

## Semaphorins III and IV repel hippocampal axons via two distinct receptors

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### SUMMARY

The semaphorins are the largest family of repulsive axon guidance molecules. Secreted semaphorins bind neuropilin receptors and repel sensory, sympathetic and motor axons. Here we show that CA1, CA3 and dentate gyrus axons from E15-E17 mouse embryo explants are selectively repelled by entorhinal cortex and neocortex. The secreted semaphorins Sema III and Sema IV and their receptors Neuropilin-1 and -2 are expressed in the hippocampal formation during appropriate stages. Sema III and Sema IV strongly repel CA1, CA3 and dentate gyrus axons; entorhinal axons are

only repelled by Sema III. An antibody against Neuropilin-1 blocks the repulsive action of Sema III and the entorhinal cortex, but has no effect on Sema IV-induced repulsion. Thus, chemorepulsion plays a role in axon guidance in the hippocampus, secreted semaphorins are likely to be responsible for this action, and the same axons can be repelled by two distinct semaphorins via two different receptors.

Key words: Semaphorin, Neuropilin, Hippocampus, Cortex, Mouse

### INTRODUCTION

Mounting evidence indicates that in the developing central nervous system, growth cones can be guided at a distance by diffusible molecules secreted by non-target cells (Tessier-Lavigne and Goodman, 1996). Many of these factors function as chemorepellents: they induce growth cone collapse and oriented axonal outgrowth away from the source of the factor. Since the demonstration 5 years ago by Pini (1993) that the septum in the embryonic rat produces a diffusible factor that repels olfactory bulb axons, several studies have indicated that chemorepulsive molecules are produced in a variety of central nervous system (CNS) regions, such as the ventral spinal cord (Fitzgerald et al., 1993), the floor plate (Colomarino and Tessier-Lavigne, 1995; Tamada et al., 1995; Varela-Echavarría et al., 1997) or the thalamus (Tuttle et al., 1998). Potential chemorepellents have been identified in two gene families, the netrins and the semaphorins (Culotti and Kolodkin, 1996; Tessier-Lavigne and Goodman, 1996). Floor plate-derived Netrin-1 has been shown to repel motor axons from the trochlear, trigeminal, facial and glossopharyngeal cranial nuclei (Colomarino and Tessier-Lavigne, 1995; Varela-Echavarría et al., 1997). Most of the secreted chemorepellents identified to date belong to the semaphorin family, which also contains transmembrane members (Kolodkin et al., 1993; Mark et al., 1997). Sema III/D (hereafter referred to as Sema III) and its avian ortholog Collapsin-1, which is expressed in the embryonic ventral spinal cord, are known to cause in vitro the collapse of sensory axons growing from explants of dorsal root

ganglia (Luo et al., 1995) and can also repel these axons (Messersmith et al., 1995; Püschel et al., 1995). Sema III has also been reported to act as a chemorepellent for most rat cranial motor axons (Varela-Echavarría et al., 1997) and for olfactory axons (Kobayashi et al., 1997). Two other secreted semaphorins, Sema A and Sema E/Collapsin-3, can repel and cause the collapse of axons from sympathetic ganglia (Adams et al., 1997; Koppel et al., 1997). Recently, the transmembrane protein Neuropilin-1 has been shown to be a receptor, or a component of a receptor, for Sema III (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Other studies have demonstrated that the secreted semaphorins Sema E/collapsin-3, Collapsin-2, Collapsin-5 and Sema IV also bind to Neuropilin-1 with apparently equivalent affinities (Chen et al., 1997; Feiner et al., 1997). Neuropilin-2, a homolog of Neuropilin-1, exists in at least six isoforms, that are probably generated by alternative splicing (Chen et al., 1997; Kolodkin et al., 1997). Neuropilin-2 can bind Sema E and Sema IV, but not Sema III with high-affinity (Chen et al., 1997) but the involvement of Neuropilin-2 in mediating a semaphorin response remains to be demonstrated.

Although semaphorins and their receptors are expressed in many brain regions during development, functional responses have been reported only for sensory, sympathetic and motor axons (see above for references). In the present study we have investigated the role of diffusible semaphorins in the patterning of the main hippocampal connections. We have selected this region not only because Neuropilin-1 (Kawakami et al., 1996), Neuropilin-2 (Chen et al., 1997) and several semaphorins (Zhou

et al., 1997; Skaliora et al., 1998) are expressed in this area, but also because the hippocampus has a simple pattern of afferent connections. The main afferents arise from the entorhinal cortex and from the CA3/hilar hippocampal fields, and terminate in a layer-specific fashion. Entorhinal axons, through the perforant pathway, innervate the outer dendritic segments of the principal neurons (the stratum lacunosum-moleculare and the outer molecular layer), and commissural/associational afferents from CA3/hilar regions innervate inner dendritic segments in the stratum oriens, stratum radiatum and inner molecular layer (see Fig. 1A; Amaral and Witter, 1995).

Tracing studies in mouse embryos have shown that developing hippocampal afferents invade their appropriate target region and layers in a highly specific fashion (Supèr and Soriano, 1994; Supèr et al., 1998). Such stereotyped growth suggests the involvement of both long-range cues influencing early axonal trajectories, and membrane- or substrate-anchored cues, providing layer-specific positional information. Analyses of mutant mice indicate that the diffusible factor Netrin-1 and its receptor, Deleted in Colorectal Cancer, are involved in the formation of commissural connections (Serafini et al., 1996; Keino-Masu et al., 1996). In addition, it has been shown that the Cajal-Retzius cells and the extracellular protein Reelin regulate the elaboration and targeting of entorhinal afferents (Del Rio et al., 1997; Supèr et al., 1998; Borrell et al., unpublished). In the present study, we show in the entorhinal cortex and neocortex of mouse embryos the presence of long-range repellent cues, which repel hippocampal axons. We also demonstrate that several members of the semaphorin and neuropilin families are expressed in the hippocampal formation when connections are being formed. We further show that the secreted semaphorins *Sema III* and *Sema IV*, but not *Sema A*, *Sema E* or *Sema H*, exert potent repulsive effects on hippocampal axonal growth. Finally we found that *Sema III*-induced repulsion is blocked by anti-Neuropilin-1 antibodies, which have no apparent effect on *Sema IV*-induced repulsion.

## MATERIALS AND METHODS

### Animals

OF1 albino mice (IFFA-Credo, Lyon, France) were used for the culture experiments and for *in situ* hybridization studies. The day on which a vaginal plug was detected was considered embryonic day 0 (E0), and the day of birth postnatal day 0 (P0).

### In situ hybridization

E15-E18 embryos and P0 mice (2-3 animals each) were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PFA). Brains were postfixed in 4% PFA, cryoprotected with 30% sucrose, and sectioned at 50  $\mu$ m. Antisense riboprobes were labelled with digoxigenin-d-UTP (Boehringer-Mannheim) by *in vitro* transcription of mouse cDNAs encoding *netrin-1* (Keino-Masu et al., 1996), *sema III* (Messersmith et al., 1995), *sema IV* (a kind gift of Dr Harry Drabkin), *sema E* (Chen et al., 1997), *sema A* (gift of Dr Andreas Püschel; Püschel et al., 1995) and *sema H* (a gift of Dr Christensen; Christensen et al., 1998), or *neuropilin-1* (He and Tessier-Lavigne, 1997) and *neuropilin-2* (Chen et al., 1997). *In situ* hybridization was performed on free-floating sections as described elsewhere (de Lecea et al., 1997). Controls including hybridization with sense riboprobes prevented hybridization signals.

