

# The Repulsive Guidance Molecule RGMA Is Involved in the Formation of Afferent Connections in the Dentate Gyrus

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In the developing dentate gyrus, afferent fiber projections terminate in distinct laminae. This relies on an accurately regulated spatio-temporal network of guidance molecules. Here, we have analyzed the functional role of the glycosylphosphatidylinositol (GPI)-anchored repulsive guidance molecule RGMA. *In situ* hybridization in embryonic and postnatal brain showed expression of RGMA in the cornu ammonis and hilus of the hippocampus. In the dentate gyrus, RGM immunostaining was confined to the inner molecular layer, whereas the outer molecular layers targeted by entorhinal fibers remained free. To test the repulsive capacity of RGMA, different setups were used: the stripe and explant outgrowth assays with recombinant RGMA, and entorhino–hippocampal cocultures incubated either with a neutralizing RGMA antibody (Ab) or with the GPI anchor-digesting drug phosphatidylinositol-specific phospholipase C. Entorhinal axons were clearly repelled by RGMA in the stripe and outgrowth assays. After disrupting the RGMA function, the specific laminar termination pattern in entorhino–hippocampal cocultures was lost, and entorhinal axons entered inappropriate hippocampal areas. Our data indicate an important role of RGMA for the layer-specific termination of the perforant pathway as a repulsive signal that compels entorhinal fibers to stay in their correct target zone.

**Key words:** axon; dentate; development; guidance; hippocampus; neuron

## Introduction

In the hippocampus, extrinsic and intrinsic afferent projections terminate in a nonoverlapping way in distinct laminae. Entorhinal axons terminate on distal dendritic segments of their target neurons in the outer molecular layer of the fascia dentata and stratum lacunosum moleculare of the hippocampus proper. Complementary to the termination patterns of entorhinal axons, hippocampal commissural–associational fibers are restricted to the inner third of the molecular layer of the dentate gyrus and stratum radiatum of the hippocampus proper. This well described anatomical construction implies that specific guidance molecules expressed in the various laminae determine the ingrowth and targeting of these fiber systems. Recent studies on guidance molecules in functional *in vitro* assays or loss-of-

function models have provided direct evidence for an impact of several guidance cues on hippocampal pathfinding and target recognition (Skutella and Nitsch, 2001). A number of diffusible and membrane-associated attractive or repulsive axon guidance molecules have been identified and tested for their role in hippocampal development. These include semaphorins (Chen et al., 2000; Cheng et al., 2001; Pozas et al., 2002; Gu et al., 2003), netrins (Steup et al., 2000; Heimrich et al., 2002), ephrins (Stein et al., 1999), slits (Nguyen Ba-Charvet et al., 1999), and ECM molecules (Förster et al., 2001; Zhao et al., 2003).

The repulsive guidance molecule (RGM), a protein of 33 kDa, has been cloned and functionally characterized by Monnier et al. (2002) as a molecular determinant for the retinotectal map formation of the chick embryo (Bayer and Altman, 1987; Walter et al., 1987; Stahl et al., 1990). Three genes in the mouse genome with homology to chick have been isolated (Schmidtmer and Engelkamp, 2004), and their expression patterns have been analyzed (Oldekamp et al., 2004). Mouse RGMA is closely related to chick RGM (80% identity), whereas the two other members of the RGM family are more distantly related. In general, RGMA is a membrane-associated glycoprotein that shares no sequence homology with any other known guidance cues. It is attached to membranes by a glycosylphosphatidylinositol (GPI) anchor. In the chick embryo RGM, mRNA is distributed in an increasing

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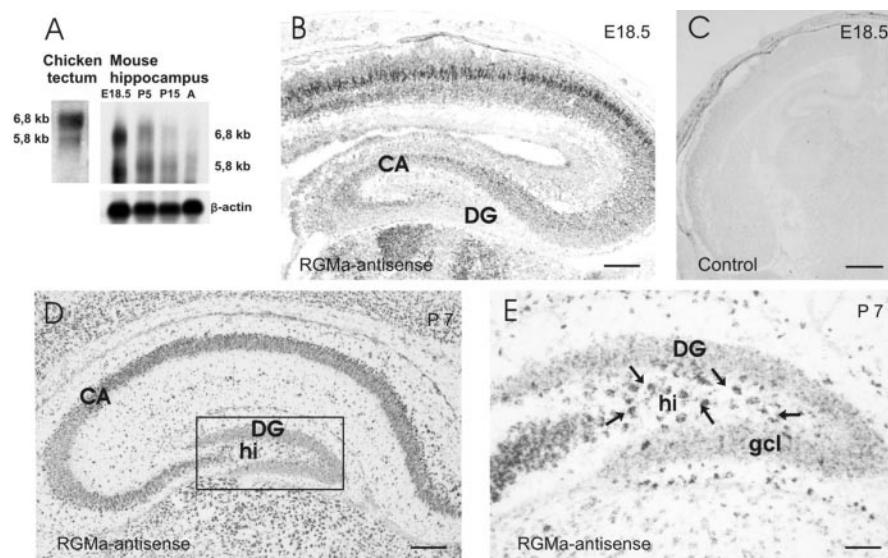
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**Figure 1.** Northern blot analysis of RGMA expression in chicken tectum and mouse hippocampal tissues and distribution of RGMA mRNA in the developing mouse entorhino–hippocampal system. *A*, Chicken embryonic optic tectum mRNA blots were hybridized with a chicken RGMA-specific probe (aa 869–1030), revealing major transcripts of 5.8 and 6.8 kb. *B*, Mouse hippocampus mRNA blots were also hybridized with an RGMA-specific probe (aa 869–1030), revealing major transcripts of 5.8 and 6.8 kb. Total RNA was obtained from E19, P5, P15, and adult (*A*) hippocampus. A hybridization with a 2 kb  $\beta$ -actin probe was used to confirm that identical mRNA levels are given in all lanes. Positions of RGMA mRNA are labeled with the respective transcript bands. *B*, At E18.5 the cortical plate is stained intensively. In the developing hippocampus the principal cell layers of CA1 and CA3 are labeled. *C*, In a control section from E18.5 incubated with an RGMA sense probe, no signal can be detected. *D*, In the hippocampus at P7, strong mRNA expression is observed in the CA1 region, subiculum, and hilus. The dentate gyrus and the CA3 region show weak staining. *E*, This micrograph is a high-power magnification of the dentate gyrus shown in *D*. Note the intense staining of hilar mossy cells (arrows), the population of cells giving rise to the commissural–associational projection to the inner molecular layer. CA, Cornu ammonis; DG, dentate gyrus; gcl, granule cell layer; hi, hilus. Scale bars: *A*, 500  $\mu$ m; *B*, 400  $\mu$ m; *C*, 600  $\mu$ m; *D*, 300  $\mu$ m; *E*, 100  $\mu$ m.

