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Función de Netrina1 y Semaforinas secretables en la guía neuronal y axonal en el hipocampo y el cerebelo

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RESULTADOS

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CAPÍTULO I

Netrina1 ejerce un efecto quimiorepulsivo independiente de Dcc en interneuronas cerebelares en migración

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Resumen

Las interneuronas GABAérgicas de la corteza cerebelar han sido poco estudiadas. Datos recientes demuestran que su desarrollo empieza en el epitelio germinal cerebelar, situado encima del cuarto ventrículo, y continúa en la sustancia blanca del cerebelo a edades postnatales, desde donde migran hasta su posición definitiva en la corteza del cerebelo. Sin embargo, todavía no se sabe con certeza como las interneuronas realizan esta última etapa del viaje a través de la corteza cerebelar. Este estudio muestra que Netrina1 tiene un efecto quimiorepulsivo en interneuronas cerebelares postnatales in vitro, y el patrón de expresión de Netrina1 y de sus receptores Dcc y Unc5h sugiere una posible función de Netrina1 en la migración de interneuronas cerebelares in vivo. Además, los resultados obtenidos en este estudio señalan a las fibras de la glía de Bergmann como posibles sustratos para la migración de las interneuronas hacia la capa molecular del cerebelo. Finalmente, experimentos con anticuerpos bloqueantes demuestran que Dcc, aunque es expresado por interneuronas cerebelares a edades postnatales, no está implicado en la respuesta repulsiva desencadenada por Netrina1

Netrin-1 exerts a chemorepulsive effect on migrating cerebellar interneurons in a Dcc-independent way

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Netrin1 repels migrating cerebellar interneurons

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1.1. Abstract

Few studies have addressed GABAergic interneurons in the cerebellar cortex. Recent data show that their development starts at the cerebellar germinal epithelium on top of the fourth ventricle. These interneurons continue to develop in the postnatal cerebellar white matter and later migrate to their final position in the cerebellar cortex. Here we report the chemorepulsive action of Netrin1 on postnatal cerebellar interneurons in vitro, and also show the expression pattern of Netrin1 and its receptors Dcc and Unc5h. Our expression results further suggest that Netrin1 is involved in the migration of GABAergic interneurons in vivo. Moreover, our data point to Bergmann glial fibres as possible tracks for these cells en route to the molecular layer. Finally, experiments using blocking-antibodies allow us to conclude that Dcc, although expressed by postnatal cerebellar interneurons, is not involved in the repulsive response triggered by Netrin1 in these cells.

1.2. Introduction

Inhibitory interneurons in the cerebellum comprise mainly five cell types: stellate and basket cells, located in the molecular layer; Golgi and Lugaro cells, present in the internal granular layer (IGL); and candelabrum cells, found in the Purkinje cell layer (Laine and Axelrad, 1994; Sotelo, 2004). Using genetic fate-maps, it was recently reported that cerebellar inhibitory interneurons (along with Purkinje cells and part of the deep nuclear neurons) originate in a portion of the neuroepithelium of the fourth ventricle defined by the expression of Ptf1a (Hoshino et al., 2005). Analysis by means of retroviral injections into cerebellar folia of postnatal rats showed that molecular layer interneurons derive from a pool of progenitor cells in the postnatal white matter (Zhang and Goldman, 1996a; Zhang and Goldman, 1996b). Thus, precursors of interneurons migrate through the cerebellar anlagen along with post-mitotic Purkinje cells, and during the first two weeks of postnatal life, continue to divide in the white matter before migrating to their final position in the cerebellar cortex. Like the EGL (external granular layer), postnatal white matter could be considered a secondary proliferative zone (Schilling, 2000; Sotelo, 2004). However, little is known about how cerebellar interneurons migrate towards their final destination and the guidance cues involved in this process.

The protein family of Netrins confers directional cues to elongating axons in several regions of developing central nervous system. Netrin1 acts as an attractive or repulsive factor for axons from several regions of the central nervous system, including spinal commissural, hippocampal, retinal, thalamic and dopaminergic axons (Barallobre et al., 2000; Braisted et al., 2000; Colamarino and Tessier-Lavigne, 1995; Kennedy et al., 1994; Lin et al., 2005; Shewan et al., 2002). Netrin1 acts through the Dcc (*Deleted in Colorectal Cancer*) and Unc5h receptor families. By alternative splicing, *Dcc* encodes several type I glycoproteins that mediate Netrin1 attraction (Mehlen and Mazelin, 2003). The Unc5h protein family (homolog of *C.elegans* UNC5) comprises type I transmembrane proteins involved in long-range chemorepulsive events triggered by Netrin1. Amongst members of this family, Unc5h1, 2 and 3 bind Netrin1 in vitro (Mehlen and Mazelin, 2003). Netrin1 binds specific extracellular domains of Dcc and UNC5 (Geisbrecht et al., 2003). Although Dcc elicits attractive responses after binding to Netrin1, the presence of Unc5h is sufficient to turn attraction into repulsion (Hong et al., 1999). Other data indicate, however, that *Drosophila* UNC5 alone has the capacity to mediate repulsion (Keleman and Dickson, 2001).

In addition to its accepted role in axon guidance, Netrin1 has been implicated in the migration of several cell types. When expressed in the ventricular zone, this protein is a repulsive factor for subventricular striatal neurons and spinal oligodendrocyte precursors (Hamasaki et al., 2001; Jarjour et al., 2003) and an attractive cue for oligodendrocyte precursors migrating from the optic quiasm to the retina (Spassky et al., 2002). Experimental data obtained in vitro show the effect of Netrin1 on distinct cerebellar cells: it repels postnatal EGL neurons and attracts embryonic pre-cerebellar precursors from the lower rhombic lip (Alcantara et al., 2000; Yee et al., 1999). Moreover, in Netrin1 knock-out mice, several precerebellar nuclei are reduced in size or missing (Sotelo, 2004). Some of these studies have shown that Unc5h and Dcc are expressed in cells responding to Netrin1, and that this response is abolished by antibodies blocking Dcc (Hamasaki et al., 2001; Jarjour et al., 2003; Tsai et al., 2003).

Here we report that Netrin1 has a repulsive effect on cerebellar interneurons in vitro. We also show that both the time and pattern of Netrin1 expression correlate well with a possible role of this protein in guiding cerebellar interneurons in vivo. Furthermore, we demonstrate that although interneurons and their precursors express *Dcc* as well as several members of the Unc5h protein family and also *Netrin1*, the chemorepellent effect exerted by Netrin1 does not depend on Dcc function. Data presented in this study also indicate that Bergmann glia is used by molecular layer interneurons as a migration pathway to reach their final positions.

