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## **Semaphorin 3C is not required for the establishment and target specificity of the GABAergic septohippocampal pathway in vitro**

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### **Abstract**

The septohippocampal (SH) pathway comprises cholinergic and GABAergic fibers. Whereas the former establish synaptic contacts with all types of hippocampal neurons, the latter form complex baskets specifically on interneurons. The GABAergic SH function is associated with the control of hippocampal synchronous networks. Little is known about the mechanisms involved in the formation of the GABAergic SH pathway. Semaphorin (Sema) 3C is expressed in most hippocampal interneurons targeted by these axons. To ascertain whether Sema 3C influences the formation of the SH pathway, we analyzed the development of this connection in Sema 3C-deficient mice. As these animals die at birth, we developed an in vitro organotypic co-culture model reproducing the postnatal development of the SH pathway. In these SH co-cultures, the GABAergic SH pathway developed with target specificity similar to that present in vivo. SH axons formed incipient baskets on several types of hippocampal interneurons at 7 days in vitro, which increased their complexity by 18–25 days in vitro. These SH fibers formed symmetric synaptic contacts on GABAergic interneurons. This synaptic specificity was not influenced by the absence of entorhinal afferents. Finally, the absence of Sema 3C in target neurons or its blockage by neuropilin-1 and -2 ectodomains in slice co-cultures did not lead to major changes in either the target specificity of the GABAergic SH pathway or its density of innervation. We conclude that the formation and synaptic specificity of the GABAergic SH pathway relies on robust molecular mechanisms, independent of Sema 3C, that are retained in our in vitro co-culture model.

## Introduction

The establishment of specific synaptic connections is a critical step during the development of the nervous system. An increasing number of molecules have been shown to be involved in the guidance of growing axons to their final destinations [semaphorins (Semas), netrin, ephrins, etc.] (Goodman, 1996; Tessier-Lavigne & Goodman, 1996; Song & Poo, 2001), but less is known about the factors that control the recognition and selection of specific synaptic targets (Waites et al., 2005; Lu et al., 2009; Shen & Cowan, 2010). The hippocampus receives three major afferent systems, the entorhino–hippocampal pathway, commissural-associational pathway, and septohippocampal (SH) connection (Amaral & Witter, 2004). The SH pathway, which arises from the medial septum/diagonal band (MS/DB) complex, includes the cholinergic and GABAergic fibers (Frotscher & Léránth, 1986; Freund & Antal, 1988; Gulyás et al., 1990). The cholinergic SH pathway establishes synapses with all of the types of hippocampal neurons. In contrast, the GABAergic SH fibers establish complex synaptic baskets exclusively around the perisomatic region of GABAergic interneurons (Frotscher & Léránth, 1986; Freund & Antal, 1988; Gulyás et al., 1990). Hippocampal interneurons are inhibitory neurons responsible for modulating the neuronal excitability of hippocampal networks. The high degree of target cell specificity of the GABAergic SH fibers confers on this pathway high efficiency in controlling hippocampal interneuron activity. Thus, for instance, the GABAergic SH pathway has been implicated in the synchronization and regulation of hippocampal rhythmic activity (Buzsáki, 2002; Hangya et al., 2009).

Previous studies have shown that, during embryonic development, both types of septal fibers respond to the similar guidance cues that direct their growth from the septum to the hippocampus (Pascual et al., 2005). Thus, for instance, *Sema 3A* and *3F*, expressed in regions near the septum, have a chemorepulsive effect on septal axons, whereas *netrin-1*, expressed in the fimbria, chemoattracts these axons (Pascual et al., 2004b). However, nothing is known about the factors that govern the exquisite target selection of GABAergic interneurons by the GABAergic SH pathway in the hippocampus. A large percentage of GABAergic SH target cells express the class III *sema 3C*, and SH neurons in the MS/DB complex co-express the *Sema 3C* receptors, *neuropilin-1* and *-2*, at postnatal stages (Pascual et al., 2005). Because *Sema 3C* is a secreted guidance cue implicated in repulsion and attraction in various kinds of axons (Bagnard et al., 1998; Chen et al., 1998; Niquille et al., 2009), we hypothesized that this *Sema* might be involved in target cell selection by the GABAergic SH pathway.

Here we studied the relevance of *Sema 3C* in the establishment of the GABAergic SH pathway. However, because this molecule is critical for correct embryonic heart development, mice deficient in *Sema 3C* die at birth (Feiner et al., 2001). Thus, in order to analyze the role of *Sema 3C* in the maturation of the GABAergic SH pathway, we first developed an *in vitro* system that allows the reconstruction and analysis of the SH pathway in organotypic slice co-cultures of septal and hippocampal tissue.

## **Materials and methods**

### **Animals**

The Sema 3C+/- mice were maintained in a CD1 background and mated to obtain Sema 3C-/- embryos (Feiner et al., 2001). Control mice (OF-1 strain; Charles River, France) were also used. The genotype of the offspring was determined by polymerase chain reaction as described previously (Feiner et al., 2001). The day on which a vaginal plug was detected was designated as embryonic day (E)0. The day of birth was considered as postnatal day (P)0. All of the experiments using animals were performed in accordance with the European Community Council directive and the National Institute of Health guidelines for the care and use of laboratory animals. Experiments were also approved by the local ethical committees (C.E.E.A., Comitè Ètic d'Experimentació Animal, University of Barcelona).

### **Detection of septohippocampal axons**

To detect SH GABAergic fibers, we deeply anesthetized [mixture 10/1 Ketolar (Parke-Davis)/Rompun (Bayer), 0.003 mL/g] OF-1 mice and injected them with an anterograde tracer by iontophoresis (Freund & Antal, 1988). The P5 (n = 4), P10 (n = 5), P18 (n = 3) and adult (n = 3) mice received midline injections of 10% biotinylated dextran-amine (BDA) (10 000 MW, Molecular Probes) in the medial septum at two anteroposterior levels and at two dorsoventral points by iontophoresis (7  $\mu$ A positive direct current, 7 s on/off cycle). Stereotaxic coordinates for the injections were (from Bregma): adult animals, anteroposterior + 0.5 and + 0.9, dorsoventral 3.0 and 3.7; P15 mice, anteroposterior + 0.6, dorsoventral 2.6 and 3.2; and P5 mice, anteroposterior + 0.5, dorsoventral 2.5 and 3. This protocol results in strong BDA labeling in the medial septum and the diagonal band area that contains the highest proportion of GABAergic SH neurons (Freund & Antal, 1988; Gulyás et al., 1990; Pascual et al., 2005). After 3 or 4 days, the animals were deeply anesthetized with Ketolar (Parke-Davis)/Rompun (Bayer) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were dissected and frozen, and 30- $\mu$ m-thick sections were obtained and stored in a cryoprotectant solution at -70 °C until use. SH fibers were detected by BDA immunodetection. After washing and blocking, sections were incubated overnight at 4 °C with the avidin-biotin peroxidase complex (1 : 100; Vector Laboratories, Burlingame, CA, USA). Peroxidase activity was developed with 0.03% 3,3'-diaminobenzidine intensified with ammonium nickel sulfate and cobalt chloride, and 0.005% H<sub>2</sub>O<sub>2</sub> yielding a black reaction product. Sections were mounted onto gelatinized slides, Nissl stained, dehydrated, and coverslipped with Eukitt (Panreac). In some sections, following the detection of BDA, the immunostaining of distinct neuronal markers was performed (see below).

