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Hyaluronan-associated adhesive cues control fiber segregation in the hippocampus

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SUMMARY

In various brain regions, particularly in the hippocampus, afferent fiber projections terminate in specific layers. Little is known about the molecular cues governing this laminar specificity. To this end we have recently shown that the innervation pattern of entorhinal fibers to the hippocampus is mimicked by the lamina-specific adhesion of entorhinal cells on living hippocampal slices, suggesting a role of adhesion molecules in the positioning of entorhinal fibers. Here, we have analyzed the role of extracellular matrix components in mediating this lamina-specific adhesion. We show that hyaluronidase treatment of hippocampal slices abolishes lamina-specific adhesion as well as layer-specific growth of entorhinal fibers to the dentate outer molecular layer in organotypic slice cultures. We conclude that hyaluronan-associated molecules play a crucial role in the formation of the lamina-specific entorhinal projection to the hippocampus.

Key words: Cell adhesion, Hippocampus, Entorhinal cortex, Axon pathfinding, Hyaluronan, Rat

INTRODUCTION

In the hippocampus ingrowing afferents terminate in specific layers. Lamina-specific fiber termination also develops in organotypic slice cultures suggesting the preservation of specific laminar recognition cues for distinct fiber systems under these in vitro conditions (Yamamoto et al., 1992; Yamamoto et al., 1999; Frotscher and Heimrich, 1993; Li et al., 1993; Li et al., 1994; Li et al., 1995; Li et al., 1996; Del Río et al., 1997). In recent years, a variety of experimental strategies have been used to identify these layer-specific recognition cues for growing axons in the hippocampus. After ablation of hippocampal Cajal-Retzius (CR) cells in hippocampal slice cultures, entorhinal fibers fail to innervate their appropriate target layers, indicating that CR cells play a key role in guiding entorhinal fibers to distinct hippocampal laminae (Del Río et al., 1996; Del Río et al., 1997; Frotscher, 1998; Ceranik et al., 1999, Ceranik et al., 2000). CR cells synthesize the extracellular matrix (ECM) glycoprotein reelin (d'Arcangelo et al., 1995; d'Arcangelo et al., 1997), but reelin is unlikely to be the molecule governing the correct lamina-specific termination of entorhinal fibers. In the hippocampus of *reeler* mice lacking reelin, entorhinal fibers terminate with correct laminar specificity (Del Río et al., 1997; Borrell et al., 1999; Deller et al., 1999). Membrane preparations of hippocampal cells have been found to guide entorhinal axons in vitro, indicating that membrane-associated molecules play a role in target layer recognition by entorhinal fibers (Skutella et al., 1999). Pathfinding of entorhinal fibers may also involve diffusible guidance cues (Chédotal et al., 1998; Steup et al., 1999; Stein et al., 1999; Barallobre et al., 2000). We have recently shown that dissociated entorhinal neurons and microspheres coated with entorhinal membranes adhere with laminar specificity on living slices of hippocampus (Förster et al., 1998) suggesting a role of these adhesive cues in the positioning of entorhinal fibers to the hippocampus.

In the present study, we took advantage of our finding that lamina-specific adhesion of dissociated cells is mimicked by fluorescent microspheres coated with isolated membranes from these cells (Förster et al., 1998). We used the microsphere adhesion assay to analyze the role of different extracellular matrix (ECM) components for a potential function in lamina-specific adhesion and entorhinal fiber growth. We show that lamina-specific adhesion on hippocampal slices is specifically abolished by the hyaluronan (HA) degrading enzyme hyaluronidase. This dramatic loss of lamina-specific adhesion was not seen after treatment of the slices with the enzymes neuraminidase or chondroitinase. Furthermore, we provide evidence that not hyaluronan itself, but hyaluronan-associated molecules mediate lamina-specific adhesion. Hyaluronidase treatment of entorhino-hippocampal cocultures also abolished target layer recognition of entorhinal fibers to the hippocampus in vitro but did not interfere with pathfinding. We conclude that hyaluronan-associated molecules play a crucial role in the segregation of fiber projections to the hippocampus.

MATERIALS AND METHODS

Preparation of hippocampal slices

Brains were removed from Sprague-Dawley rat pups, aged 4-6 days, decapitated under hypothermic anaesthesia. All experiments were

performed in agreement with the institutional guide lines for animal care. The hippocampi were dissected using fine spatulas and sliced perpendicular to their longitudinal axis with a McIlwain tissue chopper. Section thickness was 400 μm. Slices were placed in a drop of 50 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. Until seeding of membrane-coated microspheres, slices were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Preparation of membranes and coating of microspheres

Membranes and membrane-coated microspheres were prepared as described (Förster et al., 1998). Briefly, 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 (Boehringer Mannheim) was added. Cortices were homogenized with 20 strokes of a dounce homogenizer. Cell nuclei and unlyzed 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 1 mg/ml (Bradford, 1976). Fluorescent microspheres (Molecular Probes, 4 μm diameter) were coated with membrane fractions before use, according to the manufacturer's instructions. Membrane-coated microspheres were resuspended in 0.1 M phosphate buffer (PB) and sonicated before use.

Enzyme treatments of hippocampal slices

Enzymes were added to the slice incubation medium in the following concentrations: 200 TRU/ml *Streptomyces* hyaluronidase, protease free (Calbiochem No. 389561) or 2 units/ml chondroitinase, protease free (Calbiochem No. 230681) or 0.25 units/ml neuraminidase, protease free (Calbiochem No. 480712), and a cocktail of protease inhibitors (Boehringer Mannheim). Hippocampal slices were then incubated individually in a drop of incubation medium for 3 hours at 37°C. To confirm the involvement of proteins in lamina-specific adhesion, slices were incubated for 10 minutes in 0.1 M PB containing 0.01% trypsin before the adhesion assay. After the protease treatment the slices were rinsed in incubation medium and the adhesion assay was performed. As a control, slices were incubated under the same conditions, however, without addition of enzyme. After incubation, the slices were carefully rinsed in 0.1 M PB and then transferred to a large volume of incubation medium. Membrane-coated microspheres were then seeded onto each hippocampal slice, as described previously (Förster et al., 1998; Förster and Kaltschmidt, 1999). Slices with adherent microspheres were carefully transferred to a large volume of incubation medium. To remove non-adherent microspheres, the slices were washed by carefully pipetting them up and down (standardized to 10× up and down) with a 1 ml Eppendorf pipette (pipette tips were cut to a large diameter with a razor blade).

