorhinal projection in this mutant. Lamina-specific cell adhesion is abolished by fixation of the slices.

Development of the entorhinal projection and the pattern of adhesive cues in the hippocampus

The laminated segregation of entorhinal and hippocampal afferents in the dentate molecular layer also forms when slices of hippocampus and entorhinal cortex are cocultured (Frotscher and Heimrich, 1993; Frotscher and Heimrich, 1995; Li et al., 1993, 1994, 1995, 1996; Del Rio et al., 1997). The fact that the lamina distribution of entorhinal and hippocampal afferents is undisturbed even by the reversal of the relative times of arrival of different afferents strongly suggests the involvement of intrinsic recognition signals (Frotscher and

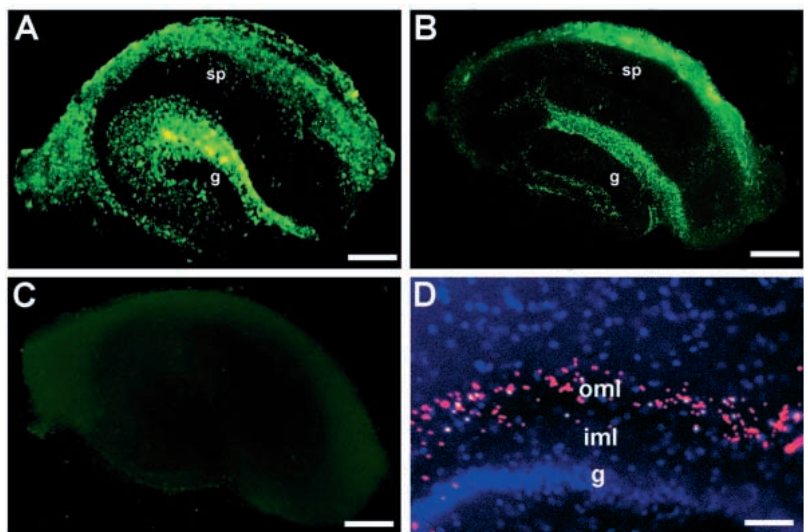


Fig. 4. Lamina-specific adhesion of membrane preparations from cortical cells to slices of hippocampus. Fluorescent microspheres were coated with membrane preparations from cortical cells (P4) and plated on hippocampal slices in the absence of divalent cations. After 2 hours incubation, non-adherent particles were removed. (A) Adhesion of membrane-coated microspheres on a hippocampal slice (P4). Membrane-coated microspheres adhere exclusively to the stratum oriens, stratum lacunosum-moleculare, the outer molecular layer, and the hilar region, but not to the stratum pyramidale, stratum radiatum, the granule cell layer and inner molecular layer. See Fig. 1A for orientation. Bar, 200 μm . (B) Lamina-specific adhesion of fluorescent microspheres coated with cortical membranes to a hippocampal slice from an adult rat. Bar, 500 μm . (C) As a control, fluorescent microspheres were coated with bovine serum albumine (BSA). After 2 hours of incubation, under the same conditions as membrane-coated microspheres, BSA-coated particles did not adhere to the slice. Bar, 200 μm . (D) Lamina-specific adhesion of fluorescent microspheres (4 μm diameter) to the outer molecular layer of a DAPI-stained hippocampal slice. Red microspheres were coated with cortical membranes, yellow microspheres with BSA. Initially, an equimolar ratio of red and yellow microspheres was seeded on the slice. After washing of the slice, almost all of the BSA-coated microspheres are removed, whereas membrane-coated microspheres still adhere to the outer molecular layer. Bar, 25 μm .