### **Immunodetection**

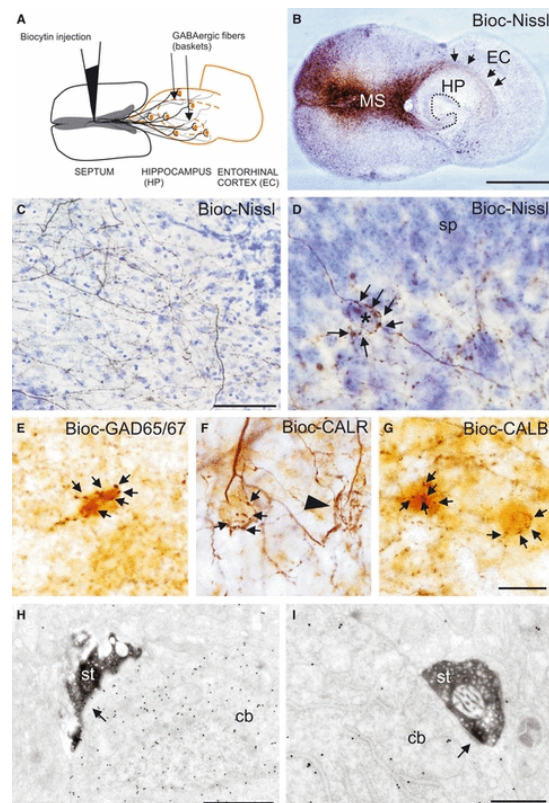
For all of the histological procedures, mice were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and postfixed in the same solution, cryoprotected with 30% sucrose and sectioned at 30  $\mu$ m as previously described for BDA-injected mice.

Immunohistochemistry was performed as described elsewhere (Pascual et al., 2005). After blocking, or after the detection of BDA tracer, the tissue was incubated overnight with well-characterized rabbit polyclonal antibodies against parvalbumin (PARV, 1 : 3000, Swant Antibodies, Bellinzona, Switzerland), calbindin (CALB, 1 : 1000, Swant Antibodies), calretinin (CALR, 1 : 1000, Swant Antibodies), Sema 3C (1 : 1000, Abcam plc, Cambridge, UK), glutamic acid decarboxylase (GAD)65/67 (1 : 1000, Chemicon International, Temecula, CA, USA) or goat anti-choline acetyltransferase antibodies (1 : 150, Chemicon International, Temecula, CA, USA). Primary antibodies were visualized by sequential incubation with biotinylated secondary antibodies (1 : 200) and the avidin-biotin peroxidase complex (diluted 1 : 100) (2 h each, Vector Laboratories). The peroxidase reaction was developed with 0.03% 3,3'-diaminobenzidine and 0.005% H<sub>2</sub>O<sub>2</sub>, yielding a brown end-product. The sections were mounted onto gelatinized slides, dehydrated and coverslipped with Eukitt (Panreac). For double immunofluorescent detection of Sema 3C and PARV, after blocking, the sections were incubated overnight with rabbit anti-Sema 3C (1 : 500) combined with goat anti-PARV (1 : 1000, Swant Antibodies, Bellinzona, Switzerland) and then incubated with

swine anti-rabbit fluorescein isothiocyanate (FITC, 1 : 250, DAKO, Glostrup, Denmark) and Alexa Fluor 568 donkey anti-goat (1 : 750, Invitrogen). The sections were mounted onto slides with Mowiol 4-88 (Merck) and viewed under a confocal microscope. All of the slices were permeabilized with Triton X-100 (0.5%) during the immunohistochemistry procedure, except for the GAD 65/67 immunodetections, where a freeze/thawing protocol was performed before the blocking.

### Organotypic septohippocampal cultures

Mouse pups (P1) were decapitated and brains dissected out of the skull. Brains were kept in cold dissection medium (minimum essential medium containing glutamine) during the dissection procedure. Slices (250  $\mu$ m) of the hippocampus–entorhinal complex were cut using a McIlwain tissue chopper. For the dissection of the septum, the anterior half of the brain was embedded in 3% low-melting agarose (Cambrex Corporation, USA) and cut into 250  $\mu$ m slices using a vibratome (VT100S; Leica, Nussloch, Germany). The tissue was kept cold during all of this process. After further dissection, the septum and hippocampus–entorhinal cortex were placed onto a plate insert (Millipore Corporation, Billerica, MA, USA) and arranged as shown in Fig. 1A. The ventral region of the septum was positioned in contact with the fimbria. Inserts were placed in a six-well plate containing 1 mL of culture medium per well [minimum essential medium containing 25% horse serum, 35% neurobasal, 12.5% Hank's Balanced Salt Solution, 2 mM glutamine, penicillin/streptomycin (1 U/1  $\mu$ g per mL), 0.04% NaHCO<sub>3</sub>, B27 and N2 (all from Gibco Life Technologies), and 0.55%D-glucose (Sigma-Aldrich)].



**Figure 1**

Reconstruction of the GABAergic SH axons in vitro. (A) Diagram showing the components of the in vitro model of the SH connection. The organotypic co-cultures include the septum, hippocampus (HP) and entorhinal cortex (EC) slices taken from P1 mice. (B) Photomicrograph of a Nissl-stained 15 DIV organotypic culture showing biocytin (Bioc) injection (black) in the medial septum (MS) and diagonal band complex, and some stained fibers in the stratum oriens (arrows) of the HP. (C) Higher magnification photomicrograph illustrating the massive septal innervation (black fibers) throughout the layers of the hippocampus in a 15 DIV culture. (D) Photomicrograph of a 15 DIV culture showing a thick fiber forming a basket with abundant boutons (arrows) contacting a neuron located close to the stratum oriens (sp). (E) Bioc-GAD65/67 staining. (F) Bioc-CALR staining. (G) Bioc-CALB staining. (H) Bioc-ST staining. (I) Bioc-CB staining.