Analysis of microsphere adhesion

Slices were mounted on microscope slides and were analyzed under a Zeiss fluorescence microscope using a 10×, 20× or 40× objective lens. Hippocampal laminae such as the dentate molecular layer, the granule cell layer or the pyramidal cell layer, were discernable under the microscope. Slices were counterstained with the fluorescent dye DAPI (Boehringer Mannheim) to confirm the laminar boundaries under UV fluorescence as described previously (Förster et al., 1998).

Microsphere aggregation assay

To test whether HA has the capacity to mediate adhesion, membrane-coated microspheres were incubated for 30 minutes in 0.1 M PB containing soluble purified HA (Serva) in concentrations ranging from 50 μg/ml to 500 μg/ml. To test the influence of CSPGs on microsphere adhesion, membrane-coated microspheres were incubated in 0.1 M PB containing 500 μg/ml HA + 100 μg/ml of a CSPG mixture (Chemicon CC117; major components of the mixture are neurocan, phosphacan, aggrecan and versican), or in 0.1 M PB containing only 100 μg/ml CSPGs without addition of HA. As a control, membrane-coated microspheres were incubated in 0.1 M PB, without addition of HA. After the incubation, samples of the incubated microspheres were carefully pipetted onto microscope slides and analyzed under the fluorescence microscope.

Preparation and hyaluronidase treatment of entorhino-hippocampal cocultures

Slices containing the hippocampus and the adjacent entorhinal cortex were cultured as static cultures (Stoppini et al., 1991; Ceranik et al., 1999) for 7 days. Briefly, newborn mouse pups (P0) were decapitated and the hippocampus plus entorhinal cortex dissected. 300-μm-thick slices were cut perpendicular to the longitudinal axis of the hippocampus as described previously (Li et al., 1993; Ceranik et al., 1999). Tissue sections were then 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 7.3). Slice cultures were incubated in 5% CO_2, 37°C. Medium was changed every 2 days. Hyaluronidase (Calbiochem No. 389561; final concentration 70 TRU/ml) was added to the medium at 2 days in vitro (DIV) and at 4 DIV. No enzyme was added to control cultures.

Tracing of entorhino-hippocampal fibers

Tracing was performed as previously described (Frotscher and Heimrich, 1993). Briefly, a crystal of biocytin (Sigma) was placed on the entorhinal cortex after 5 DIV. After 7 DIV, the cultures were fixed with 4% paraformaldehyde, sectioned (50 μm), and incubated with avidin-biotin-peroxidase complex (Vector Laboratories). Sections were developed with diaminobenzidine/nickel (DAB-Ni) and counterstained with Cresyl Violet.

Analysis of proteins released from enzyme-treated hippocampal slices

Hippocampal slices were first carefully rinsed in 0.1 M PB. The buffer was then replaced by fresh PB containing either 200 TRU/ml hyaluronidase or 2 U/ml chondroitinase or, as a control, no enzyme and a cocktail of protease inhibitors. Slices were then incubated for 3 hours at 37°C. After the incubation, the slice supernatants were pipetted into 1.5 ml Eppendorf tubes and insoluble cell debris was removed by centrifugation for 10 minutes (14000 rpm, 4°C, Eppendorf centrifuge 5402). The remaining soluble proteins in the supernatants were separated by electrophoresis on 3-8% polyacrylamide (PAA) gradient gels (Novex) for 3 hours**,** 75 V in Tris-acetate buffer, pH 7. Protein bands were visualized by subjecting the PAA gels to a silver-staining procedure according to Blum et al. (Blum et al., 1987).

Western blot analysis with antiserum against neurocan

Supernatants from hyaluronidase-treated slices were subjected to PAA gel electrophoresis as described above. An aliquot of the hyaluronidase-treated supernatant was additionally treated with 2 U/ml chondroitinase ABC for 3 hours before electrophoresis, to obtain neurocan core proteins. Blotting of separated proteins on polyvinylidene difluoride membranes (Boehringer Mannheim) was performed with the NUPAGE electrophoresis system (Invitrogen) according to the manufacturers' instructions. For the detection of neurocan core proteins, a polyclonal rabbit antiserum against

neurocan (NC-1) was used (Haas et al., 1999; the antiserum against neurocan was kindly provided by Drs R. U. Margolis and R. K. Margolis, New York University, New York). Immunoreactive bands were developed with the BM chromogenic western blotting kit (Roche Diagnostics).

RESULTS

Lamina-specific adhesion on hippocampal slices is abolished by hyaluronidase treatment

To examine whether HA is involved in mediating lamina-specific adhesion, freshly cut hippocampal slices from young postnatal rats (P6; n>60) were incubated for 3 hours with 200 TRU/ml hyaluronidase from *Streptomyces hyaluronlyticus* at 37°C, which specifically degrades HA (see Methods). This hyaluronidase concentration was shown to remove most of the HA from embryonic (Emerling and Lander, 1996) or postnatal neural tissue (Köppe et al., 1997) within a few hours. As a control, hippocampal slices (P6; n>30) were incubated under the same conditions, however, without addition of hyaluronidase. After the incubation, the slices were transferred to a large volume of 0.1 M phosphate buffer (PB) to remove the enzyme. Tissue integrity was confirmed under a microscope. Then, slices were transferred into incubation medium and membrane-coated microspheres were seeded onto the slices and allowed to settle (see Methods; Förster et al., 1998). After 30 minutes of incubation, the slices were gently rinsed to remove unattached microspheres and counterstained with the fluorescent dye DAPI. On untreated control slices, the characteristic lamina-specific adhesion pattern of adherent microspheres was seen under the fluorescence microscope, confirming our previous observations (Fig. 1A-C; Förster et al., 1998). The distribution of adherent microspheres reflected the distribution of entorhinal fibers projecting from the entorhinal cortex to the hippocampus in vivo (Blackstad, 1958) and in vitro (Frotscher and Heimrich, 1993; Li et al., 1993). In contrast, on hyaluronidase-treated slices microsphere adhesion was abolished (Fig. 1D). For quantification, the densities of adherent microspheres on different hippocampal laminae were determined for 10 hyaluronidase-treated slices and 10 control slices (Fig. 2A,B). As previously described, the outer molecular layer/stratum lacunosum-moleculare (oml/slm) and inner molecular layer/granule cell layer (iml/g) were grouped together, since on untreated slices the microsphere densities were very similar on these laminae (Förster et al., 1998). On control slices, the highest densities were counted on the stratum lacunosum moleculare and the dentate outer molecular layer (slm/oml) with 3400-7200 microspheres/mm^2, followed by a density of 80-2600 microspheres/mm^2 on the hilar region (h). The lowest densities of microspheres were counted on the stratum oriens (so) with 20-980 microspheres/mm^2, on the inner molecular layer and the granule cell layer (iml/g) with 80-1080 microspheres/mm^2, the stratum radiatum (sr) with 100-1040 microspheres/mm^2, and the pyramidal cell layer (p) with 60-600 microspheres/mm^2. Thus, the quantification of adherent microspheres on untreated control slices confirmed our previously obtained results (Förster et al., 1998).