gradient along the anterior–posterior axis of the embryonic tectum (Monnier et al., 2002). In the same study the authors have demonstrated that recombinant RGMA induces collapse of temporal but not nasal growth cones of retinal ganglion cells and guides temporal retinal axons *in vitro*. These results point to a repulsive and axon-specific guiding activity of RGMA.

In the present study, we have examined the role of RGMA in the development of the entorhino–hippocampal system in rodents. *In situ* hybridization and immunohistochemistry show that RGMA is expressed during perinatal development in the hippocampus. Stripe and outgrowth assays reveal that entorhinal neurites are repelled by recombinant RGMA. Both separation of RGMA from its GPI anchor and specific blockade of the RGMA protein did not impair the outgrowth of entorhinal axons but resulted in an abnormal innervation of the hippocampal target. These results support a role for RGMA in the developmental construction of the precise entorhino–hippocampal connectivity pattern.

## Materials and Methods

### Northern blotting

Total RNA was prepared from frozen mouse hippocampus [embryonic day (E) 16.5, postnatal day (P) 5, P15, adult] by column purification (RNeasy, Qiagen, Hilden, Germany). RNA was size-separated on 1% denaturing agarose gels (2.5  $\mu$ g per lane) and blotted onto nylon membranes (Hybond-N+, Amersham Biosciences, Braunschweig, Germany). Blots were hybridized with random-primed ( $^{32}$ P)dCTP DNA probes encompassing the coding region of RGMA (accession number BC023870). Overnight hybridizations were performed at 60°C in the presence of 50% formamide, 5 $\times$  Denhardt's solution, 5 $\times$  SSC, 0.5% SDS, and 100 ng/ml probe. The final washing stringency was 0.1%

SSC and 0.1% SDS at 60°C. Blots were exposed to Amersham Hyperfilm ECL.

### In situ hybridization

For *in situ* hybridization experiments, E16.5, E18.5, and P7 mouse brains were directly frozen in O.C.T. compound (Tissue-Tek). Sections of 20  $\mu$ m were prepared on a cryostat. Hybridization was performed with a digoxigenin (Dig)-labeled antisense riboprobe corresponding to nucleotides 245–629 of the mouse RGMA cDNA. A sense probe served as control. *In situ* hybridization was performed as described (Oldekamp et al., 2004).

### Immunohistochemistry

Postnatal mice (P7) were killed by transcardiac perfusion with 4% paraformaldehyde, pH 7.4. Brains were removed and postfixed overnight in the same fixative before vibratome sectioning. Immunohistochemical analysis was performed on coronal free-floating vibratome sections (70  $\mu$ m) incubated with a polyclonal rabbit antibody (Ab) against the RGMA peptide EEVNVAVEDR spanning residues 279–289. This peptide appears in mouse as well as in chicken, and the resulting Ab has been demonstrated to only recognize RGM when used in Western blotting (Monnier et al., 2002).

All sections were incubated with 0.02% hydrogen peroxide for 10 min to block endogenous peroxidases and rinsed in PBS. Sections were then incubated with blocking solution [PBS containing 0.2% Triton X-100 and 10% normal goat serum (NGS)] for 30 min. The RGMA Ab was diluted 1:500 in PBS, 0.1% Triton X-100, and 1% NGS, and sections were incubated at 4°C overnight. After washing with

PBS, sections were incubated with a secondary Ab (biotinylated goat anti-rabbit IgG, diluted 1:250) with 1% NGS and 0.1% Triton X-100 for 2 hr at room temperature. Sections were then washed and transferred to ABC solution (ABC-Elite, Vector Laboratories, Burlingame, CA) for 2 hr. After final washes in PBS, sections were stained with 0.07% 3,3'-diaminobenzidine tetrahydrochloride (DAB) activated with 0.001% hydrogen peroxide in PBS, mounted onto gelatin-coated slides dehydrated through an ascending series of ethanol, and coverslipped. Sections were digitally photographed (Olympus BX-50).

### RGMA-alkaline phosphatase fusion protein binding

RGMA-alkaline phosphatase (RGMA-AP) binding to receptor molecules was assayed using a ligand-affinity probe. Chicken RGMA-AP, which consisted of the extracellular domain of RGMA, was fused in-frame with a heat-stable human placental alkaline phosphatase (Genhunter). To detect expression of RGMA receptor proteins, mouse sections (20  $\mu$ m thickness) from P1 were prepared on a cryostat and fixed with methanol at  $-80^{\circ}$ C for 5 min. After the sections were rehydrated in PBS, they were equilibrated in HBSS without  $\text{Ca}^{2+}/\text{Mg}^{2+}$  for 5 min and incubated in HBSS supplemented with 20% fetal calf serum (FCS) for 2 hr. The sections were then overlaid with concentrated conditioned medium containing the recombinant protein diluted in HBSS plus 20% FCS for 90 min. After one wash with HBSS and three washes with TBS (20 mM Tris-HCl, 135 mM NaCl, pH 7.5) for 5 min each, the sections were equilibrated with PBS for 5 min and fixed in 3.7% formaldehyde in PBS for 5 min. After one wash with PBS, endogenous phosphatases were heat-inactivated at 65°C for 50 min. After equilibrating with AP-buffer (100 mM Tris, 100 mM NaCl, 5 mM  $\text{MgCl}_2$ , pH 9.5), bound RGMA-AP fusion proteins were visualized with a staining solution containing 34 mg/ml Nitro-blue-tetrazolium and 18 mg/ml 5-bromo-4-chloro-3-indolylphosphate (Boehringer Mannheim, Mannheim, Germany) in AP-buffer. The specificity of the RGMA-AP binding was determined by

competition through an excess of unlabeled RGMA peptide, which abolished the binding.