stratum pyramidale (sp) of CA3. (E) Immunostaining for the enzyme GAD65/67 allows the detection of all GABAergic hippocampal interneurons (brown). The combined detection of GAD65/67 and biocytin shows biocytin-positive septal baskets (black) with abundant boutons (arrows) contacting GAD65/67-immunoreactive cells in the cultures. (F) Micrograph showing the hippocampal region of a 15 DIV organotypic culture in which biocytin detection (black) was combined with the immunodetection of CALR (brown). Several CALR-positive cells receive GABAergic SH contacts with some boutons (arrows) around the soma of CALR-immunopositive neurons. Some complex baskets containing biocytin can be seen in distinct layers of the hippocampus; some of them contact neurons that are immunonegative for this marker (arrowhead). (G) Double detection of biocytin and calbindin (CALB) showing a GABAergic SH basket contacting the perisomatic region of CALB-positive interneurons. (H) Electron micrograph showing a biocytin-containing septal synaptic terminal (st) that establishes synapses on the cell body (cb) of a GABAergic postembedded hippocampal interneuron in the organotypic culture. (I) High-magnification electron photomicrograph showing the synaptic terminal of a medial septum neuron establishing a symmetric synapse (arrow) with the cell body of a GABA-positive interneuron. Scale bars: 1000  $\mu\text{m}$  in B; 50  $\mu\text{m}$  in C; 20  $\mu\text{m}$  in G also applies to D-F; 1  $\mu\text{m}$  in H and 50 nm in I.

Co-cultures were maintained for 5–25 days in a 37  $^{\circ}\text{C}$ , 5%  $\text{CO}_2$ , humidified atmosphere and the medium was changed three times per week. For the Sema 3C blocking experiments, neuropilin-1 ectodomain and neuropilin-2 ectodomain (Ecto-NRP1 and Ecto-NRP2)-enriched media or control medium (Pascual et al., 2004b) were applied to the cultures every time the medium was changed. For the Sema 3C experiments, the protocol was as described above except that the co-cultures consisted of septa taken from P1 wild-type mice and hippocampi taken from either Sema 3C $^{+/+}$  or Sema 3C $^{-/-}$  E18 mice. For the establishment of the co-cultures without the entorhinal cortex, this region was removed from the entorhino-hippocampal slices before plating them on the culture inserts.

#### **Detection of septohippocampal fibers in organotypic cultures**

To trace the SH component in the organotypic co-cultures, a small crystal of biocytin (Sigma-Aldrich) was placed in the MS/DB complex region of the co-culture, in order to label septal neurons projecting to the hippocampus. The culture medium was changed several times per day. After 24 h, co-cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 1 h and kept in phosphate buffer until sectioned at 30–40  $\mu\text{m}$  using a vibratome.

Biocytin immunodetection was performed to detect SH fibers in the co-cultures. After washing, sections were permeabilized with increasing concentrations of dimethylsulfoxide and blocked. Free-floating sections were incubated overnight at 4  $^{\circ}\text{C}$  with the avidin–biotin peroxidase complex (1 : 100; Vector Laboratories). Peroxidase activity was developed with 0.03% 3,3'-diaminobenzidine intensified with ammonium nickel sulfate and cobalt chloride, and 0.005%  $\text{H}_2\text{O}_2$  yielding a black reaction product. Sections were mounted onto gelatinized slides, Nissl stained when needed, dehydrated, and coverslipped with Eukitt (Panreac). In some experiments, BDA detection was combined with the detection of PARV, calbindin (CALB), calretinin (CALR), Sema 3C or GAD65/67 using the protocol described for tissue sections. The double-immunofluorescent detection of Sema 3C and PARV was performed as described above.

#### **Quantification of the septohippocampal innervation**

The complexity of the GABAergic SH baskets was assessed in the CA3 area of sections from P5, P10, P18 and adult mice, and sections from co-cultures maintained for 7, 10, 16 and 25 days in vitro (DIV). Briefly, the number of boutons per basket was quantified in some sections ( $n = 2$ –3 per condition) and the average was calculated for the in vivo and in vitro situation for each age. The percentage of hippocampal interneurons contacted by GABAergic axons was calculated in organotypic cultures lacking entorhinal cortex, in the co-cultures established for Sema 3C experiments, and in the co-cultures treated with Ecto-NRP1 and Ecto-NRP2 or control media. For this purpose, the total number of GAD65/67-, PARV- or CALR-positive cells and the number of these cells contacted by SH baskets were quantified in 14–15 DIV co-cultures ( $n = 4$ –10 co-cultures per experimental group). Cajal–Retzius, mossy and immature granular cells were excluded from CALR quantification. Overall

comparisons and posthoc two-sample Student's t-test (two-tailed) were used to examine differences between the experimental groups, with  $P < 0.05$  considered as significant.

#### **Detection of cholinergic neurons**

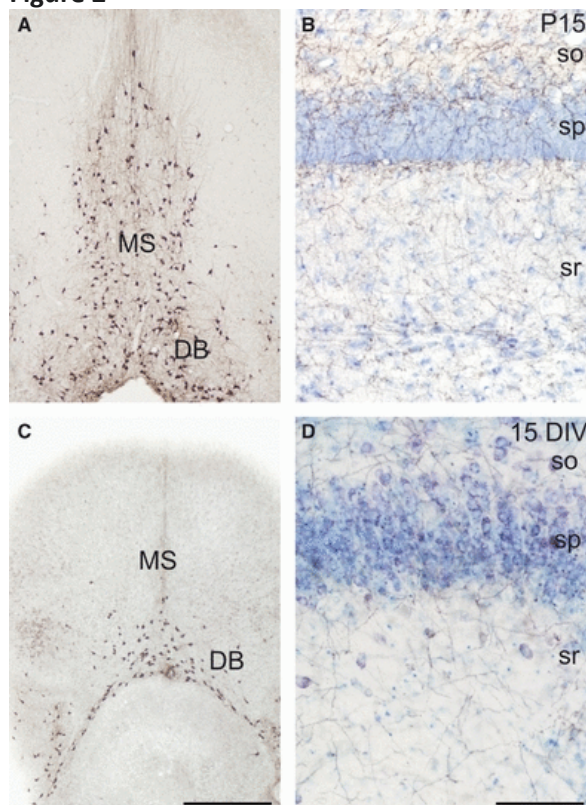
Acetylcholinesterase histochemistry was performed as described by Tago et al. (1986) with some modifications. After inactivation of the endogenous peroxidase activity, tissue was rinsed in 100 mM maleate buffer and incubated for 40 min in a solution of maleate buffer containing 20  $\mu$ M acetylthiocholine iodide, 0.002% tetraisopropyl pyrophosphoramidate (both from Sigma-Aldrich), 5  $\mu$ M potassium ferricyanide, 50  $\mu$ M sodium citrate dihydrate (both from Merck) and 30  $\mu$ M copper sulfate (Panreac). After washing in 50 mM Tris-HCl buffer (pH 7.5), the peroxidase activity of the precipitate was developed with 0.04% 3,3'-diaminobenzidine intensified with 0.3% ammonium nickel sulfate and 0.01% H<sub>2</sub>O<sub>2</sub>, yielding a black reaction product. Sections were mounted onto gelatinized slides, Nissl stained when needed, dehydrated, and coverslipped with Eukitt (Panreac).