After hyaluronidase treatment, the density of adherent microspheres in the oml/slm was dramatically reduced (Fig. 1D). On these layers now only 320-2200 microspheres/mm^2 were counted (Fig. 2B). On the iml/g, up to 860 microspheres/mm^2 were counted, on the stratum radiatum up to 660 microspheres/mm^2, on the pyramidal cell layer up to 240 microspheres/mm^2, on the hilar region up to 2200 adherent microspheres/mm^2, and on the stratum oriens up to 1620 microspheres/mm^2. The massive reduction in the number of adherent microspheres in the oml/slm demonstrates that the relevant adhesive cues in these layers are susceptible to hyaluronidase treatment, suggesting a role of hyaluronan in mediating lamina-specific adhesion.

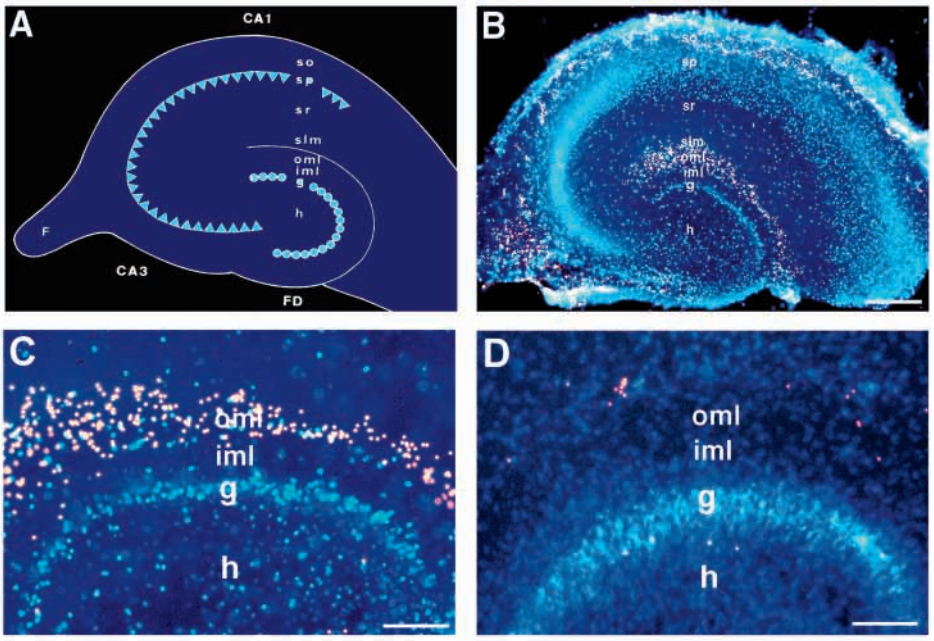

Fig. 1. (A) Schematic illustration of hippocampal lamination. So, stratum oriens; sp, pyramidal cell layer (areas CA1 and CA3 are indicated); sr, stratum radiatum; slm, stratum lacunosum-moleculare; ml, dentate molecular layer (oml=outer ml; iml=inner ml); g, granule cell layer; h, hilus; FD, fascia dentata; F, fimbria. (B) Fluorescent microspheres (4 μm diameter) were coated with membrane preparations from cortical cells (P0) and plated on hippocampal slices (P6). After incubation, non-adherent particles were removed. Microspheres adhere only to the stratum oriens, the stratum lacunosum moleculare, the outer molecular layer and the hilar region. Microspheres do not adhere to the pyramidal cell layer, the granule cell layer, and to the stratum radiatum. Bar, 200 μm. (C) Lamina-specific adhesion of membrane-coated fluorescent microspheres to the outer molecular layer of a DAPI-stained hippocampal control slice (P6). Bar, 50 μm. (D) Lamina-specific adhesion of microspheres is abolished on a hippocampal slice (P6) which was treated with hyaluronidase before the adhesion assay. Bar, 50 μm.

Neuraminidase treatment of hippocampal slices does not affect lamina-specific adhesion

To find out whether other glycosidases may similarly abolish lamina-specific adhesion, hippocampal slices (P6; *n*=30) were incubated with 0.25 units/ml neuraminidase for 3 hours, 37°C before the adhesion assay (see Materials and Methods). Neuraminidase catalyzes the release of sialic acid residues, a frequent posttranslational modification of glycoproteins, such as NCAM (Cremer et al., 1994), or of chondroitinsulfate proteoglycans (CSPGs), such as neurocan (Retzler et al., 1996), both being molecules known to be expressed in the oml/slm (Miller et al., 1993; Haas et al., 1999). After neuraminidase treatment, the microsphere adhesion assay was performed as described above. When looking at the slices under the fluorescence microscope, the characteristic lamina-specific pattern of adherent microspheres was seen, similar to that on the untreated control slices (not shown). Quantification confirmed that microsphere adhesion on neuraminidase-treated slices was not reduced when compared to untreated control slices (Fig. 2C). Thus, laminar adhesive cues are not susceptible to neuraminidase treatment. This finding is in line with previously obtained results by Nakanishi (Nakanishi, 1983), demonstrating that neuraminidase treatment of brain tissue slices does not affect the staining of the ECM glycans HA and chondroitinsulfate.