### Explant cultures and cocultures

The hippocampus, entorhinal cortex and dorsal parietal cortex, all

from E15-E17 embryos, were dissected out in a single piece, and 250-350  $\mu$ m thick horizontal slices were obtained using a tissue chopper. Selected slices containing the hippocampal region were further dissected using fine tungsten needles to get small tissue pieces (approximately 300-500  $\mu$ m across) from the entorhinal cortex, dentate gyrus, and CA3 and CA1 areas; in most cases the fimbria and the marginal zone were taken out and excluded from the explants (see Fig. 1B). Other brains were sectioned coronally to obtain neocortical explants. Cocultures consisted either of all combinations between entorhinal cortex, CA3, CA1, dentate gyrus and neocortex, or entorhinal cortex, CA3, CA1 and dentate gyrus cocultured with aggregates of COS cells transfected with secreted alkaline phosphatase (AP), using the AP-Tag-4 vector (gift of Dr J. Flanagan), or *Human sema III-myc* (Messersmith et al., 1995), *Human sema IV-AP* (Chen et al., 1997), *mouse sema E-AP* (Chen et al., 1997), *mouse Sema A-myc* or *mouse Sema H-myc*. Those last two constructs were obtained by amplifying by PCR cDNAs encoding *mouse sema A* and *sema H*, then subcloned into PCDNA3.1 myc/his (Invitrogen). Expression was controlled on western blots using the monoclonal 9E10 anti-myc antibody or a polyclonal anti-AP antibody (Dako). Explants were embedded in rat-tail collagen gel as previously described (Lumsden and Davies, 1986), and cultured for 48-72 hours in Neurobasal medium supplemented with L-Glutamine, NaHCO<sub>3</sub>, D-glucose and B27 supplement (all from Gibco Life Technologies). Cocultures were incubated either with 5% or 1% horse serum, or serum-free, in a 5% CO<sub>2</sub>, 95% humidity incubator at 37°C. No differences were observed whether explants were cultured in 1% or 5% serum or in serum-free medium, so most cocultures were incubated in a low-serum medium.

Explants were fixed in ice-cold 4% PFA. For the visualization of neuronal processes, cultures were fixed for 1 hour, rinsed several times in 0.1 M PBS, blocked with 10% normal goat serum, incubated with a neuron-specific anti-class III  $\beta$ -tubulin antibody (1:3000; clone TUJ-1, Babco; Moody et al., 1989), followed by an HRP-conjugated goat anti-mouse antibody (1:2000; Jackson Immunological Laboratories), and developed with a diaminobenzidine reaction. Other cocultures were kept in 4% PFA and one explant in each pair was injected with a small crystal of lipophilic tracer DiI (1,1' dioctadecyl-3,3,3',3' tetramethylindocarbocyanine; Molecular Probes). After 4-6 days in the dark to allow the diffusion of the tracer, explants were recorded and photographed under rhodamine fluorescence optics.

For function-blocking experiments, explants were cultured in the presence of 10  $\mu$ g/ml of anti-Neuropilin-1 immunoglobulins (IgG). At such a concentration this antibody has been previously shown to abolish completely *Sema III*-induced collapse of DRG axons (He and Tessier-Lavigne, 1997). For control experiments either no IgGs were added, or some FitC-conjugated rabbit IgGs (Jackson Immunological Laboratories) at a similar concentration.

### Quantification

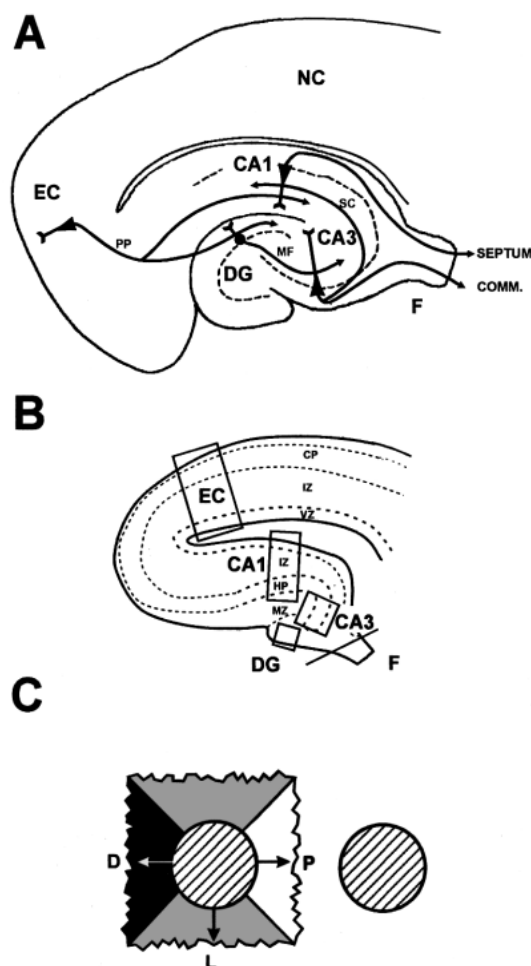
After  $\beta$ -tubulin immunostaining or DiI labeling the neurite length of the explants was measured in the proximal, distal and lateral quadrants (four different axons among the longest ones were measured in each quadrant for each culture) using a millimetric eyepiece (Messersmith et al., 1995). Data were statistically analyzed using the LSD test, Student's *t*-test or Mann-Whitney Rank Sum test. Individual cultures were additionally classified as described in Table 1.

## RESULTS

### Hippocampal axons are repelled by diffusible factors released from the entorhinal cortex

To investigate the existence of long-range repulsive influences in the growth and patterning of hippocampal connections we took advantage of the assay system in three-dimensional

collagen gels (Lumsden and Davies, 1986) and cocultured explants from the entorhinal cortex and from several hippocampal regions (Figs 2, 3; Table 1). We selected E15-E17 hippocampal tissue because these stages correspond to the



**Fig. 1.** Schematic illustrations of the pattern of hippocampal connections, the explant dissection procedure used, and the explant coculture assay. (A) Representation of the adult hippocampal formation showing the main hippocampal connections and neuronal cell types. The entorhino-hippocampal axons (the perforant pathway, PP) arise in the upper layers of the entorhinal cortex (EC) and project to pyramidal neurons in the CA1 and CA3 regions and to granule cells in the dentate gyrus (DG). Granule cell axons, the mossy fibers (MF), synapse on CA3 pyramidal neurons. These neurons, in turn, give rise to the commissural pathway (COMM.) via the fimbria (F) and to associational connections innervating the CA1 region (Schaffer collaterals, SC). Pyramidal neurons in the CA1 subfield project to the septal area among other fields. NC, neocortex. (B) Diagrammatic representation of the murine embryonic hippocampus (E16-E17) illustrating the areas from which the hippocampal and entorhinal explants were dissected out. The following embryonic layers are indicated: CP, cortical plate; VZ, ventricular zone; IZ, intermediate zone; HP, hippocampal plate; MZ, marginal zone; F, fimbria. (C) Schematic representation of the coculture assay used and method of analysis. Tissue explants were cocultured at a distance in collagen gel for 2-3 days. After  $\beta$ -tubulin immunocytochemistry or DiI injection in one of the two explants, axonal outgrowth (neurite length) was measured in the proximal (P), lateral (L) and distal (D) quadrants.

period of maximal axonal growth (see Discussion). In most cases, the marginal zone-layer I was dissected out since in preliminary control experiments we found that this procedure enhances radial axonal outgrowth from the explants. Also, the marginal zone-layer I, where the Cajal-Retzius cells are located, may exert chemoattractive influences which could interfere with the assay (Del Río et al., 1997; Soriano et al., 1997; J. A. D. R., V. B. and E. S., unpublished observations).

In a first set of experiments we confronted explants from the entorhinal cortex with explants from the hippocampal regions CA3, CA1 or dentate gyrus, and monitored entorhinal axonal outgrowth by injections of DiI. Axonal outgrowth from entorhinal cortex explants was predominantly radial (36 out of 45; Figs 2A,B, 3A; Table 1) although in some cocultures entorhinal axons exhibited a weak attraction for CA3 and CA1 tissues (9 out of 45). This chemoattraction may be due to the persistence of Cajal-Retzius cells in the explants (see above). We never found entorhinal axons being repelled by hippocampal explants (Table 1). We thus conclude that embryonic hippocampal tissue does not express long-range repellent signals for entorhinal axons.