## Results

### Target specificity in the generation and reconstruction of the GABAergic septohippocampal pathway in organotypic slice cultures

As Sema 3C-deficient mice die at around birth, we developed an *in vitro* SH slice culture model to analyze the relevance of the absence of Sema 3C in the postnatal development of the SH pathway. We obtained slices from the septal area and the hippocampus–enthorinal cortex complex from newborn mice (P0–1). The ventral septum was positioned near the fimbria of hippocampal slices to facilitate innervation of the target (Fig. 1A). After 15–25 DIV, septal fibers were traced by biocytin injection in the medial region of the septum (Fig. 1B). As described previously *in vivo* (Freund & Antal, 1988; Rocamora et al., 1996; Pascual et al., 2005), the organotypic culture showed fibers formed in the medial septum entering the hippocampus through the fimbria and invading all layers of the hippocampus, mainly the stratum oriens and the hilar region close to the granule cell layer (Fig. 1B). Closer inspection allowed us to identify two types of biocytin-labeled fibers. One group of axons was small and displayed numerous small ‘en-passant’ varicosities, these possibly corresponding to cholinergic fibers. To confirm these observations, some cultures were immunostained for choline acetyltransferase or histochemically reacted for the enzymatic detection of acetylcholinesterase. In agreement with *in vivo* studies, neurons immunoreactive for choline acetyltransferase were present in the MS/DB complex *in vitro* (Fig. 2A and C). Moreover, cholinergic fibers had similar distributions and shapes in sections from P15 brains and in SH organotypic co-cultures (Fig. 2B and D). These data indicate that the establishment of organotypic SH co-cultures leads to a pattern of cholinergic innervation similar to that observed *in vivo*.

**Figure 2**



The SH cholinergic component is maintained in the *in vitro* model. (A) Photomicrograph illustrating the distribution of cholinergic neurons in the medial septum (MS) and diagonal band (DB) of P15 mice. (B) High-magnification photomicrograph showing the cholinergic innervation (in black) of the CA3 hippocampal region in a Nissl-stained section from a P15 mouse; a myriad of thin fibers with some ‘en-



passant' boutons innervate the entire hippocampus, mainly close to the stratum pyramidale (sp). (C) Choline acetyltransferase immunodetection in an SH organotypic culture shows a similar distribution of cholinergic septal neurons as illustrated in vivo. (D) Enzymatic detection of acetylcholinesterase in a 15 DIV organotypic culture reveals a distribution of cholinergic fibers similar to the in vivo situation, with massive innervation of cholinergic septal afferents onto all of the hippocampal layers, mainly close to the pyramidal cell layer. so, stratum oriens; sr, stratum radiatum. Scale bars: 1000  $\mu$ m in C (also applies to A); 100  $\mu$ m in D (also applies to B).

We identified a second group of biocytin-labeled axons. These were thicker and formed complex baskets characteristic of the GABAergic SH component (Fig. 1C and D). Some of these baskets exhibited a high degree of complexity, resembling the perisomatic contacts described in mice (Freund & Antal, 1988; Gulyás et al., 1990; Pascual et al., 1998). To analyze the degree of specificity of GABAergic septal fibers in organotypic cultures, biocytin detection was combined with immunohistochemistry against several specific markers for hippocampal interneurons. These experiments showed that about 40% of GAD65/67-positive neurons ( $n = 25$  111 cells, 17 co-cultures) in the hippocampus received specific input from the GABAergic SH fibers (Fig. 1E). Remarkably, we did not detect SH baskets surrounding the somata of GAD65/67-negative cells, thus indicating that the GABAergic SH pathway generated in vitro maintains a high degree of target specificity for GABAergic interneurons. Further immunohistochemical analyses with the interneuronal markers CALR, PARV and CALB demonstrated that all types of hippocampal interneurons were contacted by the GABAergic SH pathway (Fig. 1F and G). These markers identify non-overlapping populations of GABAergic hippocampal interneurons.

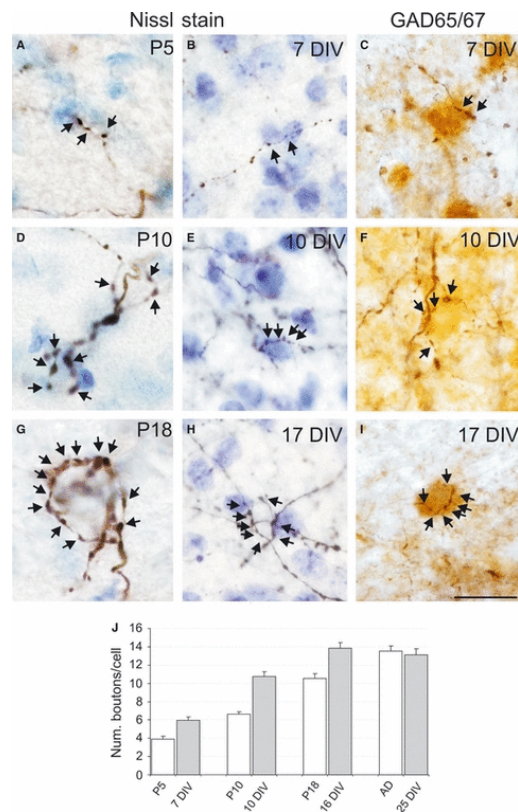
Quantitative analyses revealed that approximately 31% of PARV-positive interneurons ( $n = 3717$  cells, 6 co-cultures) and 50% of CALR-positive cells ( $n = 2221$  cells, 7 co-cultures) in the hippocampus of the co-cultures received GABAergic SH input. Finally, and to study whether GABAergic SH baskets reconstructed in vitro established synaptic contacts, we performed an immunohistochemical ultrastructural analysis, combining the pre-embedding detection of biocytin and postembedding immunogold detection of GABA. These analyses showed that the GABAergic septal axon terminal established symmetric synaptic contacts with cell bodies immunopositive for GABA antibodies (Fig. 1H and I). We conclude that the GABAergic SH pathway reconstitutes in vitro with target and synaptic specificity similar to that in vivo.

### **Pattern of postnatal maturation of the GABAergic septohippocampal pathway in vivo and in vitro, and its independence from the entorhinal cortex inputs**

Previous tracing studies in mice have shown that septal axons reach the hippocampus at around E16 (Super & Soriano, 1994). At early postnatal stages (P0–P5), SH fibers invade their target hippocampal layers, although pericellular baskets were not noticeable at these stages. To determine the onset and maturation of the GABAergic SH pathway in vitro, we first analyzed the postnatal maturation of this connection in mice at P5, P10, P18 and in adults. For this purpose, we performed iontophoretic injections with BDA in the MS/DB complex. As previously described (Pascual et al., 2005), at P5, thick septal fibers, presumably corresponding to the GABAergic SH component, invaded the hippocampus and occasionally formed incipient pericellular baskets formed by three to four boutons around the somata of interneurons (Fig. 3A and J). At P10, the number and complexity of the baskets increased throughout all hippocampal layers (Fig. 3D and J). At P18, the complexity of the GABAergic SH baskets was increased, with some formed by more than 10 boutons (Fig. 3G and J). In adult mice, complex baskets formed by an average of 14 boutons were present in all hippocampal layers but were concentrated near the main cell layers (Fig. 3J). These baskets were similar to the pericellular SH baskets described previously (Freund & Antal, 1988; Gulyás et al., 1990; Rocamora et al., 1996; Pascual et al., 1998). Thus, during early postnatal stages, GABAergic septal fibers establish specific contacts

with the perisomatic region of hippocampal interneurons, which achieve maximal complexity in adult mice.