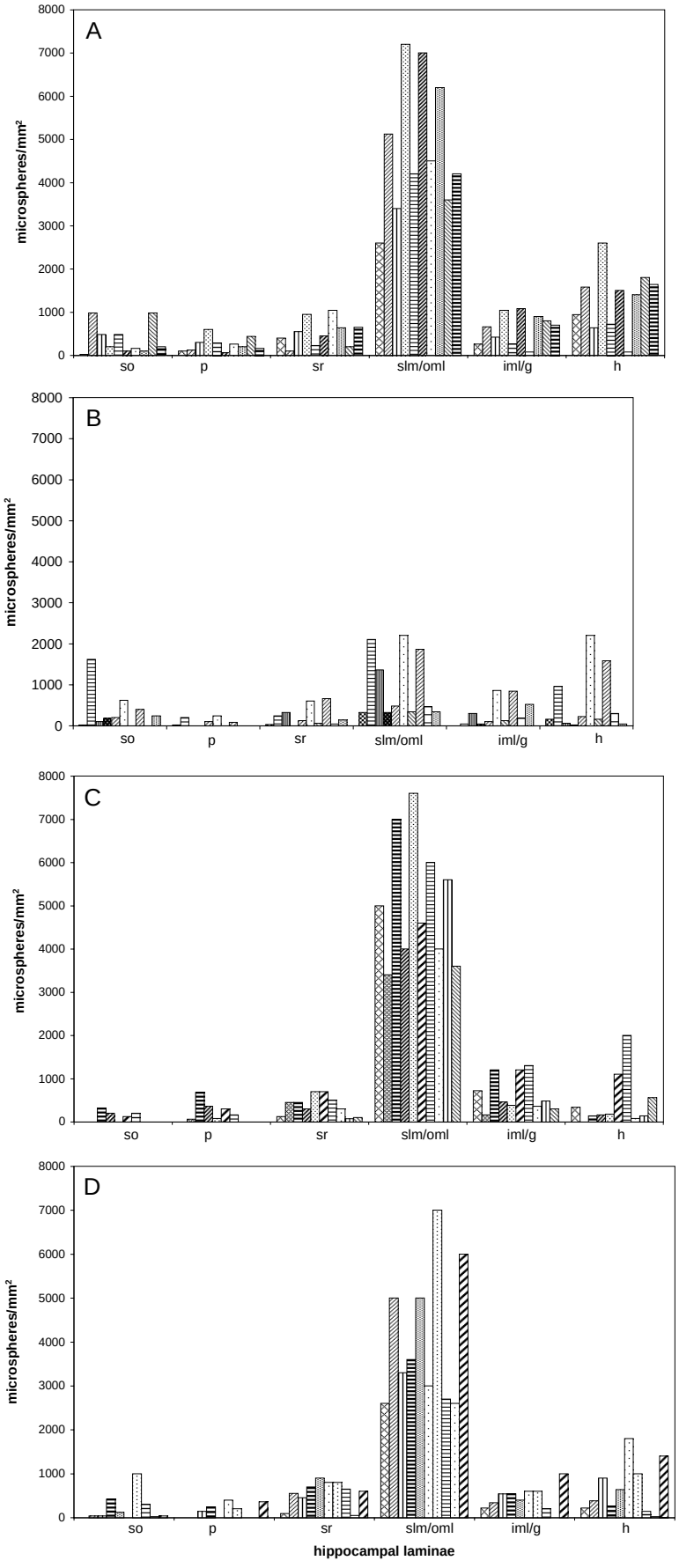

Fig. 2. Influence of different enzyme treatments on microsphere adhesion to hippocampal slices. Densities on the different hippocampal laminae, stratum oriens (so), stratum pyramidale (sp), stratum radiatum (sr), stratum lacunosum-moleculare + dentate outer molecular layer (slm/oml), inner molecular layer + granule cell layer (iml/g), hilus (h), were determined for 10 hippocampal slices (individual columns). (A) Densities of adherent microspheres on different hippocampal laminae on untreated control slices. Densities of adherent microspheres differed significantly between adjacent laminae (Wilcoxon Rank Sum Test, two tailed: $P<0.05$). (B) Hippocampal slices were incubated with 200 TRU/ml hyaluronidase before the adhesion assay. Note the massive reduction of microsphere adhesion in the layers oml/slm when compared to control slices. Densities of adherent microspheres still differed significantly between oml/slm and iml (Wilcoxon Rank Sum Test, two tailed: $P<0.05$). (C) Hippocampal slices were incubated with neuraminidase before the adhesion assay. Densities of adherent microspheres on different hippocampal laminae were similar to those on untreated control slices (compare with A) and differed significantly between adjacent laminae (Wilcoxon Rank Sum Test, two-tailed: $P<0.05$). (D) Hippocampal slices were incubated with 0.25 U/ml chondroitinase before the adhesion assay. Membrane-coated microspheres adhere with laminar specificity to the layers oml/slm. However, the number of adherent microspheres in the layers oml/slm is slightly reduced when compared to untreated control slices (A) or neuraminidase-treated slices (B). (Wilcoxon Rank Sum Test, two-tailed: $P<0.05$).

Lamina-specific adhesion after chondroitinase treatment

Chondroitinsulfate (CS) is a glycosaminoglycan which is covalently linked to the core proteins of CSPGs, a class of ECM proteoglycans associated with HA (Bignami et al., 1993). The enzyme chondroitinase ABC degrades all forms of CS (and dermatansulfate). To examine whether CS may be involved in mediating lamina-specific adhesion, hippocampal slices (P6; n>30) were incubated with 2 units/ml chondroitinase ABC for 3 hours, 37°C. This treatment has been shown to remove most of the CS from embryonic (Nakanishi, 1983; Miller et al., 1995; Emerling and Lander, 1996) and from postnatal cortical slices (Köppe et al., 1997). After chondroitinase treatment, the adhesion assay was performed as described above. When analyzing the slices under the fluorescence microscope, again the characteristic lamina-specific pattern of adherent microspheres could be seen (not shown). However, quantification of adherent microspheres indicated a reduction in the number of adherent microspheres in the oml/slm when compared to untreated control slices (Fig. 2A,D). This minor reduction of microsphere adhesion could be interpreted two ways. (1) It is likely that the reduction of microsphere adhesion is due to partial degradation of HA, since hyaluronidase activity has also been reported for chondroitinase (Yamagata et al., 1968). (2) Alternatively, CS might also be involved in mediating lamina-specific adhesion, suggesting a role for HA-associated CSPGs.

HA fails to aggregate membrane-coated microspheres

To test whether hyaluronan could be the molecule that mediates adhesion, membrane-coated microspheres were incubated for 30 minutes at 37°C in 0.1 M PB containing solubilized HA in concentrations ranging from 50-500 μg/ml. As a control, membrane-coated microspheres were incubated in 0.1 M PB without addition of HA. After the incubation, microspheres were analyzed under a fluorescence microscope (Fig. 3). However, this assay did not give any evidence that HA could directly mediate adhesion of membrane-coated microspheres (Fig. 3A,B). None of the tested HA concentrations increased aggregation of microspheres when compared to controls. These results suggest that HA is not the molecule that mediates lamina-specific microsphere adhesion on hippocampal slices, but molecules that are bound to HA. Aggregates between HA and proteoglycans are formed via non-covalent binding of link proteins to HA (Bignami et al., 1993). To test the influence of proteoglycans on microsphere aggregation, membrane-coated microspheres were incubated in 0.1 M PB containing 100 μg/ml of a CSPG-mixture, or 500 μg/ml HA + 100 μg/ml CSPGs (see Materials and Methods). In the solution containing CSPGs but not HA, only a few aggregated microspheres were detected (Fig. 3C). In contrast, in the solution containing both HA and CSPGs, an increased number of aggregated microsphere was seen (Fig. 3D). Thus, HA-associated CSPGs could also play a role in mediating lamina-specific adhesion.