1.3. Results

1.3.1. Netrin1 has a chemorepulsive effect on migrating cerebellar interneurons.

During early postnatal development, interneurons destined to cerebellar cortex arise from progenitor cells located in the white matter, where they proliferate and migrate to their appropriate layer before differentiating into basket, stellate, and Golgi cells (Zhang and Goldman, 1996a; Zhang and Goldman, 1996b). During this period, Netrin1 and its receptors Dcc, Unc5h2 and Unc5h3 are expressed

in the cerebellum (Alcantara et al., 2000; Livesey and Hunt, 1997). We tested whether Netrin1 influences migration of interneurons from cerebellar cortex using the 3D collagen matrix assay system (Lumsden and Davies, 1986). Explants containing all layers of cerebellar cortex, except the EGL (i.e. IGL, Purkinje cell layer, molecular layer and white matter) were dissected out of postnatal mouse cerebella (P2, P5 and P8), and co-cultured in collagen matrices in front of aggregates of control EBNA-293 cells or cells secreting Netrin1. We used GABA and Pax2 as two markers for visualizing migrating interneurons in explant cultures. In the cortex of murine postnatal cerebellum, Pax2 protein is present only in the interneuron cell population, including its progenitors in white matter, and disappears by the time functional synaptic contacts with Purkinje cells are established (Maricich and Herrup, 1999).

In the three postnatal ages examined, explants from cerebellar cortex in front of control cells exhibited robust radial migration of cells stained either with anti-GABA and anti-Pax2 antibodies, sometimes more than 100 μm from the explant (Fig.1). However, in explants co-cultured with cells secreting Netrin1, both GABA+ and Pax2+ cells migrated preferentially in the opposite direction to the source of Netrin1 (Figs. 1 and 2). There was clear chemorepulsion even inside the explant, where cells tended to accumulate in the distal part (Figs. 1H and 2C, D). This asymmetrical pattern of migration of GABA and Pax2 cells was similar in explants from P2, P5 and P8 cerebella, with similar intensity in all three ages, and in both GABA- and Pax2-labelled interneuron population (Fig. 2). The fact that GABA and Pax2 cells have the same behaviour in front of a Netrin1 source indicates that postnatal cerebellar interneurons probably acquire responsiveness to Netrin1 early in their developmental program, when they are still precursors in the white matter and start to migrate through cerebellar cortex. To sum up, these data indicate that Netrin1 exerts a chemorepulsive effect on migrating cerebellar GABA and Pax2 cells, and that this effect occurs in a period when, in vivo, cerebellar interneurons are migrating to their final position.

1.3.2. Migratory pathways of cerebellar interneurons

Cells in cerebellum follow two main modes of migration: a radial mode is observed either in Purkinje cells and interneuron progenitors of the cerebellar anlage, or granular cells in postnatal cerebellum, and requires radial glial cells. The second is the tangential mode, shown by precerebellar neurons in the rhombic lip and cells in the EGL, which implies neurophilic mechanisms of migration (Alcantara et al., 2000; Hatten and Heintz, 1995; Sotelo, 2004; Yee et al., 1999). Little is known, however, about how postnatal progenitors in cerebellar white matter reach their final position. Explants from P5 mouse cerebella were placed in collagen gel matrices and after 48-72 h of incubation were double-labelled with anti-GABA and anti-GFAP antibodies in order to visualize interneurons and glial fibres, respectively. Numerous GABA+ cells left the explants, many gathering in GFAP+ fibre bundles (Fig. 3C); when cultures were analysed at high magnification, we observed cells with bodies apposed to single GFAP+ fibres displaying several processes that often almost totally superimposed glial extensions (Fig. 3-D). These findings are further supported by the multiple colocalization points in single confocal sections (Fig. 3 E-H). Thus, in vitro, cerebellar interneurons attach to glial cells and emit processes that faithfully follow glial cell fibres.

To examine whether cerebellar interneurons also associate with glial cells in vivo, we performed fluorescence immunostaining against Pax2 on postnatal brain sections from mice expressing GFP under the control of the GFAP promoter (Zhuo et al., 1997). In this mouse strain, all cells from astroglial lineage emitted green fluorescence, and we observed labelled soma and processes in all layers of postnatal cerebella, including fibre palisades across cerebellar cortex which belongs to Bergmann glia. When stained sections were visualized at high magnification, Pax2+ cell bodies were observed throughout the cerebellar cortex and white matter (Fig. 3, I-L). These bodies were intermingled with somata and fibres of glial cells in white matter, the IGL and Purkinje cell layer (PCL). In the incipient ML, many Pax2+ cells were close to Bergmann glial fibres, some displaying elongated nuclei, which are characteristic of migrating cells (Fig. 3-J).

Therefore, the spatial proximity of Bergmann glial processes and Pax2+ nuclei in vivo indicates a possible association between glial fibres and interneurons of the ML. To further ascertain this observation, we used brain sections from heterozygous double transgenic Ptf1a-cre/+;R26R/+ mice, which express β-galactosidase specifically in the GABAergic neuronal lineages of the cerebellum, i.e. Purkinje cells and cerebellar interneurons in the cerebellar cortex, and interneurons in the deep nuclei (Kawaguchi et al., 2002; Soriano, 1999). Double-labelling immunohistochemistry against β-galactosidase and GFAP was performed on P5 brain sections from Ptf1a/+;R26R/+ mice to visualise

GABAergic interneurons and glial processes of the cerebellar cortex (Fig. 4). Many β-galactosidase+ interneurons were detected in the ML and were close to radial GFAP+ extensions (Fig. 4 B). Again, this close association between interneurons and glial processes was corroborated by the numerous colocalization sites observed in single confocal sections (Fig. 4 C-G). Thus, the results obtained in vivo further demonstrate the capacity of cerebellar interneurons observed in vitro to associate with glial cells, and points to the possibility that GABAergic interneurons, like granular cells, use Bergmann glial palisades to ascend through the ML.

Finally, to confirm that the repulsive action of Netrin1 observed in vitro on GABA+ and Pax2+ cerebellar cells was not due to an indirect effect on glial cells, we cultured explants from P5 murine cerebella confronted to aggregates of control- and Netrin1 secreting-EBNA 293 cells in collagen matrices, and immunostained them against GFAP. No difference in the outgrowth of GFAP+ fibres between the two conditions was detected, the distribution being radial in all explants (Fig. 3A-B).