Figure 3



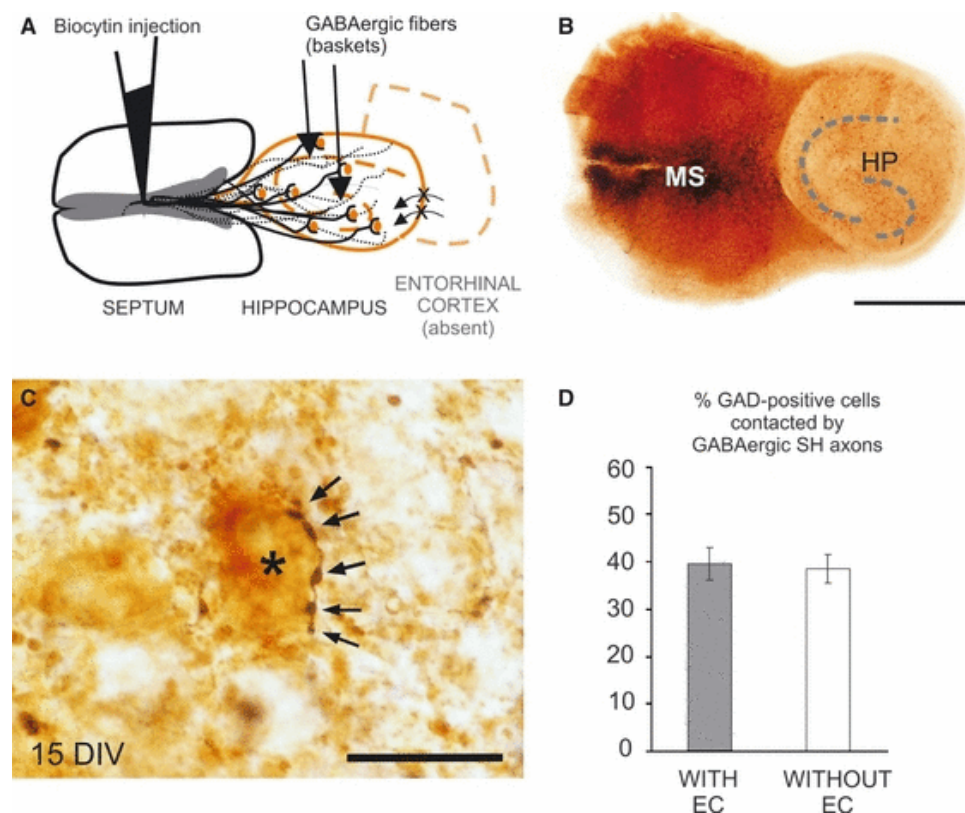
Postnatal development of the GABAergic SH contacts in vivo and in vitro. (A, D and G) In vivo tracing of the SH pathway at distinct developmental stages. (A) Photomicrograph showing incipient GABAergic SH baskets (arrows) on somata of hippocampal neurons in P5 mice. (D) At P10, the complexity has increased and prominent baskets with more and larger synaptic boutons (arrows) are established. (G) At P18, extremely complex baskets with as many as 20 synaptic boutons (arrows) around a single soma are frequent. (B, E and H) Photomicrographs of the hippocampal region of cultures simultaneously immunostained for biocytin (black) and Nissl stain or combined with GAD65/67 immunodetection (brown; C, F and I) at various time-points of the cultures. Arrows indicate synaptic boutons. (B) In 1-week-old cultures, immature baskets with few synaptic boutons (arrows) are observed. (C) These incipient baskets contact specifically with the perisomatic region of GAD65/67-positive cells. (E) In 10 DIV cultures, the complexity of the contacts has increased notably. (F) Baskets display some thick synaptic boutons (arrows) around GAD65/67-positive somata. (H) In 17 DIV cultures, the baskets are very complex. (I) GABAergic basket fibers display a large number of boutons covering GAD65/67-immunoreactive somata. (J) The number of GABAergic SH boutons per cell was quantified in the hippocampus of co-cultures at 7, 10, 17 and 25 DIV, and in the CA3 of the hippocampus of P5, P10, P18 and adult (AD) mice. Scale bar: 20  $\mu$ m in I (also applies to A–H).

In order to determine whether the temporal sequence of maturation in vitro correlates with the above in vivo pattern, we analyzed the development of the GABAergic SH pathway at 7, 10, 16–17 and 25 DIV. At 7 DIV, some thick fibers invaded the hippocampus through the stratum oriens and formed incipient baskets in distinct layers of the hippocampus, mainly close to the principal cell layers; these axons formed baskets composed of five to six boutons, which surrounded GAD65/67-positive interneurons (Fig. 3B, C and J). At 10 DIV, the number and complexity of the pericellular baskets was

markedly increased (Fig. 3E, F and J). At 16–17 DIV, GABAergic SH axons formed complex baskets containing an average of 12–14 boutons (Fig. 3H–J). Slices cultured for 25 DIV did not show apparent differences when compared with cultures aged 16 DIV. A semi-quantitative evaluation of basket complexity (number of boutons per neuron) revealed that the pattern of maturation of the GABAergic SH pathway *in vitro* was roughly similar to that observed in postnatal mice, although slightly accelerated in the *in vitro* condition (Fig. 3J).

Target-cell selection and synaptogenesis require both target recognition by specific molecules and appropriate levels of neuronal activity (Easter et al., 1985; Spitzer, 2004; Lu et al., 2009). In line with this, a previous study, in which hippocampal slice cultures did not contain the entorhinal cortex, concluded that the GABAergic SH pathway does not reconstitute in SH slice cultures (Heimrich et al., 1996). Thus, we addressed whether the high degree of target selectivity depended on entorhinal cortex inputs. To this end, we compared the formation of the GABAergic SH pathway using hippocampal co-cultures with or without the entorhinal cortex (see Fig. 4A and B). Biocytin anterograde tracing combined with GAD65/67 immunohistochemistry did not reveal any apparent difference between the two groups of co-cultures. Thus, perisomatic baskets were recognizable in both groups (Fig. 4C). To substantiate these observations, we quantified the percentage of GABAergic hippocampal interneurons contacted by septal baskets. This percentage was similar in the presence or absence of the entorhinal cortex ( $n = 13$  553 cells in 7 co-cultures and  $n = 13$  264 cells in 10 co-cultures with and without entorhinal cortex, respectively;  $P = 0.82$ , Student's *t*-test, Fig. 4D). This observation indicates that entorhinal cortex afferents do not influence the correct formation of the GABAergic SH connection.