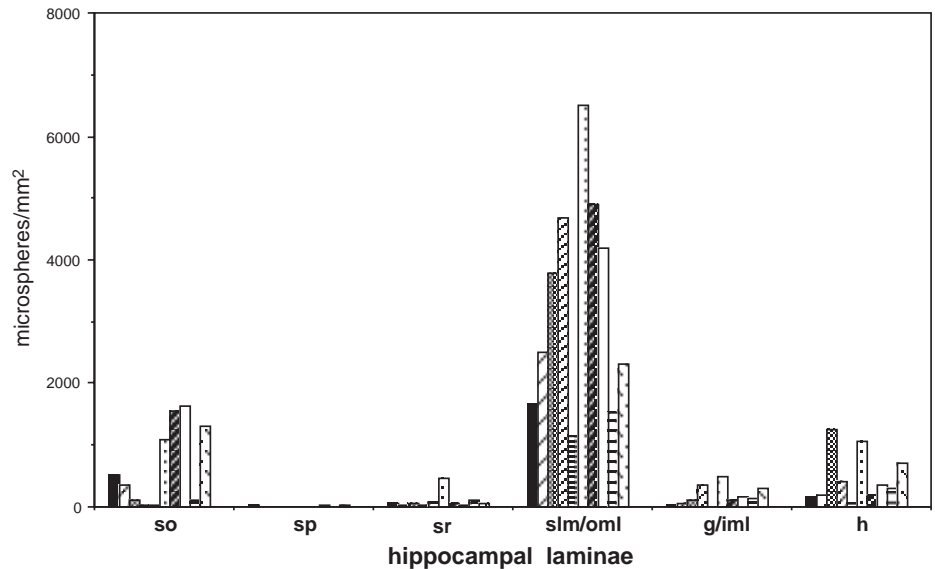


Fig. 5. Densities of adherent microspheres on different hippocampal laminae. As with adherent cortical cells, densities of adherent microspheres on the different hippocampal laminae were determined for 10 hippocampal slices (individual columns). Densities of adherent microspheres differ significantly between adjacent laminae (Wilcoxon Rank Sum Test, two tailed: $P < 0.05$).

Heimrich, 1993). Further evidence for the existence of such signals is the finding that entorhinal afferents even establish their correct lamina-specific termination patterns when subdissected slice-fragments are offered as a target (Li et al., 1996). These observations imply the existence of laminar recognition cues *in vitro*.

Malpositioning of entorhinal fibers in the *reeler* mutant corresponds to changes in adhesiveness

Recently we showed that CR cells and the ECM glycoprotein reelin, expressed in a lamina-specific manner by CR cells in the hippocampus, acts as a recognition signal for entorhinal axons (Del Rio et al., 1997). Reelin, however, cannot be the only positional cue for entorhinal fibers in the hippocampus, since in the hippocampus of *reeler* mice, a natural *reelin*^{-/-} mutant, growing entorhinal axons still innervate the hippocampus (Bliss and Chung, 1974; Stirling and Bliss, 1978; Stanfield et al., 1979; Del Rio et al., 1997). The striking correlation between the pattern of adhesive cues and the termination of entorhinal fibers in the hippocampus is even maintained in areas with an abnormal innervation pattern of entorhinal fibers in the temporal hippocampus of *reeler* mice (hilus of the fascia dentata, pyramidal cell layer of CA1). These observations suggest that distinction of different laminae by their different adhesive properties may provide a developmental mechanism that underlies the segregation of lamina-specific projections to the hippocampus.

Do adherent cells recognize positional cues for axonal growth in the hippocampus?

The idea that the distribution of positional cues might allow growing axons to recognize and adhere to their proper termination fields was first proposed by Sperry (1963) for the retinotectal system. In the retinotectal system (Barbera et al., 1973; Gottlieb et al., 1976) and also the thalamocortical projection (Emerling and Lander, 1994, 1996), selective cell adhesion to the target

region has been demonstrated. In the retinotectal system, candidate chemoaffinity tags were identified as ligands of the Eph family of receptor kinases and act as repulsive axonal guidance signals (Cheng et al., 1995; Drescher et al., 1995; Harris and Holt, 1995; Friedman and O'Leary, 1996). One argument against the hypothetical involvement of the adhesion cues on hippocampal slices in axonal pathfinding is the finding that adhesive and non-adhesive laminae are also recognized by dissociated cells from other cortical areas, indicating a wide cellular distribution of the recognition molecules for hippocampal lamination. It has been shown, however, that dissociated septal cholinergic neurons from embryonic day 17 preferentially adhere to the granule cell layer and, to a lesser extent, also to the pyramidal cell layer of living hippocampal slices (Haraldson et al., 1997). This pattern was not found when septal cholinergic neurons from postnatal stages were seeded on hippocampal slices, indicating a temporal regulation of the relevant adhesive cues in these neurons (Haraldson et al., 1997). We also regard it as an important finding that the adhesive behaviour of 3T3 cells and cerebellar granule cells on hippocampal slices differed from that of entorhinal cells. Moreover, 3T3 cells seemed to recognize specific directional