To confirm the involvement of proteins in mediating lamina-specific adhesion, we treated hippocampal slices (P6; n>20) with 0.01% trypsin for 10 minutes before the adhesion assay. Analysis of the slices under the fluorescence microscope after the adhesion assay demonstrated that this treatment was sufficient to abolish microsphere adhesion on hippocampal slices (not shown). Thus, proteins, most likely associated with HA, are candidate cues involved in mediating lamina-specific adhesion on hippocampal slices.

Laminar specificity of the entorhinal-hippocampal projection is abolished by hyaluronidase treatment

To test whether hyaluronidase treatment also affects the lamina-specific growth of entorhinal fibers to the dentate outer molecular layer, entorhino-hippocampal cocultures were prepared from newborn mice (P0; see Frotscher and Heimrich, 1993). Hyaluronidase (70 TRU/ml final concentration) was added to the incubation medium of the cocultures (n=12) after 2 and 4 days in vitro (DIV). No enzyme was added to control cultures (n=12). Thereafter, biocytin crystals were positioned on the entorhinal cocultures to trace the entorhinal fibers. Following 2 further days of incubation the cultures were processed to visualize the labeled entorhinal fibers as previously described (Del Río et al., 1997; see Methods). In control cultures, the characteristic lamina-specific termination of entorhinal fibers in the slm and oml was seen (Fig. 4A), confirming our previous results (Frotscher and Heimrich, 1993; Del Río et al., 1997). Virtually no entorhinal fibers invaded the inner molecular layer, and a sharp boundary between the iml and the oml could be discerned (Fig. 4A). In hyaluronidase-

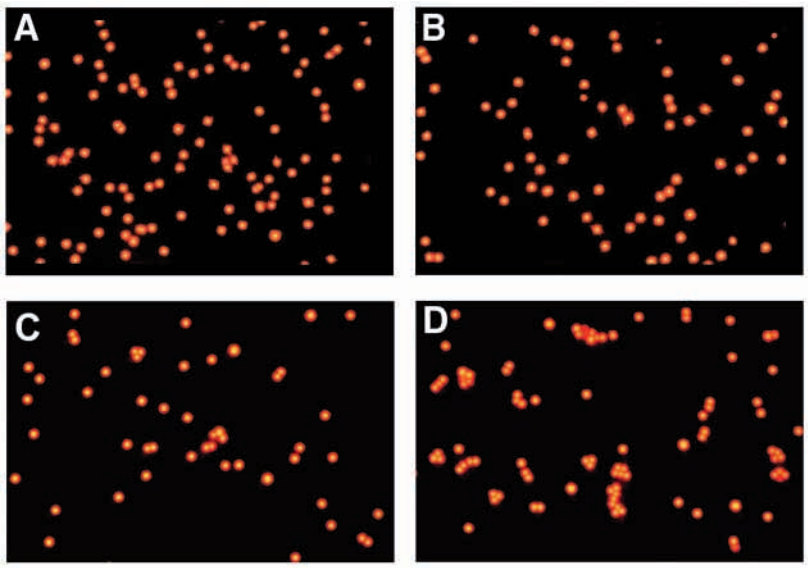

Fig. 3. Increased aggregation of membrane-coated microspheres in a solution containing hyaluronan and a mixture of CSPGs. (A) Control: Membrane-coated microspheres after 30 minutes incubation in 0.1 M PB. (B) Membrane-coated microspheres after 30 min of incubation in 500 μg/ml hyaluronan in 0.1 M PB. (C) Membrane-coated microspheres after 30 minutes incubation in 0.1 M PB containing 100 μg/ml of a CSPG mixture. (D) Membrane-coated microspheres after 30 minutes incubation in 0.1 M PB containing 500 μg/ml hyaluronan and 100 μg/ml of a CSPG mixture. Note the increased number of aggregated microspheres.