#### Explant preparation

To collect embryonic tissue from E18.5, pregnant mice were anesthetized with Ketamin (5 mg/100 mg), and embryos were removed from the uterus. Embryos were placed in cold HBSS medium (Invitrogen, Gaithersburg, MD) and decapitated, and hippocampi were dissected. Entorhinal explants were microdissected out with tungsten needles under binocular optics with 40 $\times$  magnification. The explants were placed in suspension culture in a 5.5% CO<sub>2</sub> humidified incubator in Neurobasal medium (Invitrogen) supplemented with 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 10% heat-inactivated fetal bovine serum, and B27 supplement (Invitrogen) for 2 hr for equilibrium. Thereafter, entorhinal hippocampal explants were immediately used for the assays.

#### Explant outgrowth assay

Entorhinal cortex (EC) explants were plated on confluent layers of RGMA or mock-transfected cells. The total number of  $\beta$ -III tubulin-stained (TUJ-1 Ab, Convance) processes from the entorhinal explants was scored semiquantitatively.

#### Stripe assay

**Preparation of membranes.** Membrane suspensions were prepared from stably RGMA-transfected human embryonic kidney (HEK) 293 cells. All solutions used were sterile, 4°C, pH 7.4, and supplemented with protease inhibitors (protease inhibitor mixture; Sigma, St. Louis, MO). Cells were homogenized in homogenization buffer (10 mM Tris-HCl, pH 7.4, 1.5 mM CaCl<sub>2</sub>, and 1 mM spermidine) by pressing the tissue at least 10 times through a 27 ga, 1.5 syringe needles. The homogenate was layered on top of a step gradient of 50 and 5% sucrose and centrifuged for 10 min at 28,000  $\times$  g at 4°C in a TLS 55 rotor (Beckman Instruments, Fullerton, CA). The membrane fragments were collected from the interphase layer situated between 5 and 50% sucrose and washed with PBS. Membrane suspensions were adjusted to an optical density of 0.1 (measured at 220 nm).

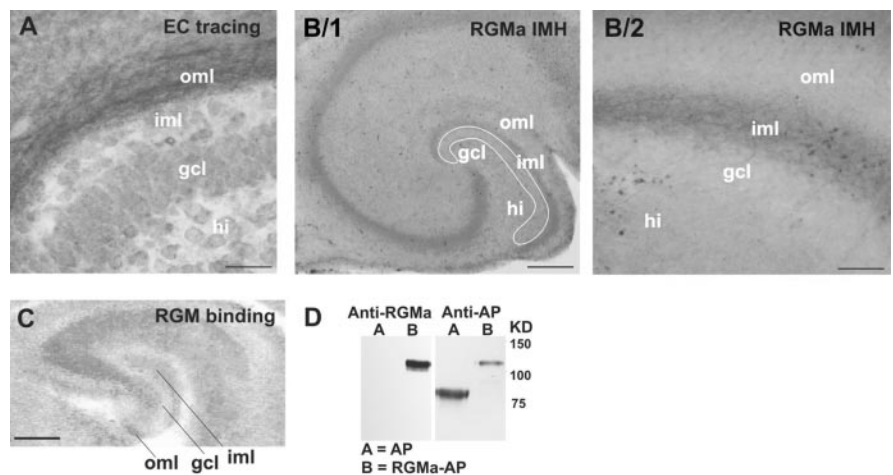
**Preparation of stripes.** Stripe preparation was performed according to the protocol described previously (Walter et al., 1987). Stripes were prepared with membranes obtained from either RGMA or mock-transfected HEK293 cells onto polyvinyl propylene filters with 0.4  $\mu$ m pores pre-coated with 20  $\mu$ g/ml laminin. The membrane carpets were then placed into cell culture inserts (Falcon) for 35 mm tissue culture dishes with 1.5 ml culture medium. Explants were positioned on the membrane carpets using sterile forceps. Cultures were maintained in a 5.5% CO<sub>2</sub> humidified incubator for 5 d.

#### Analysis of outgrowth preference

Neurites growing out from the explants were visualized by immunolabeling with a neurofilament Ab (Boehringer Mannheim) and subsequent DAB staining as described above. Culture medium was removed from the dishes 5 d after the explants were placed on the carpets. Neurite growth from the explants was examined and photographed by light microscope. Growth preferences for one or the other set of membrane stripes were evaluated using a three-class system: (1) clear-cut preference, with almost all of the fibers growing on one of the membrane lanes, (2) slight or moderate preference, with most fibers growing preferentially on one membrane lane, although others cross randomly, and (3) no choice or random outgrowth.

#### Preparation of organotypic slice cultures

Entorhinal cortex with the adjacent hippocampus was dissected from newborn mice (P0) and cut into 350  $\mu$ m sections with a tissue chopper.



**Figure 2.** Entorhinal cortex tracing, RGMA immunohistochemistry, and binding. *A*, The biocytin-labeled entorhinal projection shows its typical distribution in the dentate gyrus. Labeled axons are found exclusively in the outer molecular layer, whereas the inner molecular layer above the Nissl-stained somata of dentate granule cells in the hilus is free of any staining. *B1*, RGMA immunostaining of P7 rat labels fibers in the inner molecular layer of the dentate gyrus and cellular elements in the hilus. *B2*, This micrograph is a higher magnification of the dentate gyrus shown in *B1*. Note the intense staining of fibers belonging to the commissural-associational projection in the inner molecular layer. *C*, Localization of the RGMA receptor in the developing entorhino–hippocampal system at P1. In the hippocampus, RGMA–AP fusion protein binding was strongest in the outer molecular layer of the dentate gyrus and low in the inner molecular layer. Binding was also seen in the stratum radiatum–stratum lucidum. hi, Hilus; EC, entorhinal cortex; gcl, granule cell layer; oml, outer molecular layer; iml, inner molecular layer. *D*, An RGMA-specific Ab (anti-RGMA) recognizes the 113 kDa RGMA–AP fusion protein, whereas an alkaline phosphatase (anti-AP) Ab detects an 80 kDa protein. The size of the RGMA protein is 33 kDa. Scale bars: *A*, *B*, 50  $\mu$ m; *C*, 250  $\mu$ m.