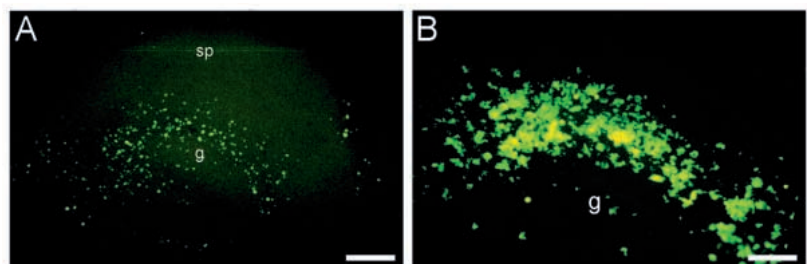


Fig. 6. (A) Adhesion of dissociated cortical cells from adult *NCAM*^{-/-} mutant mice to a slice from adult *reeler* mouse hippocampus (the low density of adherent cells in this experiment is due to the small amount of dissociated cells seeded on the slices). Bar, 500 μ m. (B) Adhesion of dissociated cortical cells from adult *NCAM*^{-/-} mutant mice to a hippocampal slice from a P4 rat. Cells adhere to the outer molecular layer and stratum lacunosum moleculare, but not to the inner molecular layer and granule cell layer. Bar, 50 μ m.

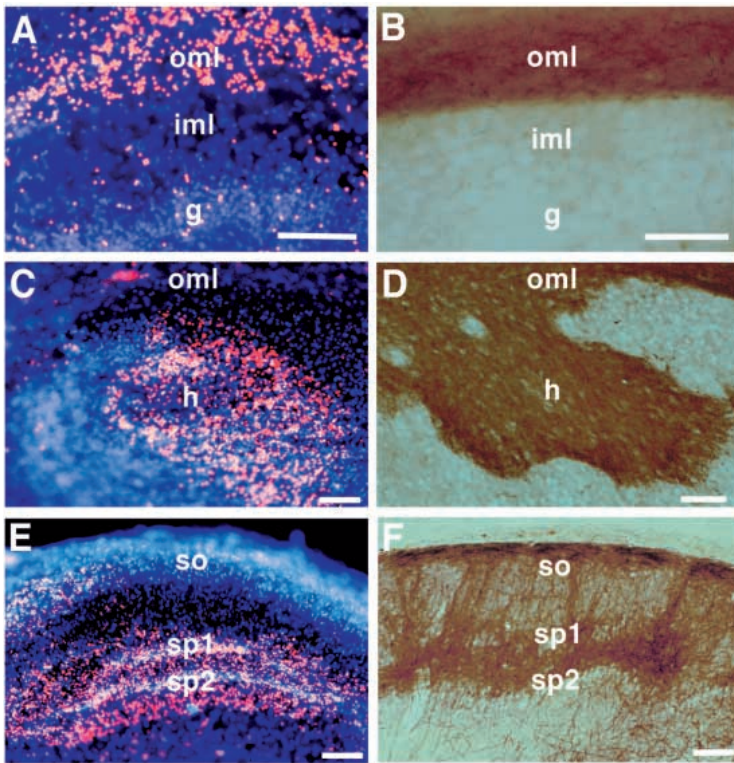


Fig. 7. Adhesion of fluorescent microspheres (4 μm diameter) coated with cortical membranes to slices from adult *reeler* mouse hippocampus. Comparison with the distribution of entorhinal fibers in the hippocampus, as revealed by the anterograde tracing with PHAL. (A,B) Lamina-specific adhesion of membrane-coated fluorescent microspheres (A) and PHAL tracing of the entorhinal fiber projection (B) to the outer molecular layer in the septal hippocampus. Bar, 100 μm . (C,D) Altered adhesion of membrane-coated fluorescent microspheres to the hilar region (C) corresponds with an abnormal hilar projection of entorhinal fibers in the temporal hippocampus (D) of the *reeler*^{-/-} mutant mouse. Bar, 100 μm . (E,F) Adhesion of membrane-coated microspheres to the abnormal CA1 region of the temporal hippocampus of the *reeler*^{-/-} mutant mouse (E) corresponds to an altered entorhinal projection to CA1 between the two pyramidal cell layers sp1 and sp2 (F) Bar, 125 μm .