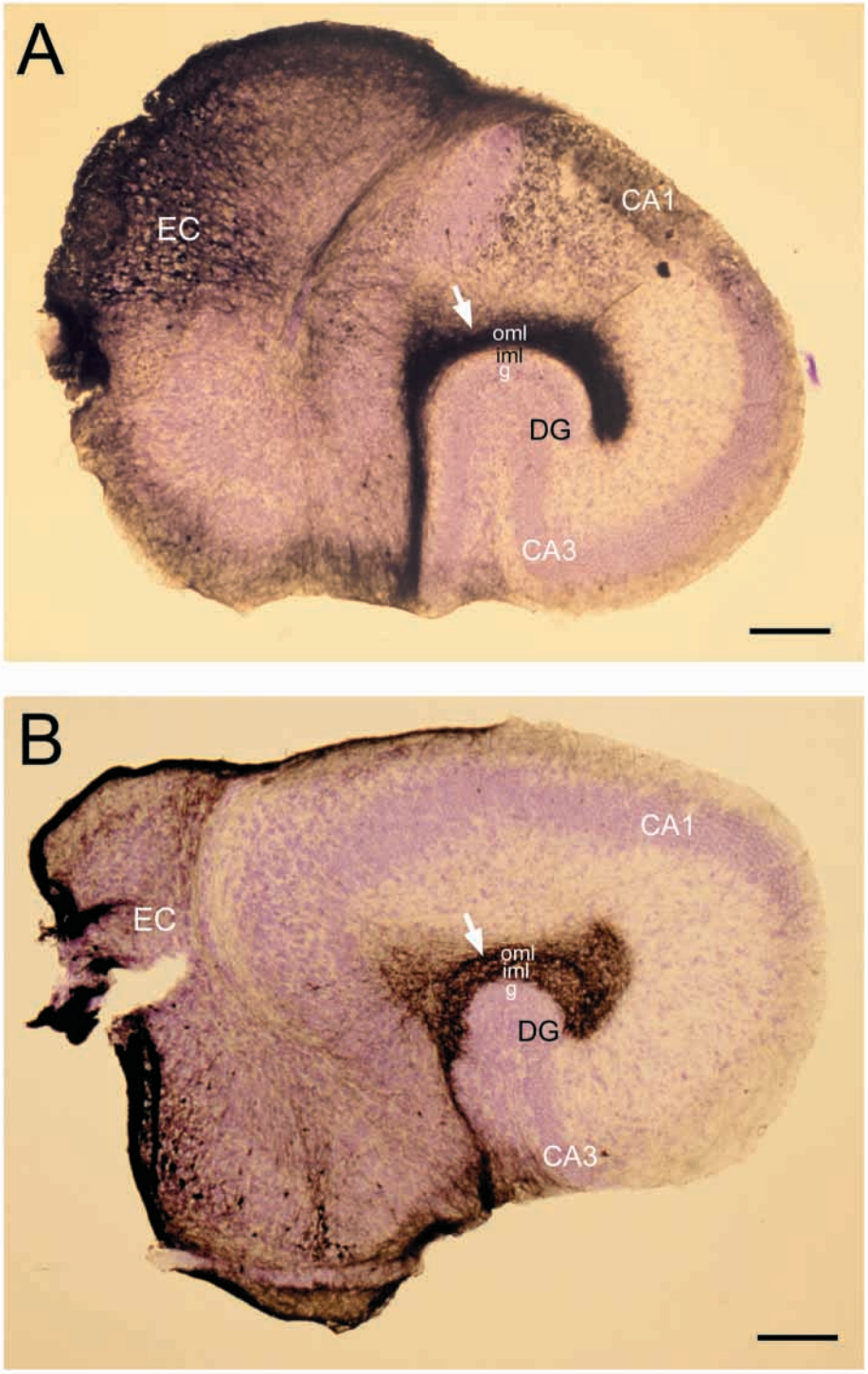

Fig. 4. Effect of hyaluronidase treatment on the lamina-specific growth of entorhinal fibers in entorhino-hippocampal cocultures. (A) Control: Entorhino-hippocampal coculture after 7 days in vitro (DIV) without addition of enzyme. The entorhinal fibers are visualized by tracing with biocytin. Entorhino-hippocampal fibers (arrow) are restricted to the stratum lacunosum moleculare (slm) and dentate outer molecular layer (oml). Note that entorhinal fibers do not invade the inner molecular layer (iml). The tissue section is counterstained with Cresyl Violet. DG, dentate gyrus; g, granule cell layer; EC, entorhinal cortex; hippocampal areas CA1 and CA3 are indicated. Bar: 100 μm. (B) Hyaluronidase-treated entorhino-hippocampal coculture after 7 DIV. As in control cultures, entorhino-hippocampal fibers project to the slm and the molecular layer (arrow). However, entorhinal fibers are not restricted to the oml but also invade the iml. Bar, 100 μm.

treated cultures, entorhinal fibers still invaded the slm and the oml, suggesting that axonal pathfinding to the hippocampus was not affected by the enzyme treatment. However, entorhinal fibers were no longer restricted to the oml but densely innervated the iml (Fig. 4B), suggesting that the distribution of laminar guidance cues, which normally keep entorhinal fibers restricted to the oml, had been disrupted by the hyaluronidase treatment. Thus, hyaluronan-associated cues play a crucial role in keeping entorhinal fibers restricted to the oml. Biocytin-filled entorhinal neurons in hyaluronidase-treated cultures displayed a normal morphology (not shown), demonstrating that hyaluronidase treatment did not cause gross alterations of these projection neurons.

Release of HA-associated proteins by hyaluronidase treatment of hippocampal slices

Since lamina-specific adhesion and lamina-specific fiber growth are abolished by hyaluronidase treatment, we wanted to know whether novel proteins or proteoglycans could be detected in the slice incubation buffer after the enzyme

treatment. Release of proteoglycans from tissue sections by hyaluronidase digestion has been previously reported (Asher et al., 1991). Hippocampal slices were incubated for 3 hours, 37°C in 0.1 M PB containing hyaluronidase, chondroitinase or a combination of both enzymes at the same concentrations as were used for the adhesion assays. Control slices were incubated without addition of enzyme. After the enzyme treatment, the supernatants were recovered and centrifuged to precipitate insoluble proteins (see Materials and Methods). The supernatants containing the soluble proteins were separated by electrophoresis on 3-8% gradient polyacrylamide (PAA) gels. Proteins were then visualized by using a silver-staining procedure. Supernatants from untreated slices displayed a wide range of protein bands, suggesting that soluble ECM proteins from the slices had diffused into the incubation buffer (Fig. 5). We wanted to know whether novel protein bands appeared after hyaluronidase treatment, representing candidate cues involved in lamina-specific adhesion and fiber growth. PAA-gel electrophoresis of supernatants from enzyme-treated slices resulted in a band pattern that appeared to be largely identical to that of control slices. Major differences were apparent in the molecular range above 200 kDa (Fig. 5A). One band of approximately 300 kDa appeared after hyaluronidase treatment, but not after chondroitinase treatment (Fig. 5A), suggesting release of this protein specifically by hyaluronidase treatment. This band was, however, not seen in supernatants of slices that were treated with both hyaluronidase and chondroitinase, suggesting that it represents a chondroitinsulfate proteoglycan (CSPG) that is released by hyaluronidase and then degraded by chondroitinase. A second band of approximately 130 kDa appeared in the supernatant after hyaluronidase treatment, but not after chondroitinase treatment of hippocampal slices (Fig. 5B), suggesting that this protein is also specifically released by hyaluronidase treatment. The band did not disappear after combined treatment of the slices with both chondroitinase and hyaluronidase, suggesting that it represents a protein not modified by CS. The otherwise identical band patterns in this molecular range (Fig. 5B) suggest that the appearance of this band is not due to a nonspecific effect, such as protease activity. Specificity is also suggested by the different sensitivities of the 300 kDa band and the 130 kDa band to the enzymatic treatment. Controls were performed to ensure that the additional bands did not represent the added enzyme or other proteins in the enzyme buffer.

A molecular mass of 300 kDa has been reported for neurocan (Meyer-Puttlitz et al., 1995), suggesting that neurocan is a candidate CSPG, released by hyaluronidase treatment of hippocampal slices. To find out whether neurocan is present in supernatants from hyaluronidase-treated hippocampal slices, western blot analysis was performed with an antiserum against neurocan core proteins (see Materials and Methods). Without further enzymatic treatment of the supernatants, no proteins were detected by the antiserum, suggesting that the supernatant did not contain neurocan core proteins (Fig. 6). To determine whether the hyaluronidase-treated supernatants contained full-length neurocan including the chondroitinsulfate side chains, the supernatants were