1.3.3. Expression of Netrin1 and its receptors in postnatal cerebellum

Next we performed non-radioactive in situ hybridisations to study the expression pattern of *Netrin1* and its receptors *Unc5h2, Unc5h3* and *Dcc* during postnatal cerebellar development (Fig. 5, A-L). At P0, *Netrin1* was strongly expressed in the EGL, and by a population of cells spreading over the cortex, a few of these cells also located in white matter. This expression pattern was maintained at P5, with a strong label in the IGL, PCL and incipient ML. At P10, expression was prominent in the cortex, with continuous label in the EGL, IGL and white matter (less intense than in the EGL), and numerous discrete cells strongly stained in ML and PCL layers were observed. *Unc5h2* expression was strong at P0, mainly in the EGL but also with less intensity in the rest of the folia, and showed discrete labelling mostly in the white matter. At P5, sections displayed robust and continuous labelling in the EGL and IGL, and slight and puctual staining in the PCL and white matter; this pattern remained unchanged until P10. *Unc5h3* was expressed at P0 in the overall extension of the cerebellar cortex and white matter, with greater intensity in the EGL; punctual labelling was also observed throughout all layers. At P5, label was strong in the cortex and diminished in the PCL and more central parts of white matter; discrete positive cells were sparsely distributed throughout the ML and IGL, and showed strong concentration in the lower part of the PCL. This pattern was maintained at P10. *Dcc* expression was intense in the EGL at P0, and much weaker in the rest of the layers, where punctual, sparse label was detected. At P5, punctual labelling occurred in white matter, and in the PCL few cells were stained. At later stages, evident labelling of the EGL and IGL was observed while the PCL and ML showed weaker staining, and white matter was almost devoid of stain. Taken together, expression data suggest that Netrin1 and its receptors influence migrating interneurons in the postnatal cerebellum.

To further confirm the expression of *Unc5h2*, *Unc5h3* and *Dcc* in interneurons, we studied the co-expression of Netrin1 receptors and Pax2 protein in vivo by double non-radioactive in situ hybridisation and peroxidase immunohistochemistry (Fig. 5, M-P). These three genes were expressed by Pax2+ cells, and in addition, in cells stained with anti-Pax2 antibody also were labelled with Netrin1 riboprobe. Therefore, in vivo, the expression pattern and co-expression results highlight the function of Netrin1 and its receptors in migration and positioning of cerebellar interneurons.

1.3.4. Anti-Dcc antibodies do not block the repulsive effect of Netrin1 on migrating interneurons

Previous studies report the requirement of the Dcc and the UNC-5 family receptors for the chemorepulsive events mediated by Netrin1, whereas Dcc family members are sufficient for attractive responses (reviewed in Barallobre et al., 2005). In addition, antibodies against the extracellular domain of Dcc prevent the repulsive response of *Xenopus* spinal axons to Netrin1 (Hong et al., 1999).

Since cerebellar interneurons express Netrin1 receptors *Dcc* and *Unc5h2, Unc5h3* during postnatal development, we performed receptor-blocking experiments, culturing explants confronted to aggregates of control- and Netrin1-secreting EBNA 293 cells in collagen matrices and applying anti-Dcc antibodies (Barallobre et al., 2000; Hamasaki et al., 2001; Tsai et al., 2003)). Surprisingly, we did not find significant differences between Netrin1-confronted explants exposed either to control anti-GST or blocking anti-Dcc antibodies (Figs. 6 and 7). In both situations, GABA+ cells migrated away from Netrin1-expressing cell aggregates, compared to explants in front of control cell aggregates, where

GABA +cells show roughly radial distribution inside and outside the explants. Initially, 10 µgr/ml of dialysed antibody was used, as at this concentration Hamasaki and colleagues (Hamasaki et al., 2001) obtained a significant reduction of the Netrin1 chemorepellent action on striatal subventricular zone cells in vitro. Nevertheless, we doubled the initial antibody concentration used in our experiments. At 20 µgr/ml, blocking anti-Dcc antibodies failed to suppress, or even to reduce, the repulsive effect of Netrin1 on cerebellar interneurons and this concentration had no effect on cell capacity to migrate. We then tested the effect of dialysed Dcc function-blocking antibody on CA3 explants dissected from embryonic murine hippocampi, where it abolishes the chemoattractive action of Netrin1 on CA3 axons (Barallobre et al., 2000). A dose of 2 µgr/ml of anti-Dcc blocking antibody solution was sufficient to suppress the chemoattraction exerted by Netrin1-expressing cells on CA3 axons (Fig. 6).

Thus, the results from Dcc-blocking experiments indicate that Netrin1 binding to Dcc is not essential in promoting the repulsion of cerebellar interneurons, and suggest that in order to trigger a chemorepellent response on those cells, Netrin1 binds to another receptor, which probably belongs to the UNC5 family.

1.4. Discussion

Several studies support the notion that cerebellar interneurons are generated in the primary germinal layer in the roof of the fourth ventricle along with Purkinje and glial cells, and during the first two postnatal weeks in mouse, they migrate, some still as undifferentiated cells, from the white matter until reaching their definitive position in the cerebellar cortex (Yamanaka et al., 2004; Zhang and Goldman, 1996a; Zhang and Goldman, 1996b). However, little is known neither about the guidance cues that direct interneurons in the postnatal cerebellum nor about the specific pathways that these interneurons take towards their final destination. Here we show that a member of Netrin family of guidance proteins has a repulsive effect on migrating cerebellar interneurons in vitro. Moreover, the expression pattern of *Netrin1* and of Netrin1 receptors *Dcc, Unc5h2* and *Unc5h3* in postnatal cerebellum and the co-localization of these proteins with the interneuron marker Pax2 further strengthens the hypothesis that Netrin1 participates in the migration of interneurons in vivo. In addition, we provide evidence that interneurons associate with glia during their migration. Finally, our results suggest that the Netrin1 receptor Dcc does not participate in the chemorepulsive response of cerebellar interneurons triggered by Netrin1.

1.4.1. Netrin1 acts simultaneously as a short- and long-range repulsive cue

Previous studies have already pointed to Netrin1 as a factor that confers directionality to several types of migrating cells; for instance, Netrin1 repels oligodendrocytes and striatal cells from the ventricular zone during embryonic development (Hamasaki et al., 2001; Jarjour et al., 2003; Spassky et al., 2002; Tsai et al., 2003); and in the cerebellar system, Netrin1 repels cells of postnatal EGL and attracts precerebellar pontine and olivary cells originated in the rhombic lip (Alcantara et al., 2000; Bloch-Gallego et al., 1999; Serafini et al., 1996; Sotelo, 2004; Yee et al., 1999). In the examples mentioned above, cells manifesting responsiveness to Netrin1 are expected to leave a particular layer or position as the first step of a migration process; and it is indeed in this same layer or adjacent to it, where Netrin1 is expressed and functions as a long-range repulsive cue, thus pushing cells out of a specific compartment and forcing them either to start or to continue through a precise migration program that will eventually bring them to their final position.