We next cocultured entorhinal cortex and hippocampal explants and monitored the axonal outgrowth from the hippocampal tissue (Figs 2C-E, 3B; Table 1). As illustrated in Fig. 2C-E, the axons from the CA3 and CA1 hippocampal subfields grew far away from the entorhinal explants. All cocultures ( $n=36$ ) showed a dramatic reduction in the length of the fibers that were confronted with entorhinal explants. In contrast, the length of hippocampal axons in the quadrant distal to the entorhinal cortex averaged a 8- to 10-fold increase (Fig. 3B). A similar, albeit less pronounced, asymmetrical pattern of axonal growth was observed for the axons of the dentate gyrus when this region was confronted with the entorhinal cortex

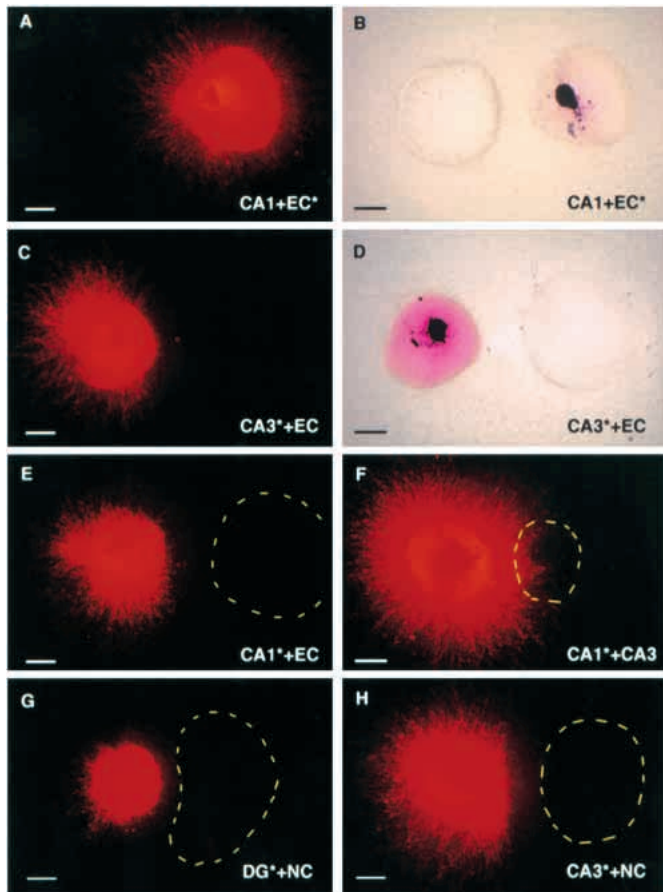
**Table 1.** Semiquantitative evaluation of axonal outgrowth in different combinations of hippocampal explants cocultured in collagen gels

Culture condition	Number of explants	Axonal outgrowth			
		+	=	-	—
<b>EC*</b> -CA3	13	2	11	-	-
<b>EC*</b> -CA1	17	3	14	-	-
<b>EC*</b> -DG	15	4	11	-	-
<b>CA3*</b> -EC	19	-	-	-	19
<b>DG*</b> -EC	14	-	3	5	6
<b>CA3*</b> -CA1	8	1	6	1	-
<b>CA1*</b> -CA3	10	1	6	1	-
<b>CA3*</b> -DG	12	-	12	-	-
<b>DB*</b> -CA3	11	2	9	-	-
<b>CA1*</b> -DG	13	-	11	2	-
<b>DG*</b> -CA1	12	-	11	1	-

Axonal growth was monitored by injection of DiI into one of the explants (labelled in bold letters and by an asterisk).

EC, entorhinal cortex; CA1, CA3, hippocampal fields CA1 and CA3; DG, dentate gyrus.

Cocultures were classified as follows: +, moderate attraction of axons (axons are 2- to 3-fold longer in the proximal than in the distal quadrant); =, radial axonal growth (axons in the proximal and distal quadrants differed by less than 2-fold in length); -, moderate axonal repulsion (axons are 2- to 3-fold longer in the distal than in the proximal quadrant); —, strong axonal repulsion (axons are more than 3-fold longer in the distal than in the proximal quadrant).



**Fig. 2.** Entorhinal cortex and neocortex release diffusible factors that repel hippocampal axons. In all figures the asterisk indicates the explant that has been injected with DiI, and in (E-H) the yellow dotted line delimits the contour of the unlabelled explant. (A) Dark-field and (B) bright-field microscopy of collagen coculture of E17 mouse CA1 and entorhinal cortex (EC) explants. A DiI crystal (black spot in B) has been placed in the entorhinal explant. Entorhinal axons grow symmetrically. (C) Dark-field and (D) bright-field microscopy of coculture of E17 mouse CA3 and entorhinal cortex explants. DiI-labelled CA3 axons are repelled by entorhinal cortex. (E) The repulsive action of E17 entorhinal cortex on CA1 axons. (F) CA1 axons are not repelled by CA3 axons. The E17 neocortex has a repulsive action on dentate gyrus (G) and CA3 (H) axons. All photographs were taken after about 2 days in vitro. Bars, 150  $\mu$ m.

(Fig. 3B; Table 1). Such a mild repulsion could be related to the late development of the dentate gyrus, or to the presence of distinct projection neurons, the granule cells and the commissural hilar neurons, which may have different responses. These findings indicate that the entorhinal cortex secretes diffusible signals that repel developing hippocampal axons.

Axons from the CA3 pyramidal neurons form the commissural pathway but they also give rise to ipsilateral associational connections (the Schaffer collaterals) that innervate the CA1 region (Fig. 1A). In contrast, axons from the CA1 pyramidal cells extend into the fimbria to innervate the septal region, but do not project to the CA3 subfield. Moreover, granule cell axons, the mossy fibers, innervate the CA3 pyramidal neurons but do not enter the CA1 subfield (Amaral

**Table 2. Semiquantitative evaluation of axonal outgrowth in different combinations of hippocampal and neocortical explants**

Culture condition	Number of explants	Axonal outgrowth			
		+	=	-	—
<b>EC*-NC</b>	14	1	4	5	4
<b>CA3*-NC</b>	10	—	—	1	9
<b>CA1*-NC</b>	6	—	—	—	6
<b>DG*-NC</b>	5	—	—	1	4
<b>NC*-EC</b>	12	1	10	1	—
<b>NC*-CA3</b>	6	1	3	2	—
<b>NC*-CA1</b>	6	1	3	2	—
<b>NC*-DG</b>	6	1	5	—	—

Axonal outgrowth was monitored by injection of DiI into one of the explants (labelled in bold letters and by an asterisk).

NC, neocortex; abbreviations and conventions as in Table 1.

and Witter, 1995). To examine whether chemorepellent cues released from the hippocampus itself influence the patterning of these connections we confronted explants from the CA1, CA3 and dentate regions. Most CA1, CA3 and dentate gyrus explants exhibited a radial axonal growth (Figs 2F, 3C; Table 1), indicating a lack of chemorepulsive or chemoattractive effects. These results suggest that the initial shaping of intrahippocampal ipsilateral connections does not depend on long-range repulsive factors.

To determine whether the chemorepellent effect we observed was caused by signals expressed only in the entorhinal cortex or present in other cortical regions, we cocultured CA3, CA1 and dentate explants with neocortical tissue (Figs 2G,H, 4A; Table 2). These experiments showed that the axons from all three hippocampal fields were similarly repelled when confronted with neocortical explants (21 out of 21). In contrast, neocortical axon outgrowth was radial when confronted with hippocampal explants (Fig. 4B; Table 2). Taken together, these findings indicate that both the entorhinal cortex and the neocortex release diffusible signals that repel embryonic hippocampal axons.

During development, entorhino-hippocampal afferents grow unidirectionally towards the hippocampus, without invading the adjacent neocortex. To determine whether this stereotyped axonal growth is influenced by repellent signals expressed in the neocortex, we cocultured entorhinal cortex and neocortex (Fig. 4B; Table 2). Whereas neocortical explants exhibited a radial axonal growth, entorhinal axonal outgrowth was repelled by neocortical explants (9 out of 14; Fig. 4B), indicating that long-range repulsive signals may prevent the ingrowth of entorhinal axons in the adjacent neocortex.