These complex slices ( $n = 62$ ) were cultivated on porous membranes (Millicell, Millipore Corporation, Bedford, MA) for 7 d *in vitro* (DIV 7) and fed with medium as described previously (Ceranik et al., 1999). The cocultures were treated in different sets of experiments either with a functional RGMA Ab (1  $\mu$ g/ml) ( $n = 18$ ) or with phosphatidylinositol-specific phospholipase C (PI-PLC) (Sigma) at two different concentrations (5 and 10 U/ml) ( $n = 36$ ). Treatment of cultures was performed during the entire incubation period. During this time, medium and drugs were replaced every second day. No substances were added to control cultures ( $n = 8$ ). At DIV 7 the developed entorhino–hippocampal projection of all cultured slices was traced using biocytin. After reincubation for 24–36 hr to allow for anterograde transport of the tracer, the cocultures were fixed with 4% paraformaldehyde, 0.1% glutaraldehyde, and 15% saturated picric acid in 0.1 M phosphate buffer (PB) at pH 7.4 for 2 hr. Thereafter, all cocultures were resliced on a vibratome (50  $\mu$ m), permeabilized with 0.1% Triton X-100 for 30 min in PB, and incubated with ABC-Elite complex (1:50; Vector Laboratories) overnight at 4°C. Sections were developed using a nickel–cobalt-intensified DAB protocol (Schwab et al., 2000). Labeled sections were counterstained with cresyl violet, dehydrated, coverslipped with Entellan, and digitally photographed. A blinded descriptive semiquantitative analysis of the entorhino–hippocampal projection was performed by three independent investigators using the following criteria: ++, layer-specific termination, +, layer-specific termination with some aberrant fibers, –, complete loss of layer specificity.

For blocking experiments, a chicken RGMA peptide spanning residues 195–349 was used to generate rabbit antibodies. For Ab purification, sera were applied to a protein-G-agarose column (Amersham Biosciences). After extensive washing with PBS, bound IgG was eluted with 0.1 M glycine, 0.15 M NaCl, pH 2.5, immediately neutralized, and dialyzed against PBS.

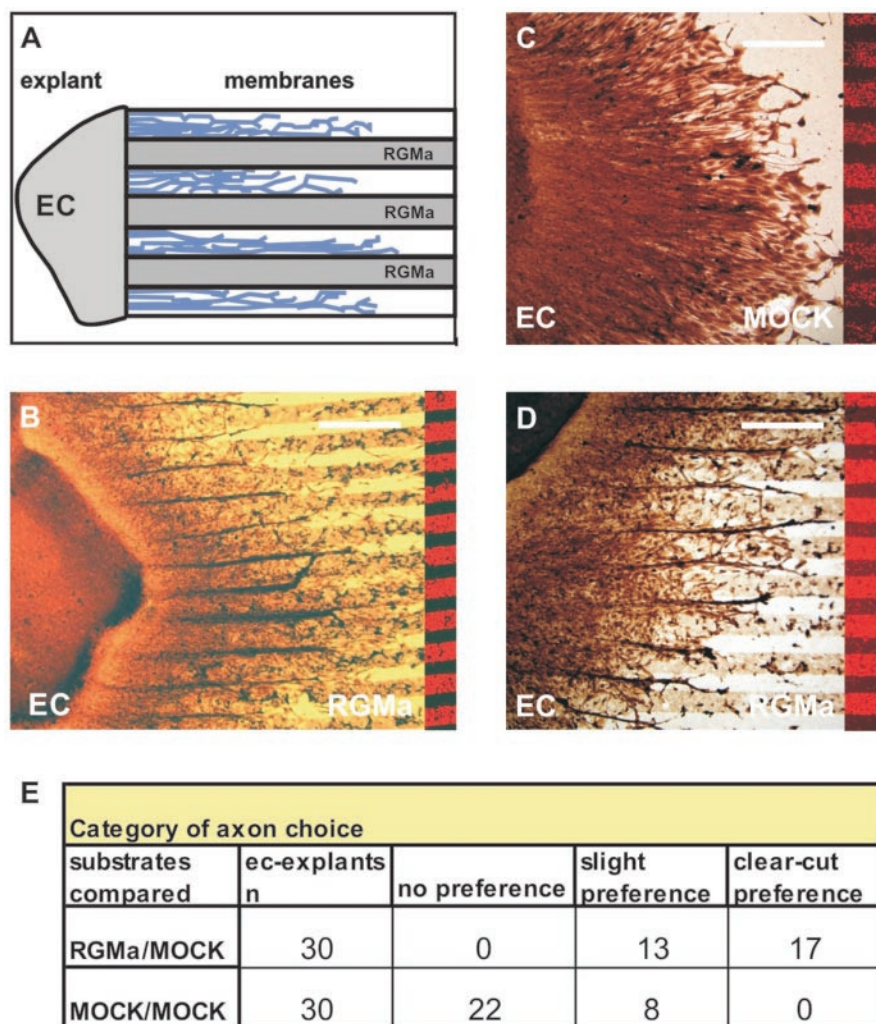
## Results

### RGMA is expressed predominantly in the hippocampus during perinatal stages of development

To determine whether RGMA is expressed during development of the CNS, we examined the sites of expression and applied Northern blotting, *in situ* hybridization, and immunohistochemistry.