Figure 4



Entorhinal cortex (EC) afferents are not essential for the development and target specificity of the GABAergic SH pathway. (A) Diagram showing the elements of the organotypic co-culture without the

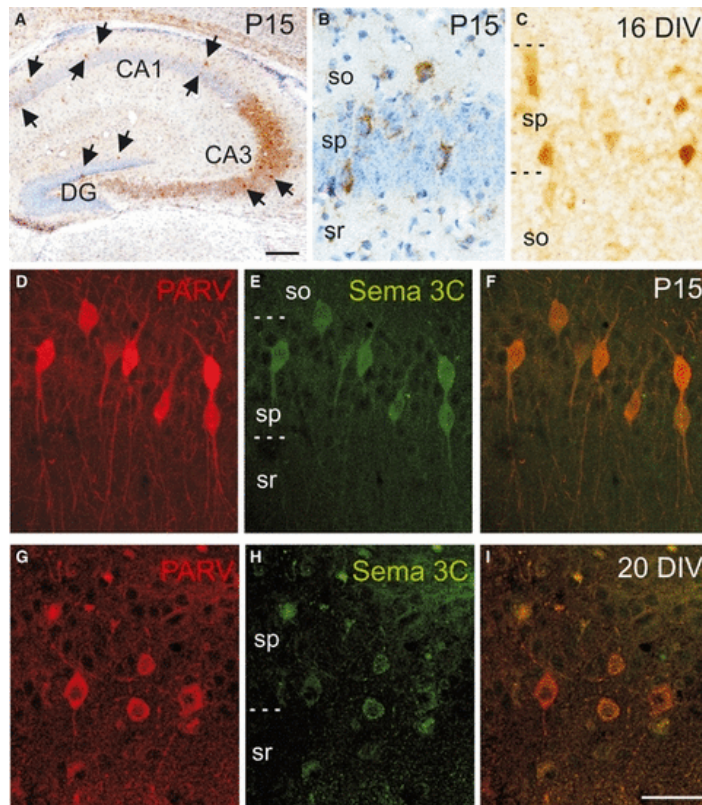
EC. The procedure is the same as in the standard culture except for the removal of the EC from the entorhino–hippocampal slice prior to culturing. As a result, the perforant pathway is removed in these cultures. (B) Micrograph showing an entire SH organotypic co-culture without the EC. Double immunohistochemical detection was performed against biocytin (black staining) and GAD65/67 (brown). (C) High-magnification micrograph showing a septal axon contacting with the somatic region of a GAD65/67-immunopositive hippocampal interneuron (asterisk) in an organotypic culture without the EC. Note the morphology of the basket contacting the interneuron, with thick synaptic boutons around the soma (arrows). (D) After immunohistochemical detection of biocytin and GAD65/67, the percentage of hippocampal interneurons received by a septal GABAergic contact was calculated. As shown in the histogram, the absence of the EC in the cultures did not affect the percentage of GABAergic hippocampal neurons contacted by septal baskets. Scale bars: 1000  $\mu\text{m}$  in B; 20  $\mu\text{m}$  in C. HP, hippocampus; MS, medial septum.

### **Semaphorin 3C is not required for the establishment and target specificity (selection) of GABAergic septohippocampal axons**

Previous data showed that sema 3C mRNA is abundantly expressed by hippocampal interneurons during the postnatal development, corresponding to the stage at which SH fibers initiate the establishment of synaptic contacts (Pascual et al., 2005). In order to substantiate the distribution of Sema 3C protein during postnatal development, we performed immunohistochemistry on P15 mouse sections. Our data showed that Sema 3C was expressed in some neurons scattered throughout all layers of the hippocampus (Fig. 5A). Sema 3C-positive neurons were located mainly in or close to the pyramidal layer and granule cell layer (Fig. 5B). We performed double-immunofluorescent detection of Sema 3C and interneuronal markers, and concluded that virtually all Sema 3C-positive neurons were also PARV-positive (Fig. 5D–F). We next examined the expression and distribution of Sema 3C in organotypic SH cultures at 16 DIV. As in the in vivo hippocampus, Sema 3C was expressed in neurons resembling interneurons (Fig. 5C). These Sema 3C-positive neurons were PARV-positive, indicating that the expression pattern of this guidance cue in vitro parallels what occurs in vivo (Fig. 5G–I).

Figure 5



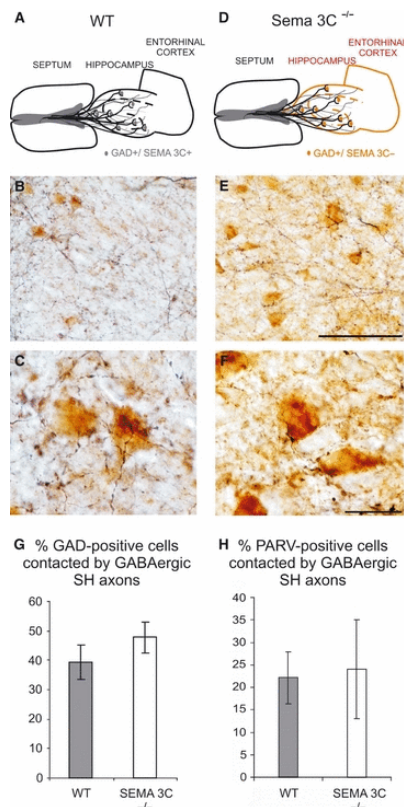


Sema 3C expression pattern at postnatal stages of development in vivo and in vitro. (A) Immunodetection of Sema 3C (brown) in a Nissl-stained hippocampus of a P15 mouse. Some hippocampal interneurons express Sema 3C (arrows). (B) A higher magnification micrograph shows Sema 3C-positive somata displaying typical interneuron morphology distributed in or close to the pyramidal cell layer. (C) Immunodetection of Sema 3C in a 16 DIV culture reveals similar morphology and distribution of the immunopositive cells compared with the in vivo situation. (D–I) Double Sema 3C and PARV immunodetection shows that most PARV-positive interneurons express Sema 3C in the hippocampus of a P15 mouse (D–F) and in a 16 DIV organotypic culture (G–I). DG, dentate gyrus; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum. Scale bars: 200  $\mu$ m in A, 50  $\mu$ m in I (also applies to B–H).