### Expression of secreted semaphorins and neuropilins in the developing hippocampus

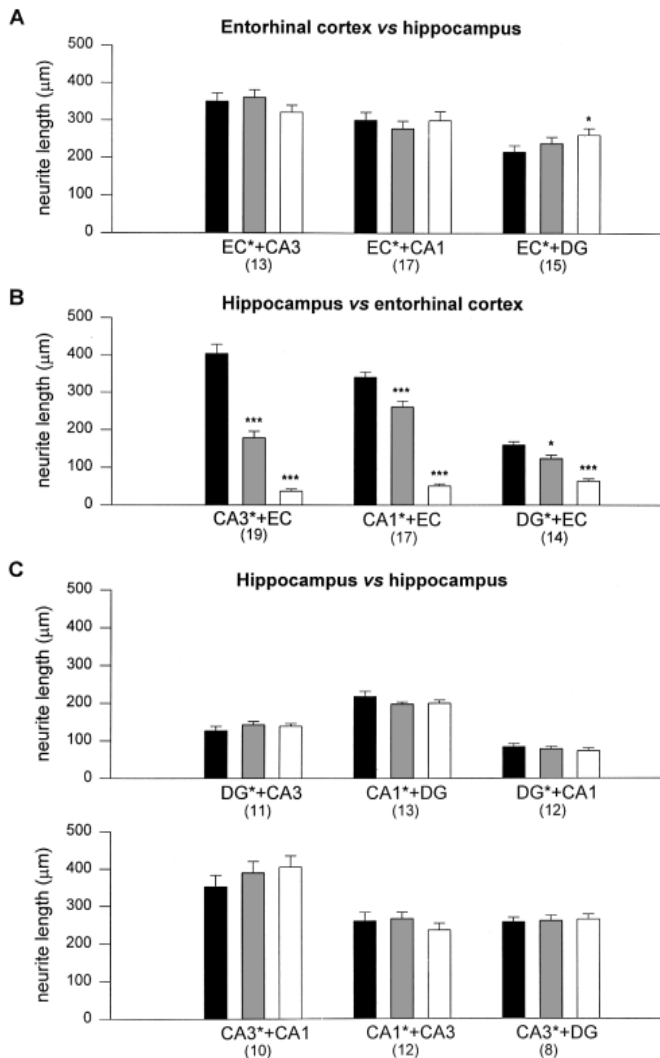
To identify the molecules that could mediate these repulsive effects, we investigated by in situ hybridization the developmental expression of known and potential chemorepellents, namely Netrin-1 and the secreted semaphorins (Sema A, Sema III, Sema E, Sema IV and Sema H). *Netrin-1* mRNA was exclusively expressed in the fimbria of the hippocampus at E15-P0, with no expression in other areas of the hippocampus, entorhinal cortex or adjacent neocortex (not shown), in agreement with earlier studies (Keino-Masu et al.,

1996). This pattern of expression indicates that Netrin-1 is highly unlikely to play a repulsive role in the patterning of the main hippocampal connections (J. A. D. R., V. B. and E. S., unpublished observations). A similar conclusion can be drawn for Sema A, whose mRNA is not expressed in the hippocampal region between E15 and P0 (not shown).

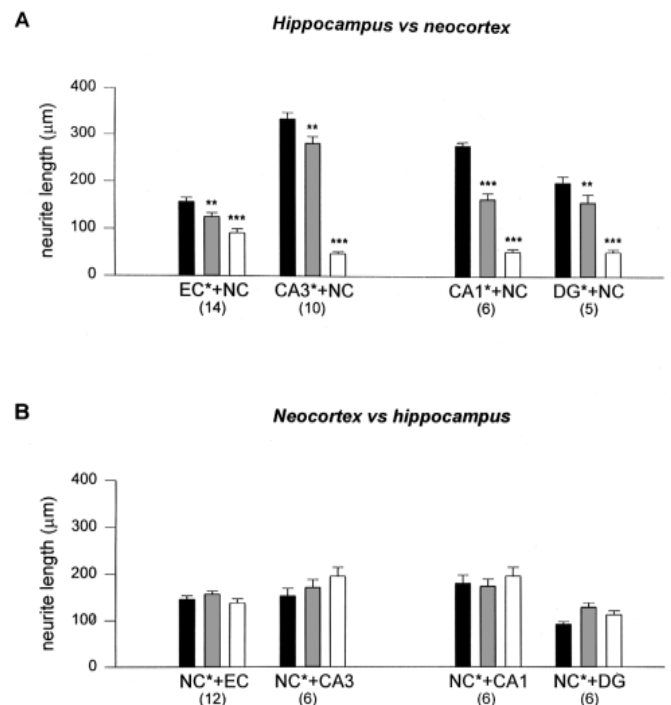
In contrast, *sema IV* mRNA was uniformly expressed

through the hippocampus, entorhinal cortex and the adjacent neocortex at E15-P0. The expression signal is low in the proliferating ventricular zone, and higher in the differentiating fields (the cortical and hippocampal plates) of these regions (Fig. 5A,B). At E15, *sema III* mRNA was widely expressed in the cortical plate and intermediate zone of the neocortex (in agreement with Catalano et al., 1998) and entorhinal area, whereas hybridization signals were virtually absent from the hippocampus (Fig. 5C). At P0 the expression pattern remained essentially the same, with high levels of expression in the neocortex and entorhinal area, and weak signals in both the hippocampus proper and dentate gyrus (Fig. 5D). At E15, *sema E* mRNA was exclusively expressed in the intermediate zone, both in the hippocampal region and neocortex (Fig. 5E). At P0, low expression was detected in the neocortex and entorhinal area, whereas hybridization signals were still very high in the hippocampus (data not shown). At E15-P0, *sema H* mRNA was expressed in the cortical plate of the neocortex, entorhinal cortex and in the hippocampus (Fig. 5F). Taken together, these expression studies show that the secreted semaphorins *sema III*, *sema IV*, *sema E* and *sema H* are expressed in the hippocampal region at the time of axonal outgrowth, suggesting that these factors could mediate the repulsive effects described above.

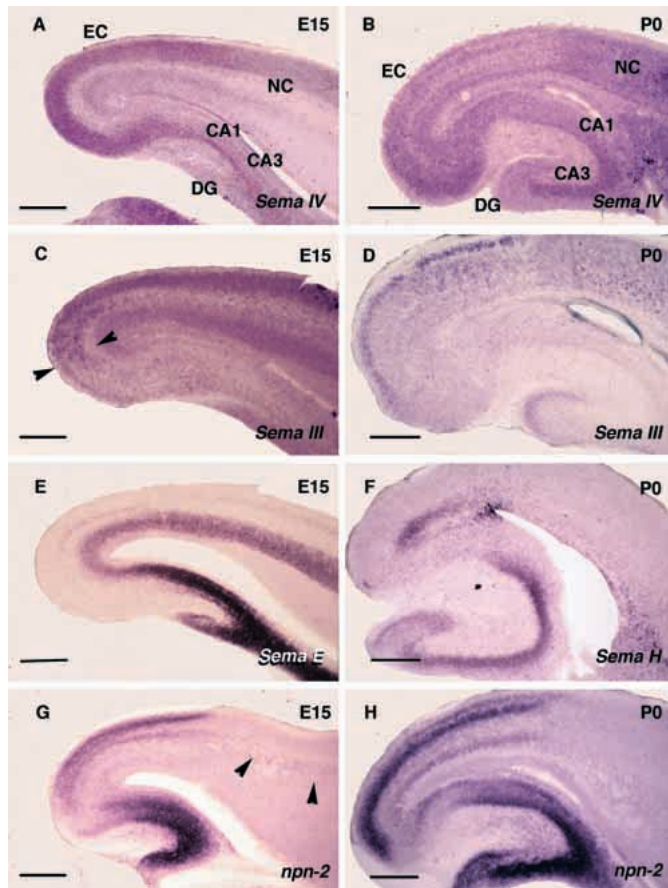
Next we investigated the developmental expression of the



**Fig. 3.** Histograms showing the axonal length (mean  $\pm$  s.e.m.) in several combinations of hippocampal and entorhinal explants labelled with DiI. Explants injected with DiI are marked by an asterisk. Axonal growth was measured in the distal (filled bars), lateral (grey bars) and proximal (white bars) quadrants. For statistical analysis, axonal growth in the distal quadrant was compared to that in the lateral and proximal quadrants. (A) entorhinal cortex axons grow symmetrically when confronted with CA3, CA1 or dentate gyrus explants. (B) On the contrary axons from CA3, CA1 and dentate gyrus explants confronted with entorhinal cortex explants were significantly shorter in the lateral and proximal quadrants compared to the distal quadrant, indicating that the entorhinal cortex exerts a repulsive action on these axons. (C) Axonal growth was measured in several combinations of hippocampal cocultures. No significant differences were observed between axonal length in the 3 quadrants, indicating the absence of long-range diffusible cues. *n* values are in parentheses. \* $P$ <0.05; \*\* $P$ <0.01; \*\*\* $P$ <0.001.