Northern blot analysis of RGMA in the mouse CNS detected





**Figure 3.** The growth preference of entorhinal neurites is influenced by RGMa. *A*, Schematic illustration of neurites avoiding RGMa lanes on a membrane carpet growing out of an entorhinal cortex explant. *B*, *D*, Entorhinal neurites of E19 explants avoid RGMa membrane lanes but do not display any preference when offered a choice of membrane lanes from mock-transfected cells (*C*). *E*, Table illustrating categories of axon choice from entorhinal neurites on RGMa and control substrates. Scale bars: *B*, 250  $\mu$ m; *C*, *D*, 100  $\mu$ m.

two bands of 5.8 and 6.8 kb with molecular weight similar to the bands detected in the chick tectum (Monnier et al., 2002) (Fig. 1*A*). In the hippocampus, strong hybridization signals were obtained at E18.5 and P5. During adulthood, the signal decreased to background levels.

To obtain further insight into the possible function of RGMa in the developing entorhino–hippocampal formation, the spatiotemporal distribution of the RGMa mRNA was investigated using a nonradioactive *in situ* hybridization. At E18.5, RGMa mRNA was strongly expressed in the parietal cortical plate, in the CA1 and CA3 regions and in the hilus of the hippocampus (Fig. 1*B*).

At P7, a strong Dig-labeling was observed, particularly in many large hilar neurons, presumably mossy cells (Fig. 1*D,E*). These cells give rise to the commissural projection to the inner molecular layer of the dentate gyrus. The RGMa signal was also found in the CA1 region, whereas other hippocampal cell layers were only faintly stained.

**Localization of the RGMa protein in the hippocampus**

On the basis of mRNA expression patterns in the developing hippocampal formation, the localization of the RGMa protein

was studied in detail. Immunostaining of the hippocampus derived from postnatal brain (P7) was most prominent in the inner molecular layer of the dentate gyrus (Fig. 2*B1,B2*). In this layer, axonal profiles appeared to be labeled, indicating an axonal localization of RGMa on commissural–associational fibers. The granule cell layer and the outer molecular layer, which is occupied by perforant path fibers, were devoid of any immunostaining.

**Localization of the RGMa ligand protein in the hippocampus**

Because RGMa was expressed in the inner molecular layer of the hippocampus, we went further on and studied the distribution of the RGMa receptor protein. To detect RGMa receptor binding in the hippocampus, we stained P1 mouse brain sections with a ligand-affinity probe, which consisted of the extracellular domain of RGMa fused in frame with a human placental AP (Fig. 2*C*). Binding in the hippocampus at P1 was most prominent in the dendritic fields of the CA3 and dentate gyrus. No staining was observed on principal neurons of the dentate gyrus and the hippocampus proper. The strongest RGMa-AP binding signal was observed in the marginal zone of the dentate gyrus, suggesting the presence of the corresponding receptor on entorhinal axons that have already entered the developing dentate gyrus. Binding also occurred in the stratum lucidum of CA3, the prospective termination zone of the mossy fiber tract, and in the stratum radiatum of CA3 and CA1 (Fig. 2*C*).

**Growth preference of EC neurites is controlled by RGMa**

We next examined whether recombinant RGMa is able to guide entorhinal axons *in vitro*. To address this question, the well characterized stripe assay was used. Growth cones were exposed to two kinds of stripes containing particles from RGMa- or mock-transfected cells. Entorhinal cortex explants obtained from E18.5 were allowed to grow on these carpets of alternating membrane stripes (Fig. 3). Outgrowing entorhinal neurites tended to fasciculate and clearly avoided membranes prepared from RGMa-expressing cells (Fig. 3*B,D*). In contrast, given the choice between a membrane lane from mock-transfected cells or laminin, embryonic entorhinal neurites did not display any preference and crossed lane borders freely (Fig. 3*C*). In all experimental groups, many migrating cells could also be observed, but these did not influence axonal outgrowth. These results support the finding that extending neurites from the entorhinal cortex are selectively repelled by RGMa. This effect was estimated semiquantitatively (Fig. 3*E*).

**Explant outgrowth assay**

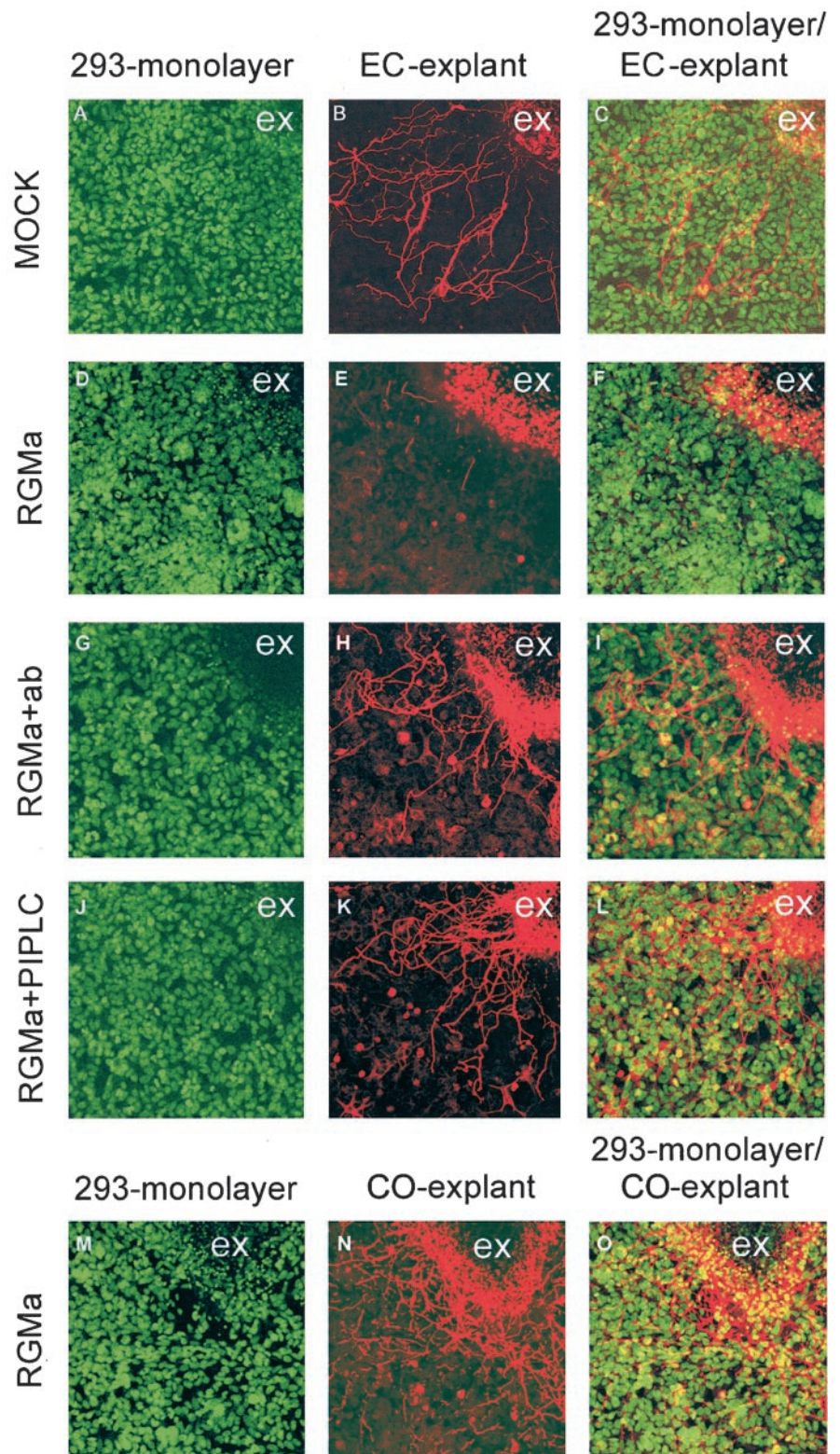
Entorhinal explants from E18.5 mouse brain were cultivated on a confluent monolayer of HEK293 cells expressing RGMa (*n* = 25) or transfected with the expression vector alone (*n* = 25) (Figs. 4,