To study the involvement of Sema 3C in the formation of the GABAergic SH pathway, we co-cultured wild-type septal slices with hippocampal slices taken from Sema 3C<sup>-/-</sup> E18 embryos or their Sema 3C<sup>+/+</sup> littermates (Fig. 6A and D). After 15 DIV, the hippocampi from Sema 3C-deficient mice were innervated by septal fibers, which reached their target regions through the fimbria, innervating all hippocampal layers in a way that was indistinguishable from that seen in wild-type slices (Fig. 6B and E). Thus, in both experimental groups, axons formed complex pericellular contacts around the somata of hippocampal interneurons (Fig. 6C and F). We further quantified the percentage of GABAergic septal baskets on hippocampal interneurons in these groups. Similar percentages of hippocampal GAD65/67-positive interneurons were targets of the GABAergic SH pathway in either the presence or absence of Sema 3C ( $n = 7151$  cells in 8 co-cultures and  $n = 11558$  cells in 10 co-cultures for the Sema 3C<sup>-/-</sup> and Sema 3C<sup>+/+</sup> condition, respectively;  $P = 0.31$ , Student's t-test, Fig. 6G). We also confirmed that the PARV-positive subpopulation of interneurons, which expressed Sema 3C, was contacted in the Sema 3C-deficient mice, with no quantitative differences from their wild-type littermates ( $n = 1897$  cells in 5 co-cultures and  $n = 1997$  cells in 4 co-cultures for the Sema 3C<sup>-/-</sup> and Sema 3C<sup>+/+</sup> condition, respectively,  $P = 0.90$ , Student's t-test, Fig. 6H).



Figure 6

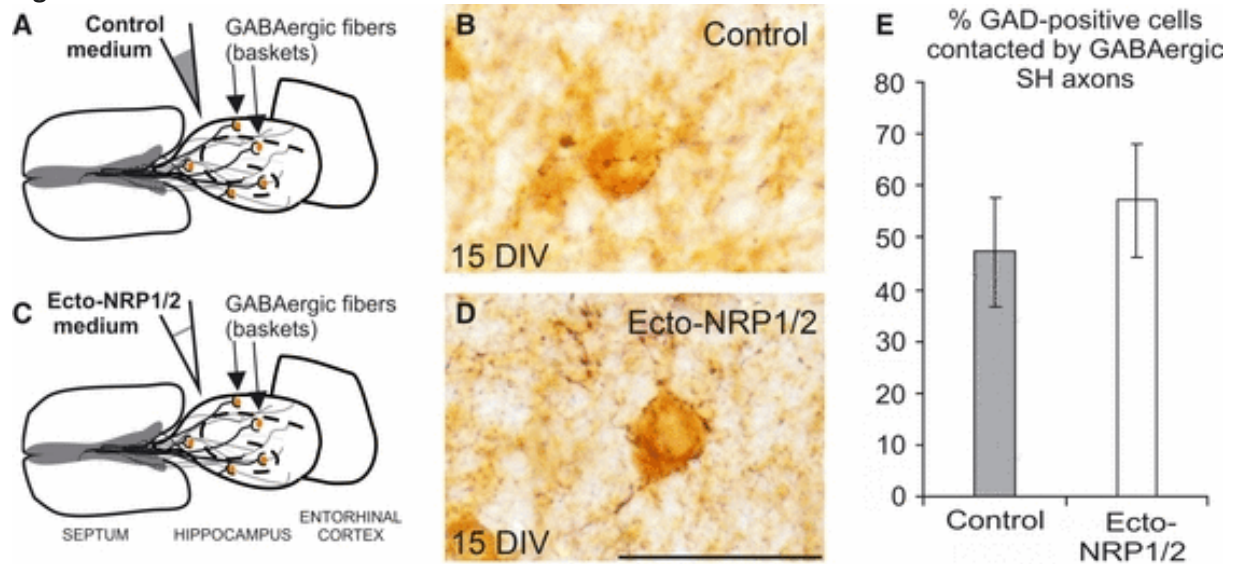


The development of the GABAergic SH connection and its target cell specificity is independent of Sema 3C expression. Comparison of the target specificity of the GABAergic SH axons in the presence [wild-type (WT), A–C] or absence (Sema 3C<sup>-/-</sup>, D–F) of Sema 3C in the hippocampus. (A) Diagram of the standard co-culture, in which GABAergic septal axons contact interneurons (gray circles) in the WT hippocampus, where Sema 3C is present. (B) Photomicrograph of a 16 DIV co-culture (biocytin staining in black, GAD65/67 in brown) displaying the dense septal innervation in the hippocampus. (C) Higher magnification micrograph shows complex SH baskets. (D) Organotypic culture including the septal region from WT mice and an entorhino–hippocampal slice from a Sema 3C<sup>-/-</sup> mouse. WT septal GABAergic axons contact GABA-positive interneurons lacking Sema 3C (orange circles). (E) The combined detection of biocytin (black) and GAD65/67 (brown) in a 16 DIV culture shows a dense innervation of the hippocampus. (F) The typical SH baskets are present in Sema 3C<sup>-/-</sup> cultures. The percentage of the total interneuronal population (GAD-positive cells) (G) or the PARV-positive interneurons (H) that are contacted by GABAergic septal axons is not affected in Sema 3C-deficient cultures in the hippocampus. Scale bars: 100  $\mu$ m in E (also applies to B); 20  $\mu$ m in F (also applies to C).

The next approach to test the role of Sema 3C in the development and maturation of the SH pathway was by functionally blocking this guidance cue with well-characterized recombinant ectodomains of its receptors, namely Ecto-NRP1 and Ecto-NRP2 (Pascual et al., 2004b). Control medium or Ecto-NRP1- and Ecto-NRP2-enriched medium (diagrams in Fig. 7A and C) were applied on top of the co-cultures. When analyzing the SH pathway in the co-cultures after the treatments, we found that the connection formed properly in both conditions. Septal axons invaded the hippocampus of the organotypic co-cultures and formed typical complex GABAergic SH baskets on the perisomatic region of interneurons (Fig. 7B and D). The percentage of the GAD65/67-positive interneurons contacted by GABAergic SH axons was not altered when Sema 3C was blocked with Ecto-NRP1 and Ecto-NRP2 ( $n = 6179$  cells in

4 co-cultures and  $n = 13$  553 cells in 7 co-cultures for Ecto-NRP1 and Ecto-NRP2 and control treatment, respectively;  $P = 0.54$ , Student's t-test; Fig. 7E). We thus conclude that Sema 3C is dispensable for the formation and maturation of the GABAergic SH pathway.

**Figure 7**



Blocking Sema 3C effects in the co-cultures does not affect the development or the target cell selection of the GABAergic SH component. Diagrams showing the application of control (A) or Ecto-NRP1 and Ecto-NRP2-enriched medium (C) in the entire co-cultures. High-magnification micrographs of 15 DIV co-cultures (biocytin staining in black, GAD65/67 in brown) allow for the detection of complex GABAergic SH baskets in the hippocampus of both control (B) and Ecto-NRP1 and Ecto-NRP2 (D) treated co-cultures. (E) Quantification of the percentage of GAD65/67-positive interneurons contacted by GABAergic SH axons reveals no differences in the cultures where Sema 3C protein is blocked (Ecto-NRP1 and Ecto-NRP2) with respect to the control. Scale bar: 50  $\mu\text{m}$  in D (also applies to B).