However, cerebellar interneurons move in the opposite direction to granule cells (also undergoing active migration in the postnatal period), that is to say, they migrate towards the EGL, where Netrin1 shows strongest expression (Alcantara et al., 2000). Therefore, it appears that the same cue affects, at the same time and in the same region, two cellular populations that are moving in opposite directions, and that this is achieved by chemorepulsion in both cases. We propose, as the most plausible explanation, that Netrin1 plays distinct roles depending on a differential sensitivity of responding cells to a given concentration of this protein: as a long-range chemorepellent cue on granule cells, making them leave the proliferative EGL and start the journey through the cortex down to the IGL; and as a restrictive, short-range cue, that will determine a inhibitory territory for the migrating interneurons, thus preventing them from occupying abnormal positions inside the EGL (Fig. 8). This hypothesis has already been proposed for developing trochlear motor axons and for granule Purkinje cells in the embryonic cerebellum (Colamarino and Tessier-Lavigne, 1995; Przyborski et al., 1998).

1.4.2. Other guidance factors may affect interneuron migration

In addition to the putative role of Netrin1 in guiding interneurons through the developing cerebellar cortex, there must be other cues that confer directionality to the migrating interneurons towards the molecular layer. In this regard, the extracellular matrix-like protein Reelin, which is produced by pre-migratory granule cells (Miyata et al., 1996), has already been proposed as a key factor for the appropriate positioning of Purkinje cells (Jensen et al., 2002), and its correct alignment to form a monolayer (Miyata et al., 1997). Moreover, the Reeler mouse shows alterations in cerebellar lamination that also affect the position of interneurons (Takayama, 1994). Similarly, members of the Ephrin-Eph family of proteins might participate in the inward migration of granule cells and in the pre-migratory zone of the EGL in chick development (Karam et al., 2000). Sema3A expressed by Purkinje cells collapses and repels cerebellar mossy fibres (Rabacchi et al., 1999), and mice deficient in Sema6A show anomalous migration of granular cells (Kerjan et al., 2005). Any of these factors may be a good candidate as a guidance cue for cerebellar interneurons. In addition, several studies on developing cerebellum suggest that granule cells attract Purkinje cells and cerebellar interneurons (Goldowitz et al., 2000; Miyata et al., 1997; reviewed in Schilling, 2000).

1.4.3. Dcc is not involved in the Netrin1-dependent response of cerebellar interneurons

Two families of receptors have been implicated in transducing Netrin1 signal: Dcc, which mediates attractive signals, and UNC5 proteins, which in combination with Dcc, trigger repulsive actions (reviewed in Barallobre et al., 2005). However, UNC5 proteins can act independently of Dcc in chemorepelling cells in *C.elegans* (Merz et al., 2001) and axons in *Drosophila* (Keleman and Dickson, 2001). Our results corroborate these previous studies. Blocking anti-Dcc antibodies did not prevent cerebellar interneurons from migrating against the source of Netrin1 in vitro, thus pointing to the possibility that Dcc does not intervene in the chemorepulsive action of Netrin1 in these cells. Moreover, the present findings reinforce the hypothesis proposed above, namely that Netrin1 has a short-range role on migrating cerebellar interneurons since it has been demonstrated in *Drosophila* that UNC5 proteins alone mediate short-range chemorepulsive events triggered by Netrin1, whereas they require Dcc for generating long-range chemorepulsive responses (Keleman and Dickson, 2001). Nevertheless, further studies should be performed to directly demonstrate the participation of UNC5 family members in the repulsive action of Netrin1 on migrating cerebellar interneurons.

1.4.4. Interneurons co-express *Netrin1* and its receptors *Dcc* and *Unc5h*

In mouse tissue sections, we found that cerebellar interneurons express Netrin1 receptors *Dcc*, *Unc5h2* and *Unc5h3* during the first two postnatal weeks. This observation strongly supports the hypothesis that Netrin1 exerts a chemorepulsive action on these cells in vivo, which has already been demonstrated in vitro. Cerebellar interneurons, however, also express *Netrin1* in vivo, which is coincident with the expression of Netrin1 receptors by the same cell population. Although further experimental evidence is required, it is plausible to assume that, during the time that immature interneurons are migrating and differentiating, the same cell expresses Netrin1 and its receptors, and is thus sensitive to Netrin1 in an autocrine or paracrine way. This hypothetical autocrine/paracrine repellent effect of Netrin1 could be to help interneurons to distribute homogeneously throughout the developing molecular layer. In this regard, supporting evidence has already been reported in cultures of cerebellar microexplants, where interneurons repel each other by contact with their growth cones, thus distributing equally in culture with time (Magyar-Lehmann et al., 1995). In addition, Netrin1 may also contribute to the pathfinding of interneuron axons inside the molecular layer, making them avoid incorrect targets (i.e. other interneurons). Another explanation for this simultaneous expression of ligand and receptor would imply the dependence function of Dcc and Unc5h receptors; in this case, Netrin1 would act in an autocrine fashion to prevent apoptosis, so a cell or a group of cells that stop producing Netrin1 would enter the apoptotic program. In support of this argument, during the first three postnatal weeks, approximately 6% of total GABAergic cells in the cerebellar cortex undergo apoptosis (Yamanaka et al., 2004).

1.4.5. Bergmann glia and other supporting elements for interneuron migration

Present knowledge about the routes taken by travelling cells in the developing cerebellum has been acquired mainly in two extensively studied cerebellar cell types: Purkinje and granule cells. Purkinje cells move through the embryonic cerebellar anlage along the radial glial system (Hatten, 1999; Yamada and Watanabe, 2002). During the first two postnatal weeks, granule cells in the cerebellar surface move inwards by means of Bergmann glial fibres to form the IGL (reviewed in Sotelo, 2004). Nevertheless, the migration of interneurons has been treated superficially. It has been proposed that, during early postnatal development, both radial glia and parallel axons from granule cells are candidates as tracks for immature interneurons (Schilling, 2000; Zhang and Goldman, 1996a; Zhang and Goldman, 1996b). However, whether they served as redundant substrates, or whether one of them is preferentially chosen has not been ascertained. Our results suggest that migrating interneurons use Bergmann glial scaffold, although we cannot exclude the possibility that, in our in vitro model of explant cultures, GABA+ cells associate with parallel axons, since the explants contained IGL (but not EGL). Neither do data obtained from cerebellar microexplants clarify this issue since they point to the presence of a range of substrates that support interneuron migration in vitro (i.e. parallel axons as well as astroglial fibres) (Koscheck et al., 2003; Magyar-Lehmann et al., 1995). Furthermore, when granule cell migration in vivo is chemically disrupted, interneurons successfully migrate through the cerebellar cortex without the presence of parallel fibres (Schilling, 2000).