5). The expression of the ligand was further confirmed by staining membrane extracts obtained from the RGMA-transfected cells with an RGMA-specific Ab, detecting a single band of the expected size (data not shown). In particular, the outgrowth of entorhinal neurites was strongly inhibited by RGMA (Fig. 4D–F) compared with the length of entorhinal explant outgrowth on mock-transfected HEK293 cells (Fig. 4A–C). In contrast, RGMA did not inhibit the neurite outgrowth from control explants ( $n = 15$ ) (Fig. 4M–O). The treatment of RGMA-transfected HEK293 cells with an RGMA-specific Ab (3  $\mu\text{g}/\text{ml}$ ) during entorhinal explant culture ( $n = 25$ ) completely abolished the outgrowth inhibitory effect of RGMA (Fig. 4G–I). Because RGMA can be removed from the membrane by PI-PLC treatment (1 U/ml), we analyzed the effect of PI-PLC on RGMA-transfected HEK293 cell inhibition of entorhinal neurite outgrowth ( $n = 25$ ). Removal of RGMA from the cell membranes restored neurite elongation from entorhinal explants (Fig. 4J–L). This observation suggests that RGMA selectively and specifically inhibits entorhinal axon outgrowth. The neurite outgrowth was analyzed semiquantitatively (Fig. 5).

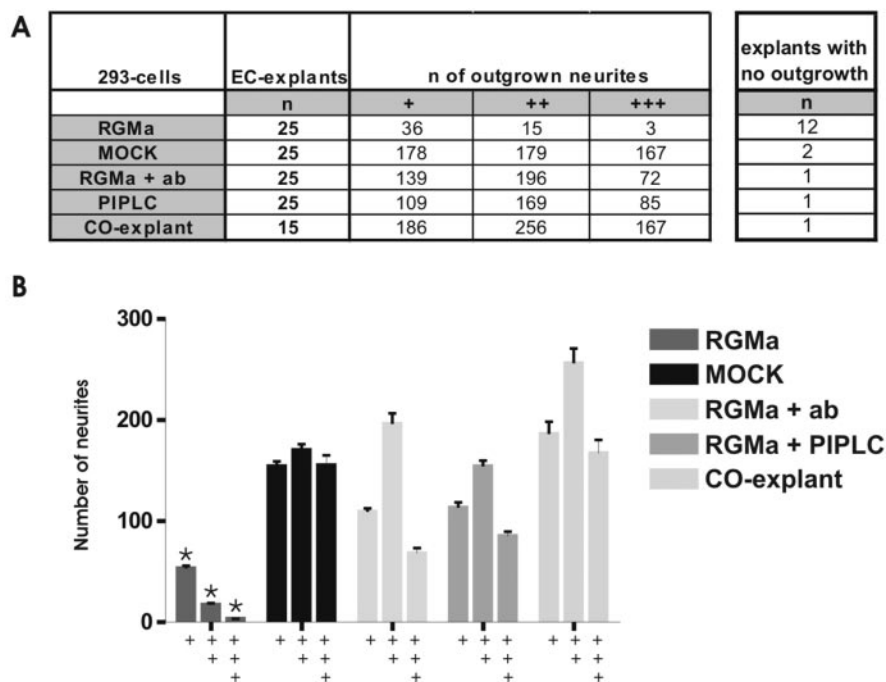
#### Blockade of RGMA leads to loss of target recognition of entorhinal axons

To address the function of RGMA in a more *in vivo*-like environment, organotypic entorhino–hippocampal complex cocultures taken at P0 were incubated for 8 d with either the enzyme PI-PLC, which cleaves the GPI-anchor, or a blocking Ab against RGMA. By use of the anterograde tracer biocytin, the entorhinal projections that had developed under control conditions and after drug treatment could be monitored in all cocultured slices (Fig. 6). Disruption of the RGMA function did not prevent entorhinal axons to find their correct route to the hippocampal target culture; however, it led to failure of entorhinal axons to recognize their appropriate target zone in the dentate gyrus. Axons were no longer confined to the outer molecular layer and invaded the inner molecular layer (Fig. 6D,E). These changes were similar at all tested concentrations of PI-PLC, as illustrated in the diagram in Figure 6F. Furthermore, when incubated with the neutralizing Ab, the entorhinal projection was massively altered, and numerous biocytin traced fibers not only invaded the inner molecular layer but also passed the granule cell layer and spread throughout the hilar region of the hippocampus. (Fig. 6E). More than 50% of all RGMA Ab-treated slices developed a completely perturbed entorhinal projection (Fig. 6G). In the control cocultures,