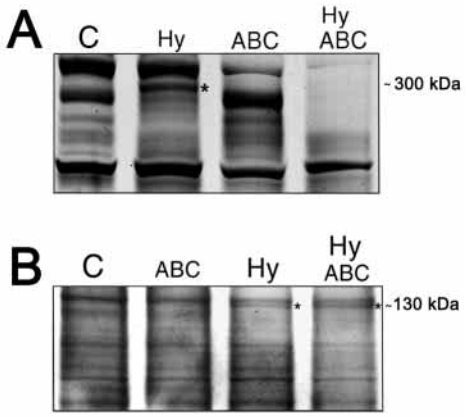

Fig. 5. Analysis of the protein content in the slice incubation buffer after hyaluronidase treatment. Silver-stained polyacrylamide gels are shown after electrophoresis of supernatants from hippocampal slices (P6): C, control without addition of enzyme; Hy, after hyaluronidase treatment; ABC, after treatment with chondroitinase ABC; Hy/ABC, after treatment with both hyaluronidase and chondroitinase. (A) Protein bands in the range between 200 kDa and 400 kDa are shown. Note the presence of an additional band (*) of approximately 300 kDa after hyaluronidase (Hy) treatment of the slices. This band is not seen in control supernatants (C) or in supernatants from chondroitinase (ABC) treated slices, suggesting specific release of this protein by hyaluronidase treatment. Absence of this band in supernatant that was treated with both hyaluronidase and chondroitinase (Hy/ABC) suggests that the band represents a protein that is modified by chondroitinsulfate. (B) Protein bands below 140 kDa are shown. Note the presence of an additional band (*) of approx. 130 kDa after hyaluronidase (Hy) treatment of the slices. This band is not seen in control supernatants (C) or in supernatants from chondroitinase (ABC) treated slices, suggesting release of this protein only by hyaluronidase treatment of the slices. However, this band is unaffected by treatment of slices with both hyaluronidase and chondroitinase, suggesting it is not modified by chondroitinsulfate.

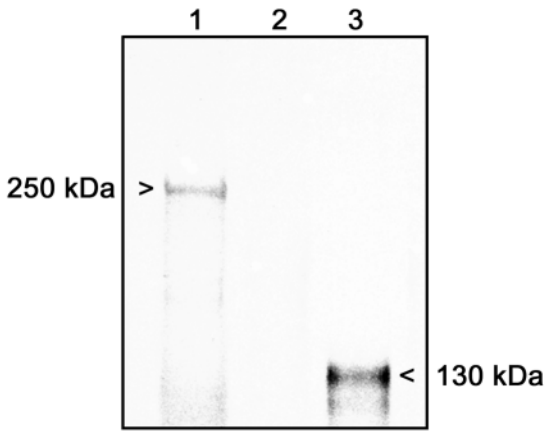

Fig. 6. Western blot for neurocan. Proteins in supernatants from hyaluronidase-treated rat hippocampal slices were separated by gel electrophoresis and analyzed with an antiserum which detects neurocan core proteins (NC-1). Lane 1: Protein size standard. Lane 2: Supernatant after hyaluronidase treatment alone; no signal was detected by the neurocan antiserum. Lane 3: The supernatant from lane 2 was subsequently treated with chondroitinase ABC to remove chondroitinsulfate side chains from CSPGs. After this double enzymatic treatment, a band of approx. 130 kDa, which is characteristic for a neurocan core protein, was detected by the antiserum. Thus, neurocan, substituted with CS, is present in hyaluronidase-treated supernatants from hippocampal slices.

additionally treated with chondroitinase ABC before gel electrophoresis. After additional chondroitinase treatment, a band of approximately 130 kDa was detected by the antiserum, most likely representing a neurocan core protein (Fig. 6; Rauch et al., 1992; Meyer-Puttlitz et al., 1995). This result suggests that full length neurocan substituted with chondroitinsulfate side chains is present in supernatants from hyaluronidase-treated hippocampal slices. Thus, the 300 kDa band, which was detected on silver-stained gels from hyaluronidase-treated hippocampal slices, most likely represents full-length neurocan (see Fig. 5). In contrast, the 130 kDa band seen on the silver-stained gels of supernatants from hyaluronidase-treated slices (Fig. 5) is not identical to the 130 kDa neurocan core protein detected by the neurocan antiserum, since without additional chondroitinase treatment of the supernatant, no signal is detected by the antiserum. Thus, the 130 kDa band seen on the silver-stained gels represents a different protein, which is released into the supernatant specifically by hyaluronidase treatment of hippocampal slices.

DISCUSSION

We found that hyaluronidase treatment of hippocampal slices eliminated laminar cues that underlie both layer-specific adhesion of membrane-coated microspheres and the laminated termination of entorhinal axons. However, solubilized hyaluronan failed to support the aggregation of membrane-coated microspheres, indicating that HA does not directly mediate lamina-specific adhesion. Taken together with the observation that lamina-specific adhesion is rapidly abolished by mild protease treatment of the slices, these results suggest that HA-associated proteins or proteoglycans are the relevant adhesive cues. Our findings indicate that neurocan is one of them. Hyaluronidase treatment of entorhino-hippocampal cocultures disrupted laminar specificity of entorhinal fiber growth but did not affect the pathfinding of entorhinal fibers to the hippocampus. Thus, hyaluronan-associated molecules play a specific role in the laminar segregation of fiber projections to the hippocampus.

Role of hyaluronan

Hyaluronic acid is thought to play a major role in the organization of brain extracellular matrix (Bignami et al., 1993). In the present study, we tested the susceptibility of hippocampal laminar adhesive cues to different enzymes that degrade ECM glycans. We found that the laminar adhesive cues in the hippocampus are abolished by the HA degrading enzyme hyaluronidase. What may be the role of HA in mediating this type of adhesion? The possibility has to be considered that HA binding sites were present in the membrane preparations that we used for coating microspheres. The family of hyaluronate receptor proteins (H-CAM or CD44; Underhill and Toole, 1979; Underhill, 1992) are expressed by various cell types including astrocytes (Vogel et al., 1992) and neurons (Sretavan et al., 1994) and might therefore be present in membrane preparations from cortical cells. It is, however, unlikely that HA is the adhesive molecule in our experimental system, since solubilized HA fails to aggregate membrane-coated microspheres. Furthermore, CD44 is only expressed by subsets of cells, which argues against a prominent role of this molecule in our adhesion experiments. Thus, it appears more likely that adhesion is mediated by hippocampal HA-associated molecules. This assumption is strengthend by the observation that laminar adhesive cues are susceptible to mild trypsinization as well as by our finding that a defined set of proteins and proteoglycans are released from tissue sections by hyaluronidase treatment. Aggregates between HA and proteoglycans are formed via noncovalent binding of core proteins containing a HA-binding domain (Bignami et al., 1993; see also Fig. 7A), and release of proteoglycans from tissue sections by hyaluronidase digestion has been reported previously (Asher et al., 1991; see also Fig. 7B).