**Fig. 4.** Histograms illustrating the axonal length (mean  $\pm$  s.e.m.) in cocultures of hippocampal and neocortical explants. Conventions as in Fig. 3. In (A) the axonal growth of hippocampal and entorhinal cortex explants was monitored when confronted with the neocortex. In all cases axons were significantly shorter in lateral and most proximal quadrants compared to the distal quadrant, indicating that the neocortex releases soluble activities that repel those axons. In (B) we recorded the growth of neocortical explants confronted with hippocampal tissue. No significant difference was observed between axonal length in the three quadrants. *n* values are in parentheses. \* $P$ <0.05; \*\* $P$ <0.01; \*\*\* $P$ <0.001.



**Fig. 5.** Expression pattern of secreted semaphorins and their receptor Neuropilin-2, in the developing entorhino-hippocampal system and adjacent neocortex. Hybridizations were performed with digoxigenin-labelled riboprobes on horizontal sections of E15 and P0 mouse brains. (A,B) Expression pattern of *sema IV* mRNA at E15 and P0 showing wide expression throughout the entorhino-hippocampal system and neocortex, mainly in the cortical plate. (C,D) Expression pattern of *sema III* mRNA at E15 and P0 illustrating high expression levels in the entorhinal cortex and neocortex and very low expression in the hippocampus itself, except at P0 in the dentate gyrus. Note that *sema III* expression stops sharply at the junction between EC and hippocampus (arrowheads). (E) *sema E* mRNA expression at E15 demonstrating high expression levels in the intermediate zone of the entorhinohippocampal system and neocortex. (F) Illustration of *sema H* mRNA expression in the hippocampus and cortex of P0 mouse. (G,H) The strong expression of *neuropilin-2* mRNA in the EC and hippocampus at E15 and P0. Also note the faint labelling in the intermediate zone of the neocortex (arrowheads) at E15. EC, entorhinal cortex; NC, neocortex; CA1 and CA3, hippocampal fields CA1 and CA3; DG, dentate gyrus. Bars, 400  $\mu$ m (A,C,E,G) and 600  $\mu$ m (B,D,F,H).

known semaphorin receptors Neuropilin-1 and Neuropilin-2. At E15-P0, *neuropilin-1* mRNA was heavily expressed in the hippocampus and neocortex, and at lower levels in the entorhinal area (not shown, but see Kawakami et al., 1996). At E15 *neuropilin-2* was heavily expressed in the hippocampus and entorhinal cortex, especially in the differentiating fields including the cortical and hippocampal plates (Fig. 5G). In contrast, low levels of expression could be detected in the intermediate zone of the adjacent neocortex. At P0 the

**Table 3.** Semiquantitative evaluation of the effect of Sema III and Sema IV on axonal outgrowth of hippocampal and entorhinal cortex explants

Culture condition	Number of explants	Axonal outgrowth			
		+	=	-	—
CA1+Sema III	23	—	—	—	23
CA3+Sema III	19	—	—	—	19
DG+Sema III	13	—	3	3	7
EC+Sema III	15	—	3	2	10
CA1+Sema IV	44	—	—	3	41
CA3+Sema IV	41	—	—	1	40
DG+Sema IV	15	—	3	2	10
EC+Sema IV	22	—	21	1	—
CA1+Control	30	—	28	2	—
CA3+Control	29	—	25	4	—
DG+Control	5	—	4	1	—
EC+Control	8	—	8	—	—

Explants were labelled using anti- $\beta$ -tubulin antibodies. Abbreviations and conventions as in Table 1.

expression pattern remained the same, though increased signals were detected in the hippocampus, particularly in the dentate gyrus (Fig. 5H). We conclude that the semaphorin receptors Neuropilin-1 and Neuropilin-2 are expressed in the hippocampus and entorhinal cortex at the time that hippocampal connections are being formed.

### Sema III and Sema IV repel hippocampal axons in vitro

To test directly whether secreted semaphorins can contribute to the chemorepulsive effect of the entorhinal cortex, we cultured CA1 and CA3 explants (from E15-E17 mouse embryos) with COS cells that had been transiently transfected with expression constructs for all five known mammalian secreted semaphorins (A, III/D, E, IV, H). We also cocultured those cells with explants from the entorhinal cortex and neocortex. Explants were cultured for 48-72 hours, fixed and stained with an anti-class III  $\beta$ -tubulin antibody that labels the entire population of axons growing from the explant (Moody et al., 1989). The expression of the diverse epitope-tagged semaphorins was verified by western blotting (data not shown).

Axons from CA1, CA3 and dentate gyrus explants grow symmetrically when cultured with control COS cells, mock-transfected or transfected with an alkaline-phosphatase expression construct (see Fig. 6C,F; Table 3 and not shown). Furthermore we could not detect any effect of COS-cells secreting Sema A, Sema E or Sema H (data not shown). In contrast, axons from CA1, CA3 and dentate gyrus explants were strongly repelled when confronted with COS cells expressing Sema III-myc (Fig. 6A,D; Table 3; see also Fig. 8A). Axons grew almost exclusively from the distal quadrant opposite the COS-cell aggregate. An equivalent strong repulsion of CA1, CA3 and dentate gyrus axons was observed when using COS cells transfected with *sema IV-AP* (Figs 6B,E, 8B; Table 3 and not shown). Axons that exited the lateral quadrant of the explants, or explants that were placed at a long distance from COS-cell aggregates producing Sema III or Sema IV, clearly turned away from the aggregates (see for example Fig. 6B), demonstrating that when present as a gradient Sema IV and

Sema III do not inhibit, but rather orientate, the growth of hippocampal axons. In addition, we found that Sema III also exercises a repulsive action on entorhinal axons, whereas Sema IV had no apparent effect on these axons (Figs 6G-I, 8A; Table 3). Axons of neocortex and cerebellar explants from E16 mouse embryos (two other regions where several secreted semaphorins and neuropilins are expressed; not shown) grew symmetrically when confronted with Sema III and Sema IV-expressing COS cell aggregates, or with cells secreting other semaphorins (not shown). These results show that CA1, CA3 and dentate gyrus axons can be repelled by two distinct secreting semaphorins, Sema III and Sema IV, whereas entorhinal axons are exclusively repelled by Sema III.

### Neuropilin-1 antibodies block Sema III and entorhinal cortex-induced chemorepulsion of hippocampal axons

It has been shown recently that members of the neuropilin family are high-affinity receptors for secreted semaphorins: Sema IV binds to Neuropilin-1 and Neuropilin-2 with high affinity, whereas Sema III only binds to Neuropilin-1 with high affinity (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). An antibody (purified IgGs) directed against the MAM and b1-b2 domains of Neuropilin-1 has been shown to abolish completely Sema III-induced repulsion and collapse of DRG axons at a concentration of 10  $\mu\text{g/ml}$  (He and Tessier-Lavigne, 1997).

We first tried to determine whether we could block Sema III-induced repulsion of hippocampal and entorhinal axons by using the anti-Neuropilin-1 antibody. In the presence of 10  $\mu\text{g/ml}$  of anti-Neuropilin-1 we found that Sema III-induced repulsion was significantly reduced (Figs 7A,B, 8A). Axonal growth was restored in the lateral and proximal quadrants to levels almost identical to that of the distal quadrant.