**Figure 4.** Outgrowth of entorhinal neurites is inhibited by RGMA. E18.5 entorhinal cortex explants were supercultivated on a confluent layer of 293 mock-transfected (A–C), RGMA-transfected (D–F), or RGMA-transfected cells treated with an RGMA Ab (G–I) or PI-PLC (1 U/ml) (J–L). Neurites were stained with  $\beta$ -III tubulin Ab (red), and cells were counterstained with Sytox-Green.

biocytin-traced entorhinal projection showed an intense labeling in the correct termination zone, the outer two-thirds of the molecular layer of the dentate gyrus (Fig. 6C). A few biocytin-labeled fibers were seen in the inner molecular layer.



**Figure 5.** Quantification of entorhinal neurite outgrowth as presented in Figure 4. 0 = no outgrowth; + = low outgrowth ( $\sim 100 \mu\text{m}$ ); ++ = moderate outgrowth ( $\sim 200 \mu\text{m}$ ); +++ = strong outgrowth ( $\sim 500 \mu\text{m}$ ); error bars indicate SEM; RGMa compared with all other groups  $p < 0.005$  (ANOVA; Mann–Whitney  $U$  test).

## Discussion

The formation of afferent connections in the dentate gyrus is controlled by an assembly of different guidance molecules. In this study we were able to identify RGMa, a novel GPI-linked axon guidance molecule in the developing entorhino–hippocampal system. *In vitro* RGMa exerts a repellent action on outgrowing entorhinal axons. RGMa mRNA expression was found in the cornu ammonis of the hippocampus and in the hilar region, the origin of the commissural–associational projection to the inner molecular layer of the dentate gyrus. Immunohistochemistry for RGMa showed that the protein is present in the inner molecular layer, presumably on commissural–associational hilar mossy cell axons. In contrast, strong RGMa binding was present in the outer molecular layer, the termination zone of the entorhinal projection. Disruption of the RGMa function led to a perturbed laminar innervation of the entorhinal projection in organotypic slice culture experiments. This is of great interest because the precise laminar segregation of extrinsic and intrinsic afferents, a common feature of the synaptic architecture in the hippocampus, is an accepted model for studying the molecular mechanisms underlying the wiring of the cortex.

In the hippocampus, there is a sequential ingrowth of afferents into the dentate gyrus as entorhinal axons precede and RGMa-positive commissural–associational axons follow (Frotscher and Heimrich, 1993; Super and Soriano, 1994). The functional stripe and outgrowth assays demonstrate a repulsive effect of RGMa on entorhinal axons. Thus, first, entorhinal axons that arrive at E19 in the RGMa-free dentate gyrus are not repelled but are enabled to occupy their target area later in development, at P2, when commissural–associational axons invade the inner molecular layer of the dentate gyrus. This is accompanied by the expansion of RGMa receptor-loaded entorhinal fibers toward the inner molecular layer, which might be prevented by RGMa expressed on the commissural–associational axons. This subsequent appear-

ance of the RGMa-containing axons suggests that in the developing dentate gyrus, RGMa is involved in both the maintenance and stabilization of the earlier-formed laminated entorhinal projection via its repulsive action on these fibers. This hypothesis is supported by the fact that blocking the RGMa protein or cleavage of the GPI anchor resulted in a remarkable loss of the layer-specific termination of entorhinal afferents in the dentate gyrus. This is demonstrated by an aberrant ingrowth of entorhinal fibers into the inner molecular layer of the dentate gyrus after PI-PLC treatment and even further into other hippocampal subfields as observed after incubation of the cocultures with the neutralizing RGMa Ab.

Our results together with previous studies by Pozas et al. (2002) and Gu et al. (2003) indicate that a network of guiding and target-related molecules are involved in the laminar segregation of EC projections to the hippocampus. Moreover, these studies suggest that EC neurons respond differentially to guidance cues: although RGMa seems to be involved in the laminar segregation pattern of entorhinal fibers terminating in the outer molecular

layer of the dentate gyrus, semaphorin (Sema) 3a seems to be involved mainly in the guidance of entorhinal projections toward the stratum lacunosum moleculare of the CA1 region, as shown in Sema 3a  $-/-$  mice (Pozas et al., 2002).

It is well known that the laminar termination of afferents in the marginal zones of the hippocampus and the dentate gyrus is a suitable model for the analysis of pathfinding, target recognition, and sprouting processes after denervation (Li et al., 1993, 1994; Woodhams and Atkinson, 1996; Del Rio et al., 1997; Frotscher, 1997). The molecular signals necessary for the formation of the normotypic entorhino–dentate projection are present in hippocampal tissue maintained in organotypic coculture with explants of the EC (Li et al., 1993, 1994; Woodhams and Atkinson, 1996; Frotscher, 1997). These experiments show that lamina-specific molecules in the hippocampal fields play important roles in the formation of layer-specific afferent projections; however, none of these studies provided information on the mechanisms guiding entorhinal axons along their normal pathways and into their target fields.