## Discussion

### Semaphorin 3C in the development and maturation of GABAergic septohippocampal axons

During embryonic development, distinct factors orchestrate the guidance of SH pathway fibers. Thus, whereas secreted Sema 3A and 3F are believed to chemorepel septal axons towards dorsal regions, netrin-1, expressed mainly in the fimbria, chemoattracts the same axons, thereby allowing their entry into the target hippocampus (Pascual et al., 2004b). Once GABAergic SH fibers reach their target region, little is known about the molecules implicated in their target selection specifically for the perisomatic region of the GABAergic hippocampal interneurons. In previous studies, we have shown that the formation of this pathway is independent of the expression of Reelin, an extracellular matrix protein expressed by some hippocampal interneurons (Pascual et al., 2004a). Conversely, GABAergic SH axons terminate preferentially onto nerve growth factor-positive interneurons, although their role is unknown (Rocamora et al., 1996; Pascual et al., 1998). In contrast to Sema 3A and 3F, Sema 3C is permissive for the growth of GABAergic SH axons and, at later postnatal stages, many pericellular SH baskets are formed on Sema 3C-expressing interneurons (Pascual et al., 2005). Moreover, Sema 3C has been described as either a chemoattractive or chemorepulsive molecule, depending on the types of axons tested and on the developmental stage studied (Bagnard et al., 1998; Chen et al., 1998; Niquille et al., 2009), and neurons in the MS/DB complex express Sema 3C receptors, neuropilin-1 and -2, at postnatal stages (Pascual et al., 2005). These observations prompted the present study, in which we used Sema 3C-deficient mice. However, we found that the absence of Sema 3C in the target neurons does not influence the formation of or target selection by GABAergic SH axons. The results obtained by blocking Sema 3C function in the entire co-cultures with Ecto-NRP1 and Ecto-NRP2 confirm that Sema 3C is not essential for the development and target specificity of the GABAergic SH pathway. We conclude that the development, maturation and synaptic connectivity of the GABAergic SH pathway occur in a Sema 3C-independent manner. The present findings, in conjunction with the observation that hippocampal interneurons express different combinations of growth factors and guidance molecules (Rocamora et al., 1996; Alcántara et al., 1998; Pascual et al., 1998), suggest that the specificity of the GABAergic SH pathway is the result of either redundant axonal guidance-related mechanisms or a still unknown crucial functional cue.

### Robust reconstruction of the GABAergic septohippocampal pathway in in vitro slice cultures

Here we set up an in vitro culture model of the GABAergic SH pathway, by using organotypic slice co-cultures. The in vitro model retains the target cell specificity of the GABAergic SH pathway and the formation of specific symmetric synaptic contacts in the perisomatic compartment. The present data obtained in vitro, particularly the percentage of interneurons contacted by GABAergic SH axons, are highly coincident with those reported in vivo for GAD65/67- and PARV-positive neurons (around 40 and 30%, respectively; Rocamora et al., 1996 and our unpublished results). However, we found that the percentage of CALR-positive neurons contacted by the GABAergic SH component in vitro is higher than that in vivo (50% vs. 25%, respectively; Rocamora et al., 1996 and our unpublished results). Some specific features of the slice co-cultures (e.g. differential neuronal survival in vitro and in vivo, altered temporal expression of some calcium-binding proteins) might account for these differences.

Regarding the temporal development of the connection in the model described here, the time-course of maturation of the GABAergic SH baskets is slightly accelerated compared with the in vivo situation but, in 3–4 weeks, they reach an identical degree of complexity to that seen in vivo. Previous studies have shown that neuronal activity-dependent mechanisms shape the fine tuning and complexity of most connections in the brain (Wang et al., 2007; Huang & Scheiffele, 2008; Huang, 2009; Blankenship & Feller, 2010). To keep the hippocampal activity as physiological as possible, our organotypic SH cultures included the entorhinal cortex, and thus entorhino–hippocampal input (Del Rio et al., 1997; Del Rio & Soriano, 2010), which is critical for the main trisynaptic hippocampal pathway (Chrobak et al., 2000; Canto et al., 2008). However, we found that the formation of the GABAergic SH pathway is independent of the entorhinal cortex. This observation suggests that the expression and biological activity of the factors responsible for orchestrating target neuron selection by GABAergic SH axons are not controlled by the synaptic activity driven by entorhinal afferents. Furthermore, the GABAergic SH

fibers formed complex pericellular baskets in our in vitro co-culture model, reaching a similar level of complexity to that in adult mice. This finding suggests that the in vivo activity-dependent related mechanisms required to finely sculpt the GABAergic perisomatic baskets were preserved in the in vitro model.

Similarly, we also show that the cholinergic SH pathway develops in a correct manner in vitro, with an axonal distribution similar to that observed in vivo (Woolf et al., 1984; Frotscher & Léránth, 1986). Taken together, our results provide evidence that the development and maturation of the GABAergic SH pathway rely on robust molecular mechanisms that are retained in our in vitro co-culture model. By terminating on most types of hippocampal interneurons, the GABAergic SH pathway is believed to inhibit their activity, thereby allowing the firing of large numbers of pyramidal neurons (Freund & Antal, 1988; Tóth et al., 1997). Such disinhibition is believed to allow the synchronous activity of large areas of the hippocampus, thereby permitting the regulation of rhythmic activities, including theta and gamma oscillatory activity (Buzsáki, 2002; Hangya et al., 2009). Furthermore, the GABAergic SH pathway and the theta and gamma rhythms are largely preserved during phylogeny, including humans (Stewart & Fox, 1991; Tesche & Karhu, 2000), and are believed to be involved in complex cognitive functions such as learning and memory and in rapid eye movement sleep, the disruptions of which have been associated with distinct stages of disease, including Alzheimer's disease (Prinz et al., 1982; Buzsáki, 2002; Cantero et al., 2003). Thus, the high degree of target specificity, the addressable manipulation of the system and the accessibility to several techniques of the co-culture model developed here lead us to propose that our model would greatly contribute to research not only into the development of the GABAergic SH pathway, but also into functional parameters related to its physiological significance.

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## Abbreviations

- BDA
- biotinylated dextran-amine
- CALR
- calretinin
- DIV
- days in vitro
- E
- embryonic day
- Ecto-NRP1
- neuropilin-1 ectodomain
- Ecto-NRP2
- neuropilin-2 ectodomain
- GAD
- glutamic acid decarboxylase
- MS/DB
- medial septum/diagonal band
- P
- postnatal day
- PARV
- parvalbumin

- Sema
- semaphorin
- SH
- septohippocampal