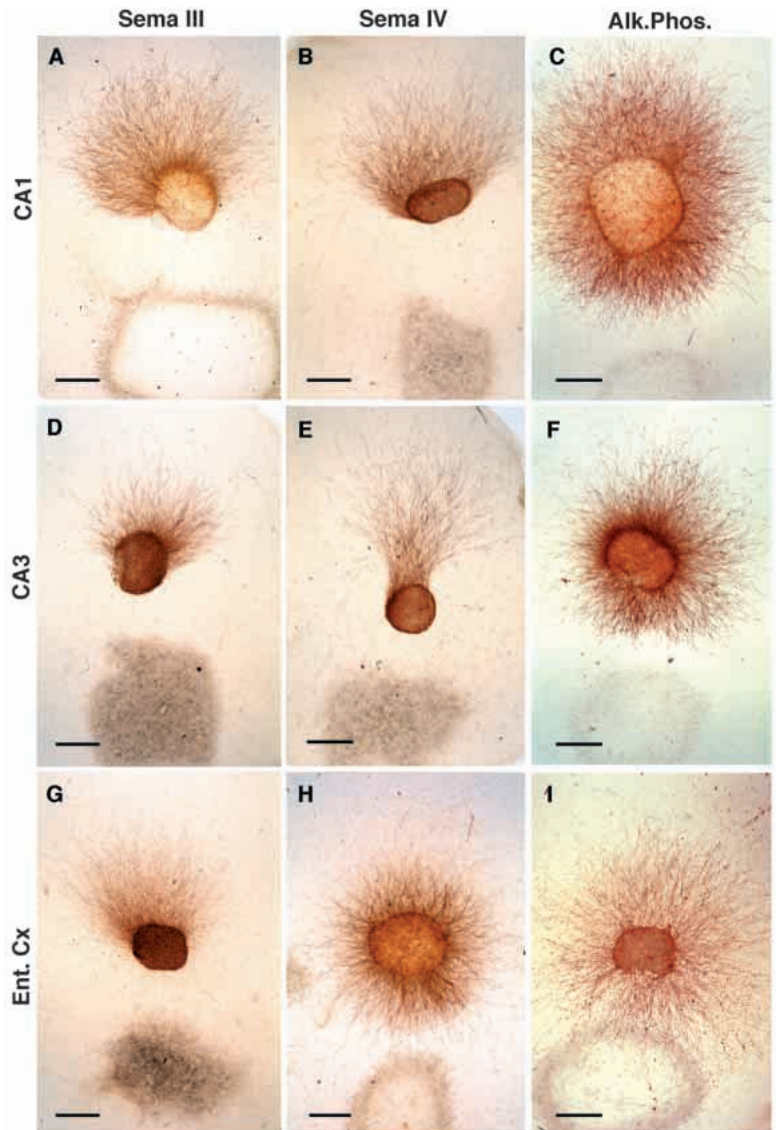
We next examined whether Neuropilin-1 was also involved in Sema IV-induced repulsion of hippocampal axons. No significant differences were observed between CA1 and CA3 explants cultured next to COS cells expressing Sema IV in the presence or absence of anti-Neuropilin-1 antibodies (Figs 7C, 8B). Thus, Sema IV induced-repulsion does not involve Neuropilin-1.

Finally, we cocultured CA1 and entorhinal explants with 10  $\mu\text{g/ml}$  of anti-Neuropilin-1 antibodies and found that explants grew almost symmetrically (Figs 7D, 8C), suggesting that Neuropilin-1 is involved in mediating to a large extent the entorhinal cortex-induced repulsion of hippocampal axons.

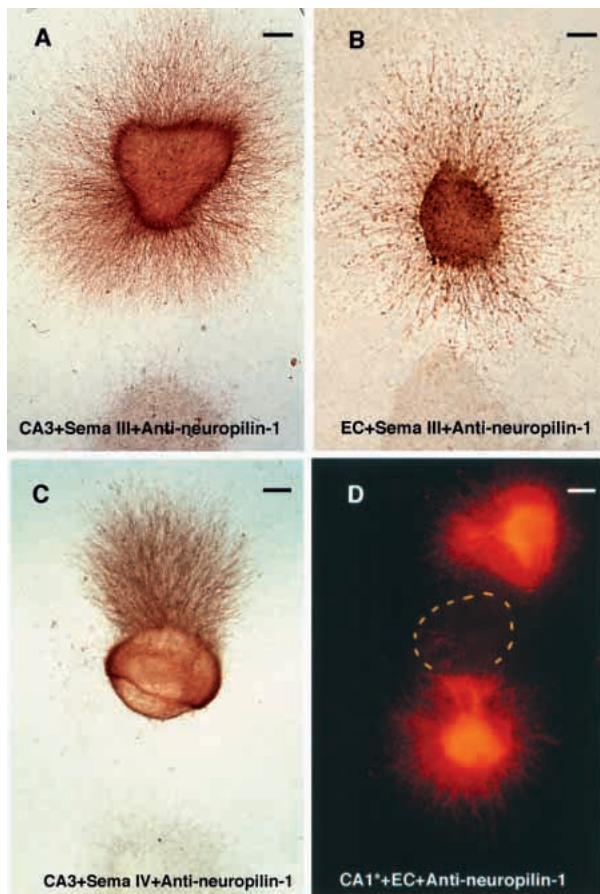
## DISCUSSION

In this study we have shown that, at the time of formation of hippocampal connections in vivo, there is a strong chemorepulsion between tissue explants from distinct hippocampal subfields. Concomitantly,

several secreted semaphorins, including Sema III and Sema IV, and their receptors neuropilin, are expressed in the developing hippocampal formation. More importantly, the repulsive effects on hippocampal axons are mimicked by Sema III and Sema IV, but not by other secreted semaphorins. Finally, the repulsive action of Sema III, but not Sema IV, is mediated by Neuropilin-1 receptors. To our knowledge the present findings are the first demonstration of the involvement of Sema III and its receptor, Neuropilin-1, in the patterning of neuronal



**Fig. 6.** Repulsion of hippocampal and entorhinal axons by Sema III and/or Sema IV. E16-E17 CA1 (A-C), CA3 (D-F) and entorhinal (G-I) explants were cocultured for 48-72 hours at a distance from aggregates of COS cells transfected with *H-sema III* (A,D,G), *H-sema IV* (B,E,H) or *alkaline-phosphatase* (C,F,I). All explants were fixed and stained with anti- $\beta$ -tubulin antibodies. CA1 (A), CA3 (D) and entorhinal (G) axons are strongly repelled by Sema III. Sema IV also exerts a robust repulsive action on CA1 (B) and CA3 (E) axons, but has no effect on entorhinal axons (H). Axons from CA1 (C), CA3 (F) and entorhinal cortex (I) grow symmetrically when confronted with COS cells secreting only alkaline phosphatase. Ent. Cx. entorhinal cortex; Alk. Phos., alkaline phosphatase. Bars, 280  $\mu\text{m}$ .