cues, since they extended processes along the pial surface. But even a wide cellular distribution of an adhesion molecule is not contradictory to a role in axonal pathfinding since axon guidance cues are often recognized by a variety of different neurons, and the same molecules may have functional significance in different contexts (Hynes and Lander, 1992; Goodman and Shatz, 1993; Rutishauser, 1993; Doherty et al., 1994; Chiba and Keshishian, 1996; Tessier-Lavigne and Goodman, 1996). As an example, integrins, which mediate cell adhesion to the ECM and are expressed by most cell types, can regulate both neuronal adhesion and neurite outgrowth (Condic and Letourneau, 1997). Moreover, lamina-specific adhesion may be only one of a variety of signals guiding ingrowing axons to their appropriate targets.

Recognition of adhesive properties of distinct hippocampal

laminae by growing axons as either a growth permissive or a non-permissive substrate may provide a simple mechanism by which lamina-specific fiber projections are segregated during development. Both lamina-specific cell adhesion and lamina-specific fiber growth are abolished on cryoslices or fixed slices, indicating that in both cases the same positional cues may be required. That alternating patterns of adhesive and non-adhesive substrates may indeed influence both attachment of neurons and neurite outgrowth has been shown by several authors (Letourneau, 1975; Kleinfeld et al., 1988; Corey et al., 1991; Matsuzawa et al., 1996). As far as the entorhinal projection is concerned, we have in fact reason to assume that the layer-specific adhesive cues are linked to the layer-specific fiber projection: in slices of the ventral hippocampus which show an abnormal entorhinal projection in the *reeler* mutant

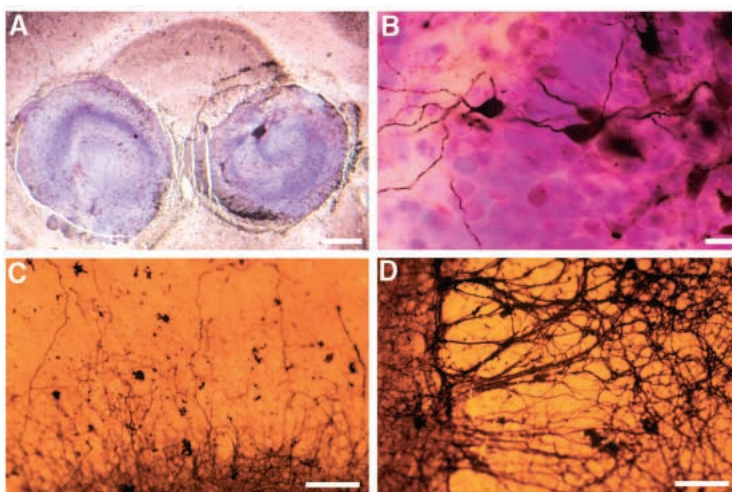


Fig. 8. Lamina-specific neurite outgrowth is abolished on hippocampal cryoslices. Living hippocampal slices (P4) were confronted with unfixed or fixed cryoslices from hippocampus (P4 or adult) and incubated for up to 14 days in vitro. (A) Example of a culture situation, where two living hippocampal slices (P4) had contact to all laminae of a hippocampal cryoslice from an adult rat. The culture is shown after biocytin tracing and cresyl violet staining. Note blue staining of only the cultured hippocampal slices since RNA is degraded in the cryoslice. Bar, 400 μm . (B) Higher magnification of hippocampal slice (P4), after biocytin tracing and cresyl violet staining. A number of neurons and their biocytin-labeled processes are shown. Bar, 25 μm . (C,D) Neurite outgrowth visualized with biocytin-tracing on a fixed cryoslice from adult rat hippocampus after 14 days in vitro. Numerous individual fibers (C) or bundles of fibers have grown over long distances but do not display laminar specificity. Bar, 50 μm .

mouse, the distribution of adhesive cues again reflects the distribution of entorhinal fibers.

Laminar recognition cues with different properties have to be assumed for the axons of commissural neurons, since their cells of origin, retrogradely prelabeled hippocampal neurons, did not specifically adhere to the dentate inner molecular layer, the termination zone of commissural afferents. The final segregated distribution of afferents and the selection of the proper postsynaptic structure may then result from a variety of attractive and repulsive factors acting on the growth cones.