Recently, Mathis and colleagues (Mathis et al., 2003) added a new candidate to the list of possible substrates for interneuron migration. Selective oligodendrocyte ablation yields defective interneuron migration within the first postnatal week; thus, immature interneurons may use myelinated axons (Purkinje cells axons, climbing and mossy fibres) as tracks in their route towards the molecular layer. Considering all the evidence, it is tempting to propose a model of migration for cerebellar interneurons, where progenitors in white matter use myelinated Purkinje cells and/or climbing axons to reach the Purkinje cell layer and, from there on, they move along Bergmann glial fibres and/or parallel axons of granule cells to reach the correct position inside the molecular layer (Fig. 8).

1.5. Materials and Methods

1.5.1. Animals and histology

OF1 embryos (E14) and postnatal mice (Charles River, Lyon, France) were used for the explant cultures, immunohistochemical procedures and non-radioactive in situ hybridizations. Glial fibrillary acidic protein (GFAP)/green fluorescent protein (GFP) transgenic mice (Zhuo et al., 1997) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and used at postnatal ages for immunohistochemical procedures. To label the lineage of cerebellar interneurons, we used heterozygous double transgenic Ptf1a-cre/+;R26R/+ mice obtained from crossing heterozygous mice of the $Ptf1a^{tm(cre)Wri}$ (Ptf1a-cre) knock-out strain (Kawaguchi et al., 2002) with heterozygous mice of the $Gt(ROSA)26Sor^{TM1Sor}$ (R26R) transgenic strain (Soriano, 1999). Ptf1a-cre/+;R26R/+ mice were used at P5, P8 for immunohistochemical procedures. In all mouse strains used, the mating day was considered E0 and the day of birth P0. Animals were kept under controlled temperature (22 ±2°C), humidity (40-60%), and light (12 h cycles) and treated in accordance with the European Community Council Directive (86/609/EEC). Animals were anesthetized with ketamine-xylazine injections (150 and 6 µgr/gr, respectively) and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.3. Brains were removed, post-fixed in the above fixative at 4ºC and cryoprotected in 30% sucrose in 0.1 M phosphate-buffered saline (PBS), pH 7.3. After freezing on dry ice, coronal or sagittal sections 30-50 µm thick were collected in a cryoprotectant solution (30% glycerol, 30% ethylene glycol, 40% 0.1M PBS) and stored at –30ºC until use.

1.5.2. Explant cultures

For postnatal cerebellar explants, brains from OF1 postnatal mice were removed from the skulls and cerebella were dissected out and cut at 250 µm in a parasagittal orientation using a tissue chopper. Slices not containing deep cerebellar nuclei (those corresponding roughly to the vermis) were selected and further dissected using fine needles. Cerebellar cortex and white matter nearby were dissected out from the slice as a single strip; the EGL was then removed and small tissue pieces (250-300 µm aprox.)

were made from the strip, each containing white matter and all layers of the cerebellar cortex except the EGL. Dissection was performed in sterile conditions using 0.6% glucose in 0.1M PBS and DMEM supplemented with L-glutamine, D-glucose, Hepes buffer (Invitrogen). For E14 hippocampal CA3 explants, the hippocampal area was dissected out, and small tissue explants (approximately 300 µm thick) were obtained from the CA3 region as described (Borrell et al., 1999; Chedotal et al., 1998).

Explants were co-cultured at 100-300 µm with aggregates of EBNA-293 cells stably transfected with a construct encoding *Netrin1-c-myc* or with the vector alone (Kennedy et al., 1994). Explants and cell aggregates were embedded in a matrix comprised of rat tail collagen or a mixture of collagen and matrigel, as previously described (Lumsden and Davies, 1986). Cerebellar explants were cultured in DMEM supplemented with L-glutamine 5mM, D-glucose 0.45%, Hepes buffer 25mM, penicillin-streptomicin and supplements B27 and N2 (Invitrogen), and hippocampal explants were cultured in Neurobasal supplemented with L-glutamine 2mM, D-glucose 0.55%, Hepes buffer 25 mM, penicillin-streptomicin and supplement B27. All experiments were performed in a 5% CO_2, 95% humidity incubator at 37 ºC for 48-72 h. In receptor blocking experiments, 2-20 µgr/ml of anti-Dcc (Oncogene, clone AF-5) or anti-GST (Santa Cruz, clone B-14) antibody was dialyzed against DMEM (for cerebellar explants) or Neurobasal (for hippocampal explants) and added to the culture medium.

1.5.3. Immunohistochemistry

For single and double labelling immunostaining procedures, polyclonal rabbit antibodies against Pax2 (Zymed), GFAP (Dako), guinea pig antibody against GABA (Biogenesis), goat antibody anti β-galactosidase (Biogenesis) and a monoclonal antibody against class III-βtubulin (clone TUJ1, Babco) were used. For all antibodies, except TUJ1 (1:2500 for immunoperoxidase reaction) and anti β-galactosidase (1:300 for immunofluorescence), dilutions were 1:500 using fluorescence secondary antibodies and 1:1000 using immunoperoxidase reaction. All solutions applied were made with 0.1M PBS, 0.2% gelatine, 5% serum and 0.5% triton. Washes between incubations were made in 0.1M PBS, 0.5% triton. Collagen matrices containing explant cultures and cell aggregates were fixed for 30 min at 4ºC in 4% PFA in 0.1 M phosphate buffer for Pax2, TUJ1 and GFAP staining, or in 4% PFA 0.1% glutaraldehyde in 0.1M phosphate buffer for GABA staining. After 3 washes in 0.1M PBS, perfused brain sections or previously fixed explant cultures were blocked in a solution containing 10% serum and 0.5% triton for 2 h at room temperature (RT). After blocking, cultures were incubated O/N at 4ºC in primary antibody at appropriate proper dilution, and for 2 h at RT in secondary antibody conjugated to Alexafluor 488 or Alexafluor 568 (Molecular Probes, 1:500) or biotinylated antibody (Vector, 1:200). For peroxidase immunohistochemistry, streptavidin-peroxidase complex (Amersham, 1:400) was applied for 2 h at RT, and after 3 washes in 0.1M PBS, peroxidase reaction was performed using diaminobenzidine (DAB, 0.06%) and 0.01% H_2O_2. Finally, sections and explant cultures were mounted on gelatinised slides with Mowiol™.