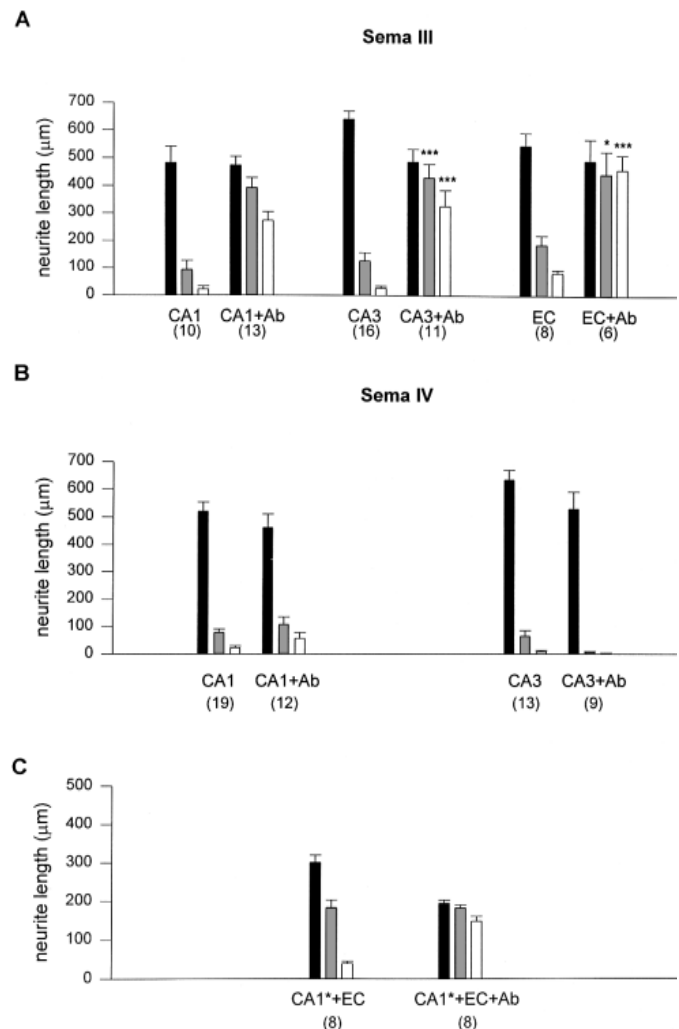


**Fig. 7.** Neuropilin-1 is involved in Semaphorin III- and entorhinal cortex-induced repulsions of hippocampal axons. E15-E17 hippocampal (A,C,D) or entorhinal (B) explants were cocultured in collagen gel next to COS cells secreting Semaphorin III (A-B) or Semaphorin IV (C), or next to entorhinal cortex explant (D), in the presence of 10  $\mu\text{g}/\text{ml}$  of anti-Neuropilin-1 IgGs. Explants were stained with anti- $\beta$ -tubulin antibodies (A-C) or DiI (D). Semaphorin III-induced repulsion of CA3 (A) and entorhinal (B) axons is prevented by the antibody. On the contrary, CA3 axons are still repelled by Semaphorin IV in the presence of anti-Neuropilin-1 IgGs (C). (D) Two DiI-labelled CA1 explants were cocultured next to an entorhinal cortex explant (dashed line) in the presence of anti-Neuropilin-1 antibody. DiI-labelled CA1 axons are growing toward the entorhinal explant, showing that neuropilin-1 is involved in entorhinal-induced repulsion of CA1 axons (compare to Fig. 2E). Bars, 100  $\mu\text{m}$  (A-C) and 180  $\mu\text{m}$  (D).

connections in the forebrain. In addition, our data also show for the first time that the secreted semaphorin, Semaphorin IV, exerts a potent repulsive response on developing axons, and that this response is likely to be mediated by Neuropilin-2.

### Involvement of chemorepulsion in the patterning of hippocampal connections

In mouse embryos the earliest entorhinal axons leave the cortex by E14, being directed towards the hippocampus; by E15 entorhinal axons reach the hippocampal intermediate zone, and 1 day later they invade their target layer, the outer marginal zone (prospective stratum lacunosum-moleculare). Concomitantly, the first axons from the pyramidal neurons in the CA3 and CA1 hippocampal fields grow rostrally through

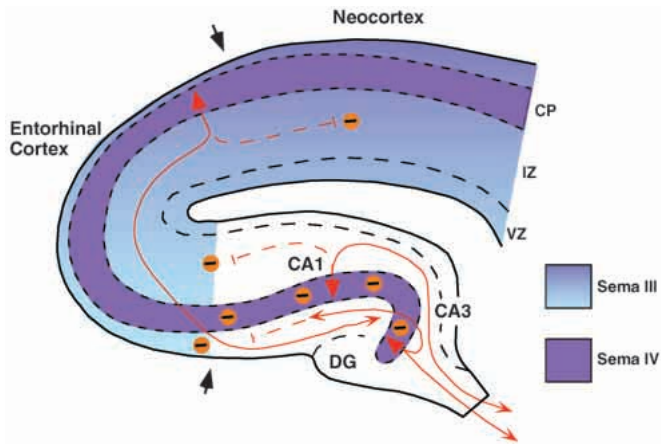


**Fig. 8.** Histograms showing the axonal length (mean  $\pm$  s.e.m.) in several combinations of hippocampal and entorhinal explants and/or COS cells expressing Semaphorin III or Semaphorin IV, in the absence or presence of anti-neuropilin-1 antibody. Explants were labelled with anti- $\beta$ -tubulin antibodies (A-B) or DiI (asterisks in C). Axonal length was measured in the distal (filled bars), lateral (grey bars) and proximal (white bars) quadrants. For statistical analysis, axonal lengths in the different quadrants, with or without antibody, were compared. Anti-Neuropilin-1 significantly inhibits Semaphorin III-induced repulsion of CA1, CA3 and entorhinal axons (A), but does not affect Semaphorin IV-induced repulsion of hippocampal axons (B). (C) Anti-Neuropilin-1 also blocks the repulsive effect of the entorhinal cortex on CA1.  $n$  values are in parentheses. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

the fimbria to form the commissural and hippocampo-septal pathways. In addition, by E16-E17, axons from the CA3 pyramidal neurons innervate the ipsilateral CA1 region (associational afferents; Figs 1A, 9; Supèr and Soriano, 1994; Supèr et al., 1998). Such a stereotyped pattern of developing connections suggest the involvement of very specific guiding cues.

We have shown here that, at these embryonic stages, the entorhinal cortex releases long-range diffusible factors that repel axons arising from several regions of the hippocampus,





**Fig. 9.** Schematic summary of the data obtained in this study on the formation of hippocampal connections in mouse embryos. Entorhinal axons project to the hippocampus intermediate zone and invade their target layer, the outer marginal zone (prospective stratum lacunosum-moleculare). Axons from the CA3 and CA1 hippocampal fields grow rostrally through the fimbria to form the commissural and hippocampo-septal pathways. In addition, axons from the CA3 pyramidal neurons innervate the ipsilateral CA1 region (associational afferents), but the axons of either the CA3 or the CA1 regions never invade the entorhinal cortex or the neocortex. Neuropilins are expressed by entorhinal and hippocampal neurons. *Sema III* mRNA is expressed at high levels in the neocortex and entorhinal area and barely detectable in the hippocampus itself. This cortical-derived *Sema III* would prevent the penetration of hippocampal axons in the entorhinal cortex and neocortex. The high expression of *Sema IV* in the hippocampal plate may direct and confine pyramidal cell axons to neighbouring layers, such as the intermediate zone and stratum radiatum. The repulsion of entorhinal axons by the neocortex is weak and could depend on the different responsiveness of distinct entorhinal projection neurons (see Discussion). –, repulsion; CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone; DG, dentate gyrus.

including the CA1, CA3 and dentate fields. In contrast, no reciprocal repulsive effect of the embryonic hippocampal tissue on entorhinal axons was observed. Since the majority of postmitotic neurons present in the CA1-CA3 hippocampus at E15-E17 are pyramidal neurons, most axons being repelled in the explant coculture assay must arise from the pyramidal projection neurons. For the dentate gyrus, repelled axons probably come from the commissural/associational neurons in the hilus and from the granule cells (Stanfield and Cowan, 1988).

These *in vitro* results are in agreement with the early patterning and trajectory followed by the developing hippocampal axons *in vivo*, in which axons from the CA3 and CA1 pyramidal neurons grow through the fimbria towards the hippocampal commissure, but never invade the entorhinal cortex (Supèr and Soriano, 1994; Amaral and Witter, 1995; Supèr et al., 1998). Thus, our data suggest that endogenous repulsive signals released by the embryonic entorhinal cortex prevent the ingrowth of hippocampal axons into the entorhinal area. In addition, the entorhinal-derived repulsive cues might contribute to pushing away the hippocampal axons and, in conjunction with chemoattractive cues present in the fimbria and hippocampal commissure such as Netrin-1 (Serafini et al.,

1996), help to direct them towards the telencephalic midline (Fig. 9).