What is the molecular identity of adhesive cues in the hippocampus?

An important criterion for the classification of cell adhesion molecules is their dependence on cations (Hynes and Lander, 1992). In the present experiments, we found that lamina-specific adhesion is maintained in the absence of divalent cations. Thus, a Ca^{2+} -independent adhesion mechanism is sufficient to mediate laminar specificity on hippocampal slices. We regard this as an important finding, since Ca^{2+} -dependent cell adhesion molecules such as cadherins, selectins or integrins may mediate adhesion of dissociated cells (Takeichi et al., 1981; Nose et al., 1988; Götz et al., 1992; Palecek et al., 1997). In contrast, molecules of the immunoglobulin (Ig) superfamily mostly mediate cell adhesion independently of divalent cations (Hynes and Lander, 1992). The majority of known cell adhesion molecules of the Ig superfamily are expressed in the nervous system (Grumet, 1991). A strong candidate for a Ca^{2+} -independent adhesion mechanism is the homophilic adhesion molecule NCAM which is widely expressed in the CNS (Edelman, 1986) including the hippocampus (Miller et al., 1993). Our experiments, however, demonstrate that NCAM cannot be a crucial factor for this type of adhesion because dissociated cortical cells from *NCAM*^{-/-} mutant mice adhered with the same laminar specificity as cells from heterozygous NCAM mutant or wild-type mice. In line with this, no differences of hippocampal cytoarchitecture were detected between wild-type mice, heterozygous and homozygous NCAM mutant mice (Cremer et al., 1994). In addition, NCAM expression is stronger in the inner molecular layer than in the outer molecular layer (Miller et al., 1993), whereas cell adhesion was preferentially observed in the outer molecular layer. Since we did not observe adhesion between membrane-coated fluorescent microspheres, a heterophilic adhesion mechanism seems to be involved in the lamina-specific cell adhesion.

Non-adhesive properties of a substrate may involve repellent cues as well (Calof and Lander, 1991; Chiquet-Ehrismann, 1991; Sage and Bornstein, 1991; Emerling and Lander, 1996; Götz et al., 1996). In the hippocampus, the stratum radiatum and the inner molecular layer of the fascia dentata express the homophilic cell adhesion molecule NCAM (Miller et al., 1993), but did not support adhesion of dissociated cortical cells. In the developing neocortex, a scaffold of chondroitin sulfate (CS)-associated proteoglycans in the ECM has been shown not only to mediate lamina-specific cell adhesion but also cell repulsion on non-adhesive layers (Emerling and Lander, 1996). Treatment with chondroitinase has been found to render the cortical plate a substratum for cell attachment and neurite ingrowth, indicating the presence of anti-adhesive factors in the cortical plate (Emerling and Lander, 1996). In slices from postnatal forebrain, however, the

involvement of CS in this laminar adhesion was not confirmed (Emerling and Lander, 1996). Similarly, CS is unlikely to play a role in cell adhesion in the postnatal hippocampus, since CS in the brain is downregulated after birth (Fernaude-spinosa et al., 1996). Furthermore, we could not detect CS expression in the hippocampus using an antibody against CS (data not shown). Cues involved in cell repulsion might include tenascin glycoproteins that have both adhesive- and anti-adhesive properties for different cell types (Erickson, 1993; Götz et al., 1996) and are expressed in the hippocampus (Ferhat et al., 1996). Distinct sites in tenascin glycoproteins are responsible for neuron binding, the promotion of neurite outgrowth, and the repulsion of cells (Götz et al., 1996).

In the developing neocortex lamina-specific cell adhesion is functionally associated with chondroitin sulfate proteoglycans (CSPGs) (Emerling and Lander, 1996). The observation that CSPGs and fibronectin are closely associated with preplate neurons indicates that subplate and marginal zone cells might be responsible for the deposition of selected ECM components, which in turn might serve as axonal guidance cues (Sheppard and Pearlman, 1997). Marginal zone cells, such as Cajal-Retzius cells, could be responsible for the formation of a specific, localized ECM in the hippocampal formation where the marginal zone is represented by the dentate gyrus outer molecular layer and the stratum lacunosum-moleculare of the hippocampus proper (Del Rio et al., 1997; Frotscher, 1997). This ECM appears to have different adhesive properties from the other layers and may at the same time be permissive for entorhinal but not commissural fibers.

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