1.5.4. In situ hybridisation

In situ hybridisation was performed on free-floating sections, essentially as described (Alcantara et al., 1998). Sections were permeabilized in 0.2% Triton X-100 (15 min), treated with 2% H_2O_2 (15 min), deproteinized with 0.2 N HCl (10 min), fixed in 4% PFA (10 min) and blocked in 0.2% glycine (5 min). Thereafter, sections were pre-hybridised at 60°C for 3 h in a solution containing 50% formamide, 10% dextran sulphate, 5X Denhardt's solution, 0.62 M NaCl, 10 mM EDTA, 20 mM Pipes (pH6.8), 50 mM DTT, 250 µgr/ml yeast t-RNA and 250 µgr/ml denatured salmon sperm DNA. *Netrin1* (Serafini et al., 1996), *Dcc* (Keino-Masu et al., 1996), *Unc5h2* and *Unc5h3* (Leonardo et al., 1997) riboprobes were labelled with digoxigenin-d-UTP (Boehringer-Mannheim) by in vitro transcription. Labelled antisense cRNA was added to the pre-hybridisation solution (250-500 ngr/ml) and hybridisation was performed at 60 ºC overnight. Sections were then washed in 2XSSC (30 min, RT), digested with 20 mgr/ml RNAse A (37°C, 1 h), washed in 0.5XSSC/50% formamide (4 h 55°C) and in 0.1XSSC/0.1% sarcosyl (1 h, 60°C). After rinsing in Trisbuffered saline (TBS)/0.1% Tween 20 (15 min), sections were blocked in 10% normal goat serum (2 h) and incubated overnight with an alkaline phosphatase-conjugated antibody to digoxigenin (Boehringer-Mannheim, 1:2000). After washing, sections were developed with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Life Technologies), mounted on gelatinised slides and coverslipped with Mowiol™.

Control hybridisations, including hybridisation with sense digoxigenin-labelled riboprobes or RNAse A digestion prior to hybridisation, yielded no alkaline phosphatase staining above background levels.

To view the expression of *Netrin1*, *Dcc*, *Unc5h2* and *Unc5h3* mRNA in cerebellar interneurons, peroxidase immunostaining using anti-Pax2 antibodies (Zymed, 1:500) was performed as described above in slices previously hybridised with the corresponding riboprobe.

1.5.5. Analysis and quantification

Hybridised and immunostained sections and explant cultures were examined under a NIKON microscope (Nikon GmbH, Germany), and the results of the effect of Netrin1 on explants were quantified using the AnalySIS™ software (Soft Imaging System GmbH, Germany). Each explant was divided into four quadrants; for postnatal cerebellar explants, total (inside and outside the explant) and outward cell number was counted for the proximal and distal quadrants; for E14 hippocampal explants, the number of axons that crossed a line placed at a distance of 100 μm from the limit of the explant was counted for the proximal and distal quadrants. The ratio of outward and total cell migration or axonal growth was obtained by dividing the number of cells/axons in the proximal quadrant by that in the distal quadrant; the proximal/distal (P/D) ratio for outward and total cell migration yields 1 for radial growth, more than 1 for attractive effect, and less than 1 for repulsive effect. Statistical analysis was done using the Student's t test and the F test for variance.

Double labelled fluorescence immunostained sections and explant cultures were examined and analysed using confocal laser scanning microscopy (LEICA, Nussloch, GmbH): LEICA SP2 (laser characteristics: Argon 458/476/488/514 nm, HeNe 543 nm). LEICA SP2 AOBS (laser characteristics: Argon 488 nm, DPSS 561 nm, Diode 405 nm, HeNe 594/633 nm) and LEICA TCS (laser characteristics: ArKr 488/ 568/647 nm); confocal section width: 0.5-1.2 μm.

1.6. Acknowledgments

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1.8. Figures

Figure 1. Netrin-1 repels migrating cerebellar interneurons in vitro. Aggregates of control EBNA-293 cells (A, C, E) or expressing heterologous Netrin1 (B, D, F) were confronted to cerebellar explants containing interneurons, which were labelled by either GABA (C, D) or Pax2 antibodies (A, B, E, F). Quantification and statistical analysis is shown in Fig. 2; schemes in G and H represent the two counting methods used (see Fig. 2). In all images, cell aggregate is to the left. Scale bar: 100 μm.