Hippocampal axons are also dramatically repelled by the embryonic neocortex, indicating that both the neocortex and entorhinal cortex release diffusible signals that prevent the ingrowth of hippocampal axons into these cortical fields. Moreover, we found that entorhinal axons are repelled by embryonic neocortical tissue, whereas neocortical axonal outgrowth is unaffected by nearby hippocampal explants. The entorhinal cortex receives convergent inputs from several neocortical areas while the entorhinal cortex projects only very sparsely to some cortical areas (Amaral and Witter, 1995). Again, the present *in vitro* findings are consistent with the pattern of neocortical/entorhinal connections in the developing and adult brain, and with the preferential growth of entorhinal axons towards the hippocampus. However, although the main output projection from the entorhinal area is the entorhinohippocampal pathway (perforant pathway), this area sends sparse projections to different subcortical nuclei (Amaral and Witter, 1995; Supèr et al., 1994), suggesting that the relatively weak repulsive response of entorhinal axons when confronted with neocortical explants (Fig. 4A), may be related to the different responsiveness of distinct entorhinal projection neurons.

In this study we did not find evidence for repulsive responses in hippocampus/hippocampus cocultures, suggesting that long-range repellent cues are unlikely to play a role in the patterning of ipsilateral hippocampal connections, such as the Schaffer collaterals or the mossy fibers. The development of these intrahippocampal connections, as well as their layer-specific targeting, could be determined by cues other than secreted semaphorins (but see below), which might include ephrins, CAMS, transmembrane semaphorins, and extracellular matrix molecules that are expressed in the developing hippocampal system (e.g. Gao et al., 1996; Zhang et al., 1996; Serafini et al., 1996; Del Río et al., 1997).

### Role of Sema III and Sema IV in the development of hippocampal connections

In insects, it is clear that semaphorins play important roles in the development of the nervous system. They function in growth cone guidance, preventing fasciculation and inhibiting axon branching, but also in synaptic terminal arbor formation (Kolodkin et al., 1993; Matthes et al., 1995; Yu et al., 1998). Interestingly, G-Sema I also apparently functions as an attractive-permissive guidance cue for growth cones of the subgenual organ in the limb bud (Wong et al., 1997). In mammals, the function of transmembrane semaphorins in brain development is still unknown, but secreted semaphorins also act as repulsive molecules. Sema III/Collapsin-1 repels motor (Varela-Echavarría et al., 1997) and sensory axons (Luo et al., 1995; Messersmith et al., 1995; Püschel et al., 1995; Kobayasi et al., 1997) *in vitro*, and Sema A and Sema E/Collapsin-3 can repel and cause the collapse of sympathetic axons (Adams et al., 1997; Koppel et al., 1997). In addition, Sema Z1a, a zebrafish semaphorin, also collapses sensory axons (Shoji et al., 1998). The present findings demonstrate for the first time that Sema III has an action on forebrain neurons, indicating a more widespread function for Sema III in axon guidance than has been shown previously. Sema IV was first identified in humans and localized to the region 3p21.3 of chromosome 3,

where several lung cancer cell lines exhibit homozygous deletions indicative of a tumor suppressor gene (Roche et al., 1996). Nevertheless, the function of *Sema IV* remained unknown. We show here that *Sema IV* has a strong repulsive action on hippocampal axons. Since *Sema III* and *Sema IV* are highly expressed in the embryonic hippocampal formation when the main axonal trajectories are established, and the repulsive responses of hippocampal and entorhinal axons are mimicked by them, we propose that both semaphorins are involved in the formation of hippocampal connections.

The pattern of *sema III* mRNA distribution, with high expression levels in the neocortex and entorhinal area and barely detectable hybridization signals in the hippocampus itself, is consistent with the notion that *Sema III* may be the cortical-derived, endogenous repulsive factor causing repulsion of developing hippocampal axons (Fig. 9). The contribution of *Sema IV* to the patterning of hippocampal connections may be more debatable, since the high expression levels of *sema IV* in the hippocampus seems to be inconsistent with the *in vitro* analyses, in which we never observed repulsive responses when hippocampus/hippocampus explants were cocultured. One major limitation of these interpretations resides in the absence of data on the localization of semaphorin proteins, their concentration level or release sites. However, in mammals, secreted semaphorins have a short stretch of basic amino acids at their carboxy terminus. This highly charged domain is likely to limit the diffusion of semaphorins, which could act as short-range or local guiding cues (Culotti and Kolodkin, 1996). If this is the case, the high expression of *Sema IV* in the hippocampal plate may direct and confine pyramidal cell axons to neighbouring layers, such as the intermediate zone and stratum radiatum (Fig. 9).

Recently two groups have obtained knock-out mice in which the *sema III* gene has been inactivated by homologous recombination (Behar et al., 1996; Taniguchi et al., 1997). The phenotypic analysis of those mice has shown that the CNS looks remarkably normal and that neurons that are strongly repelled *in vitro* by *Sema III* project normally in the mutant CNS (Behar et al., 1996; Taniguchi et al., 1997; Catalano et al., 1998). Moreover, *neuropilin-1* mutant mice also display no abnormalities in the CNS projections of sensory axons, similar to the lack of phenotype for these projections in the *sema III* mutant mice. However, when neurons from *neuropilin-1* mutant mice are tested in *in vitro* explants, they are no longer repelled by *Sema III* (Kitsukawa et al., 1997). This lack of a phenotype in the CNS of the *sema III* and *neuropilin-1* mutant mice has been tentatively explained by both the existence of other semaphorins in the developing CNS, and potentially other neuropilin/semaphorin receptors on CNS axons (or other guidance cues). Our results clearly show that two distinct semaphorins can have a similar repulsive effect on the same axons at the same time, and that two distinct receptors mediate these effects (see below). This could explain the absence of strong brain defects in *sema III* and *neuropilin-1* knock-outs.

### Involvement of Neuropilin-1 in axonal repulsion in the hippocampus

The expression pattern of the semaphorin receptors Neuropilin-1 and Neuropilin-2, with high expression in the entorhinal cortex and in the hippocampus itself, suggests that the repulsive responses described in this study are mediated by these receptors

(Fig. 9). Furthermore, the finding that anti-Neuropilin-1 antibodies block the repulsive responses of hippocampal axons versus the entorhinal cortex, indicates that this receptor is directly involved in the signaling of endogenous repulsive factors. The contribution of Neuropilin-2 receptors, however, remains more elusive. Binding studies have shown that *Sema III* only binds with high affinity to Neuropilin-1, whereas *Sema IV* binds to both Neuropilin-1 and Neuropilin-2 (Chen et al., 1997). Nevertheless, *Sema IV* appears to bind with higher affinity to Neuropilin-2 than Neuropilin-1 (the  $K_D$  is about 10-fold smaller), which suggested that the *Sema IV* signal is mediated principally by Neuropilin-2 (Chen et al., 1997). The absence of blocking effect of anti-Neuropilin-1 antibodies on *Sema IV* induced-repulsion further supports the idea that Neuropilin-2 might mediate the action of this semaphorin. But one cannot exclude the possibility that another semaphorin receptor is involved. In addition, we found that Neuropilin-1 antibodies block *Sema III*-induced repulsion of hippocampal axons therefore confirming, for the first time in a system different from the sensory axons, an essential role of Neuropilin-1 in mediating *Sema III* action. Unfortunately, *neuropilin-1* knock-out mice die around E12.5, which is too early to study hippocampal projections (Kitsukawa et al., 1997).

Finally, our results confirm that semaphorin binding is not sufficient to trigger a response in growth cones expressing neuropilins, as previously shown in the DRG (Koppel et al., 1997). Thus, the secreted semaphorins E and H, which are expressed in the hippocampus and bind neuropilins, do not have detectable guiding effects on embryonic hippocampal axons. Nevertheless, since the development of neural connections in the hippocampus proper and dentate gyrus continues postnatally, secreted semaphorins could potentially play a role at later developmental stages, for instance in axonal arbor formation and synaptogenesis.

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