Ablation of reelin-secreting hippocampal Cajal–Retzius cells (CR) prevents entorhinal fiber ingrowth into the hippocampus *in vitro* (Borrell et al., 1999). In the hippocampus of *reeler* mice lacking reelin, entorhinal fibers terminate with correct laminar specificity, indicating that the positioning of CR cells plays a key role in guiding entorhinal axons to their target layer (Förster et al., 1998; Borrell et al., 1999; Deller et al., 1999). Furthermore, experiments with membrane preparations indicate the presence of adhesive, attractive, and repulsive properties in entorhino–hippocampal tissue underlying the formation of the layered termination (Skutella et al., 1999; Förster et al., 2001). On a molecular level, the laminated distribution of hippocampal afferents is probably the result of a cooperation between attractive and repulsive guidance factors. Previously, this function was attributed to semaphorins–plexins–neuropilins (Chen et al., 2000; Cheng et



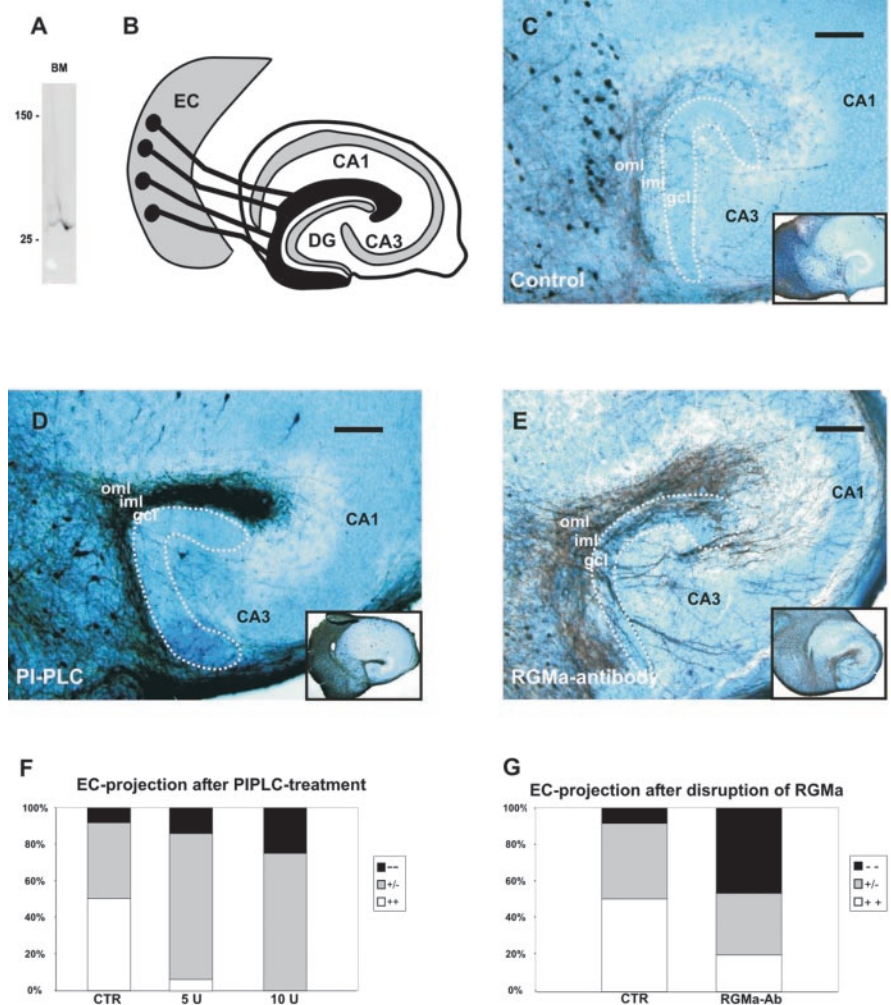
al., 2001; Pozas et al., 2002; Gu et al., 2003), the slits-robo system (Nguyen Ba-Charvet et al., 1999), netrins (Steup et al., 2000; Heimrich et al., 2002), ephrin ligands, Eph receptor tyrosine kinases (Stein et al., 1999), and ECM molecules (Förster et al., 2001; Zhao et al., 2003).

The expression pattern of RGMA in the hippocampal formation provides the first evidence that this molecule might be involved in the targeting and wiring of intra-hippocampal projections; however, it will require further investigation to determine in more detail whether subpopulations of hippocampal neurons respond differentially to this guidance cue.

In summary, our observations indicate that RGMA, first described in the tectal system of chicken, has a similar function as a repulsive signal in the rodent cortex. A close characterization of RGMA therefore improves our understanding of the formation of hippocampal connections, especially the stabilization of the laminar organization of afferent fiber systems in the dentate gyrus. This will increase our knowledge of the molecular mechanisms of axon guidance and target recognition of cortical neurons.

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**Figure 6.** RGMA controls targeting of entorhinal fibers in organotypic slice culture. *A*, To ensure Ab specificity, Western blotting was performed on membrane extracts. The Ab recognizes a 33 kDa band, which is the size of RGMA. *B*, Schema of the entorhino-hippocampal coculture approach. The anterograde tracer biocytin was placed onto the superficial layers of the entorhinal cortex explant. *C*, Biocytin-traced control coculture at DIV 9. Labeled entorhinal axons had developed their normal termination pattern and are almost confined to the outer molecular layer (oml) of the dentate gyrus. The termination zone of the commissural-associational projection, the inner molecular layer (iml), is obviously free of labeled entorhinal fibers. *D*, A PI-PLC-treated coculture reveals that an entorhino-hippocampal projection has developed. Note that labeled entorhinal axons are no longer confined to their normal termination zone. Entorhinal axons have invaded the entire marginal zone (iml + oml) of the dentate gyrus. *E*, An example of an entorhino-hippocampal coculture incubated with the RGMA Ab. Biocytin-traced entorhinal axons show a massive aberrant projection with misrouted fibers, which can be followed into various hippocampal subfields. *F*, Descriptive semiquantitative analysis of the entorhinal projection after PI-PLC treatment. Note the complete loss of the layer-specific termination of labeled fibers after treatment with 10 U PI-PLC. *G*, Descriptive semiquantitative analysis of the entorhinal projection after incubation with a specific Ab against RGMA. Approximately 50% (black bar) of all treated cultures show a massive aberrant entorhinal projection. CA1, CA3 hippocampal subfields; gcl, granule cell layer; ++, layer specific termination; +/–, layer specific termination with some aberrant fibers; – –, complete loss of layer specificity. Scale bars: *C–E*, 100  $\mu$ m.

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