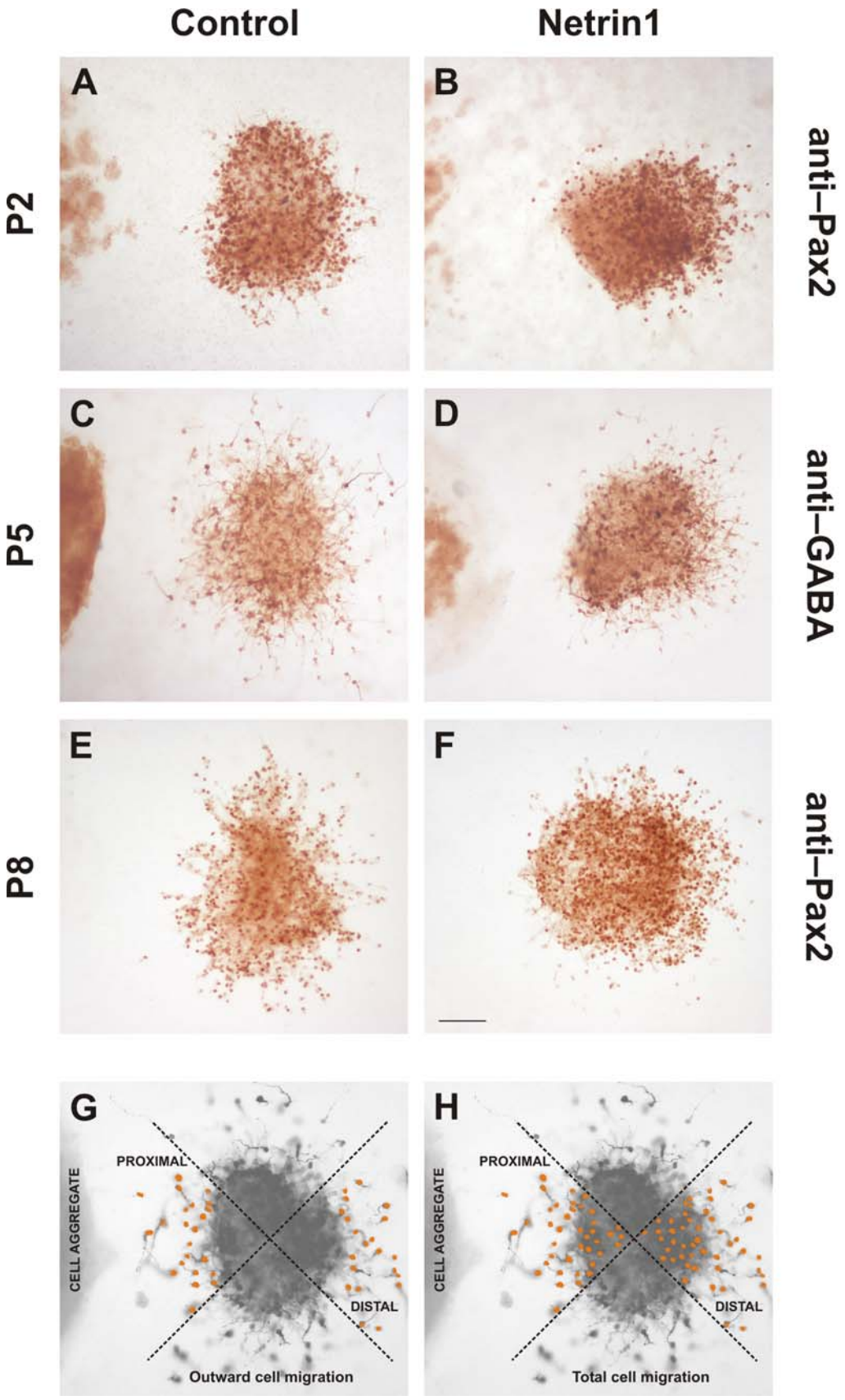

Control
Netrin1
A
B
C
D
E
F
G
H
P2
P5
P8
anti–Pax2
anti–GABA
anti–Pax2
PROXIMAL
DISTAL
CELL AGGREGATE
Outward cell migration
Total cell migration

Figure 2. Quantification and statistical analysis of interneuron migration in vitro. As shown in Fig. 1 (G, H), cerebellar explants were divided in 4 quadrants. GABA+ and Pax2+ cells were counted in proximal (in front of cell aggregate) and distal quadrants, and a ratio between proximal and distal (P/D) cell number was calculated. Interneurons in the quadrants were counted on the basis of two criteria: only those of the quadrant which were outside the borders of the explant (outward cell migration, A and B, and Fig. 1G), or all of the interneurons present in a quadrant, i.e., those which were inside and outside the explant, thus reflecting the migration of cells inside the explant (total cell migration, C and D, and Fig. 1H). 1 asterisk: $p<0.05$. 2 asterisks: $p<0.03$. 3 asterisks: $p<0.01$.

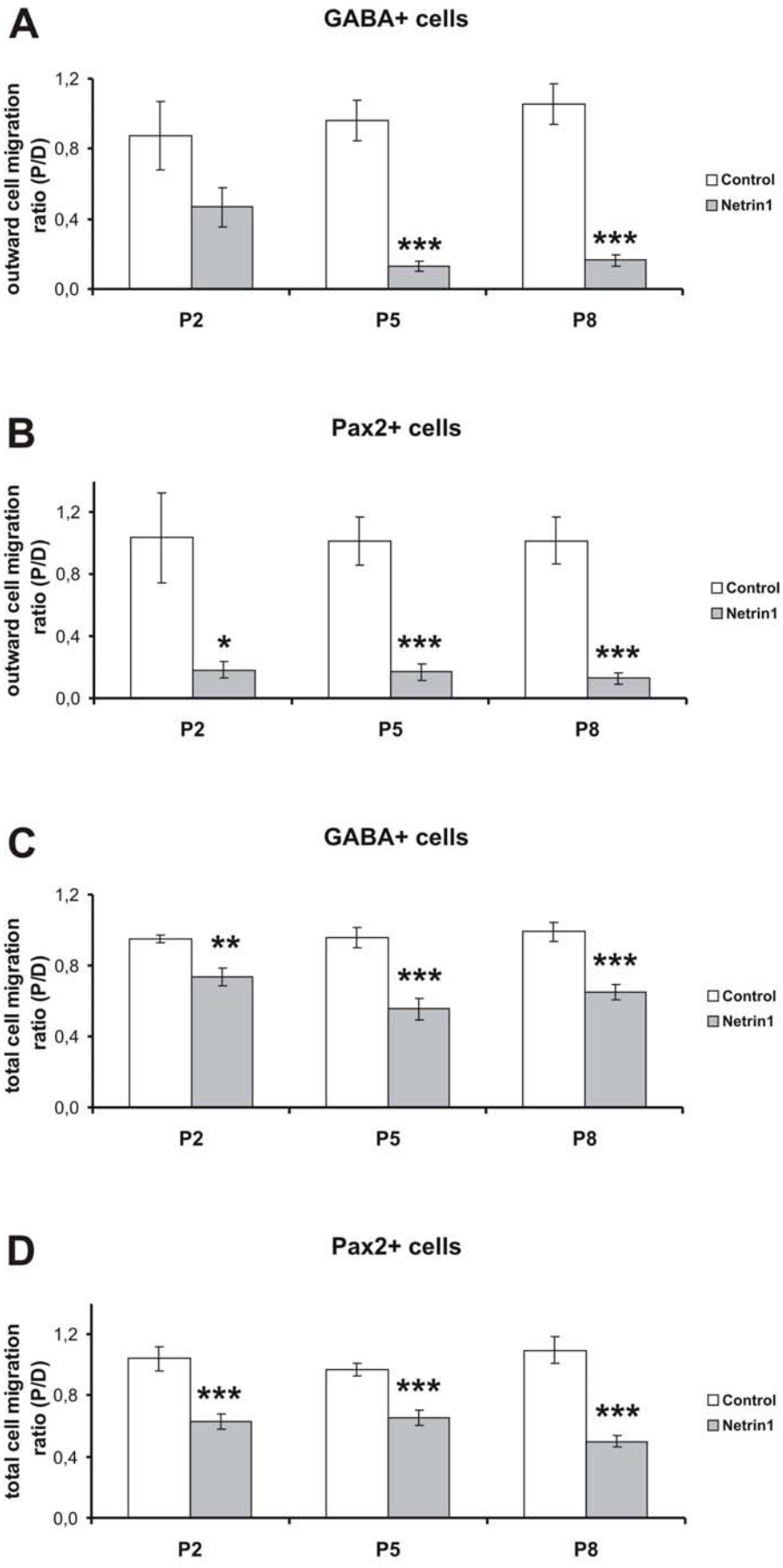

A
GABA+ cells
outward cell migration ratio (P/D)
1,2
0,8
0,4
0,0
***
***
P2
P5
P8
Control
Netrin1
B
Pax2+ cells
outward cell migration ratio (P/D)
1,2
0,8
0,4
0,0
*
***
***
P2
P5
P8
Control
Netrin1
C
GABA+ cells
total cell migration ratio (P/D)
1,2
0,8
0,4
0,0
**
***
***
P2
P5
P8
Control
Netrin1
D
Pax2+ cells
total cell migration ratio (P/D)
1,2
0,8
0,4
0,0
***
***
***
P2
P5
P8
Control
Netrin1