HA-associated cues mediate lamina-specific adhesion and play a role in lamina-specific neurite outgrowth

Comparative studies on the distribution of hyaluronidase-sensitive ECM glycosaminoglycans in wild-type and *reeler* mice suggest a role for HA-associated cues in the formation of neocortical and hippocampal lamination (Derer and Nakanishi, 1983; Nakanishi, 1983). A subset of HA-associated proteoglycans, the chondroitinsulfate (CS) proteoglycans (CSPGs), are distributed with layer specificity in the developing cortex and are colocalized with growing fiber tracts (Pearlman and Sheppard, 1996). Analysis of the distribution of ECM components in the *reeler* mouse brain indicates an important function of CSPGs in the formation of cortical lamination (Sheppard and Pearlman, 1997). CSPGs were also shown to be involved in cell-cell adhesion and cell substrate adhesion during development (Hernon and Lander, 1990;

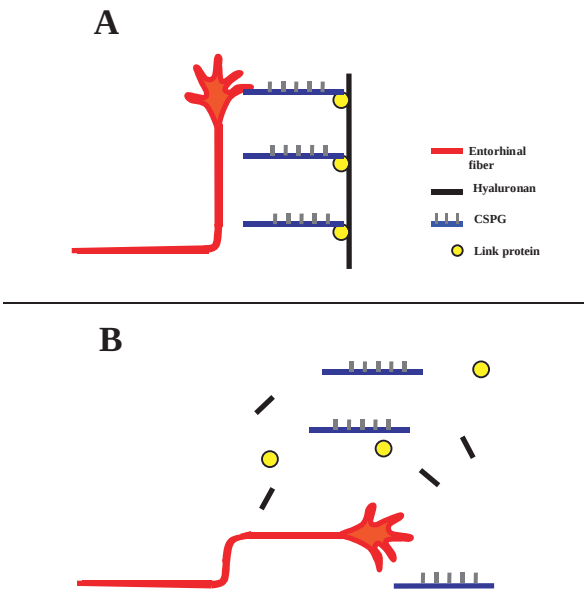

Fig. 7. Hypothetical role of hyaluronan and hyaluronan-associated molecules in the lamina-specific growth of entorhinal fibers to the dentate outer molecular layer. (A) Hyaluronan-associated cues direct a growing entorhinal fiber to the dentate outer molecular layer and prevent the growth cone from entering the inner molecular layer. (B) The boundary which is normally formed by hyaluronan-associated cues is disrupted by hyaluronidase treatment. Hyaluronan-associated cues are no longer able to prevent the entorhinal fiber from entering the inner molecular layer.

Faissner and Steindler, 1995; Margolis and Margolis, 1997; Rauch, 1997; Yamada et al., 1997). A number of HA-binding proteoglycans, including aggrecan, versican, neurocan and brevican have been found in the brain ECM (Rauch, 1997; Milev et al., 1998). In the hippocampus, the HA-binding proteoglycans neurocan and brevican were shown to be upregulated in the dentate outer molecular layer after lesion of the entorhinal cortex (ECL), and the time course of this upregulation correlates with lesion-induced neurite outgrowth (Haas et al., 1999; Thon et al., 2000; Deller et al., 2000). The proteoglycan tenascin-c, which interacts with HA binding proteoglycans (Perides et al., 1993, Grumet et al., 1994), and the proteoglycan DSD-1 that is often changed in parallel with tenascin-c (Faissner et al., 1994), were similarly shown to be upregulated with laminar specificity after ECL (Deller et al., 1997). Neurocan has recently been suggested to play a role in axon tract formation in the hippocampus (Wilson and Snow, 2000). Indeed, our present findings demonstrate that HA-associated molecules, such as neurocan, play a crucial role in both lamina-specific adhesion and the lamina-specific growth of entorhinal fibers. Which may be the relevant HA-associated molecules here? Versican and the glycoprotein tenascin were shown not to be released from tissue by hyaluronidase digestion (Bignami et al., 1993) and are therefore unlikely to function as adhesive cues in our experiments. Other HA-binding proteoglycans that were found to be present in the brain ECM are the Cat-301 proteoglycans and pgT1 (Rauch, 1997). These proteoglycans have been reported to require denaturing conditions to be efficiently solubilized from brain tissue (Rauch, 1997) and are therefore unlikely to play a role as adhesive cues in the present adhesion assay. No such limitations have been reported for the CSPGs neurocan, phosphacan and brevican. Neurocan and phosphacan were shown to have differential effects on cell adhesion (Grumet et al., 1994).

If CSPGs mediate lamina-specific adhesion and fiber growth, it has to be assumed that their core proteins are involved rather than their CS side chains, since we found that chondroitinase treatment only slightly reduced lamina-specific adhesion, probably owing to a weak hyaluronidase activity of chondroitinase (Yamagata et al., 1968). In fact, CSPG core proteins were shown to contain domains similar to those in some adhesive proteins (Zimmermann and Ruoslahti, 1989; Rauch et al., 1992). In line with this, Köppe et al. (Köppe et al., 1997) showed that chondroitinase treatment of brain slices did not alter immunolabelling of CSPG core proteins whereas staining of CS failed, suggesting that some CSPG core proteins remain in the ECM inspite of the removal of their CS side chains. We have to consider, however, that hyaluronan-associated molecules other than CSPGs may play a role in lamina-specific adhesion and fiber growth.

Release of HA-associated cues from the ECM by hyaluronidase treatment

Analysis of the slice incubation buffer after hyaluronidase treatment by polyacrylamide gel electrophoresis suggests that specific HA-associated proteins or proteoglycans are released by the hyaluronidase treatment. Two discrete novel bands could be detected on silver-stained PAA gels after hyaluronidase treatment, a chondroitinase-sensitive 300 kDa band and a chondroitinase-insensitive 130 kDa band. By western blot analysis of hyaluronidase-treated supernatants with an antiserum against neurocan, we could provide evidence that the 300 kDa band is the CSPG neurocan. In the early postnatal period, neurocan is highly expressed in the hippocampus; expression during this period has been shown in the outer molecular layer and the stratum lacunosum moleculare, suggesting a role of neurocan in axon tract formation in the hippocampus (Wilson and Snow, 2000). Furthermore, we could show that at least one additional, yet unknown protein is released into the slice supernatant, specifically after hyaluronidase treatment. It is, of course, likely that additional CSPGs and proteins are released as well by hyaluronidase treatment.

Several studies on the role of laminar hippocampal cues in the growth of entorhinal fibers have concentrated on membrane bound cues (Skutella et al., 1999; Stein et al., 1999). However, the membrane preparations used in these studies (Walter et al., 1987) do not contain CSPGs, CS or tenascin (Tuttle et al., 1995; Skutella et al., 1999). In contrast, our present experiments provide evidence that soluble, HA-associated molecules, such as CSPGs, also may play a prominent role in the lamina-specific growth of entorhinal fibers (Fig. 7).

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