Figure 3. Cerebellar interneurons may migrate along glial processes. (A, B) P5 cerebellar explants confronted to control (A) or Netrin1 (B) cell aggregates, and immunostained against glial marker GFAP show radial distribution of elongated glial processes in both conditions. (C-H) P5 cerebellar explants double-labelled with anti-GABA (red) and anti-GFAP (green) antibodies. (C) Projection of confocal sections, showing GABA+ cells that leave the explant through bundles of elongating glial, GFAP-positive fibres. (D) Magnification of dashed area in C. (E-H) Single confocal sections show GABA-positive interneurons apposed to GFAP-positive glial fibres; arrows point to colocalization spots. (I-J) Sagittal sections from GFAP-GFP transgenic mouse cerebella immunostained against Pax2 (red). Arrows show Pax2-positive nuclei close to GFAP-positive fibres in the molecular layer, which correspond to Bergmann glial cells; arrowheads indicate Bergmann glial cell bodies, and asterisks indicate Purkinje cell bodies. ML, Molecular Layer; PCL, Purkinje Cell Layer. Scale bar in A-D: 100 μm. Scale bar in E-L: 50 μm.

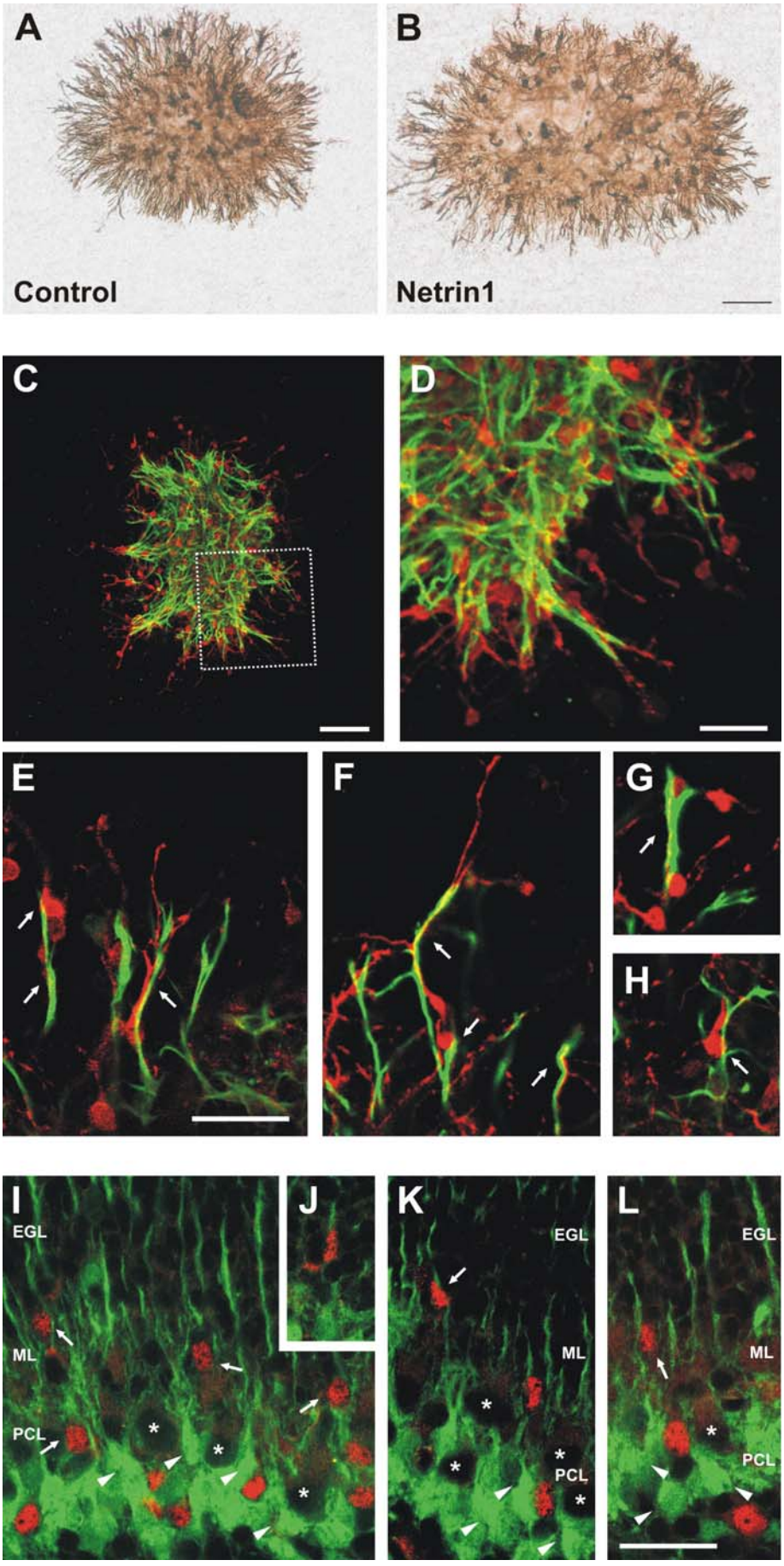

A
Control
B
Netrin1
C
D
E
F
G
H
I
EGL
ML
PCL
J
K
EGL
ML
PCL
L
EGL
ML
PCL

Figure 4. Cerebellar interneurons associate with glial fibres in the postnatal cerebellum. (A) Sagittal section from P5 heterozygous double transgenic Ptf1a/+;R26R/+ mice immunostained against β-Galactosidase (A), or immunostained against β-Galactosidase (green) and GFAP (red) (B-G). (A) Image shows labelled GABAergic interneurons in ML and IGL (arrowheads), as well as Purkinje cell bodies (asterisks) and axons in WM. (B) Projection of confocal sections, where asterisks show Purkinje cell bodies, and arrowheads point to interneurons in the ML apposed to processes of Bergmann glial cells. There may be a close association between Bergmann glial fibres and Purkinje cell dendrites (Yamada y Watanabe, 2002) (C-G) Details of single confocal sections showing interneurons in the ML associated with glial fibres. Arrowheads point to yellow colocalization sites. Scale bar: 50 μm (A), 25 μm (B), 10 μm (C-G)

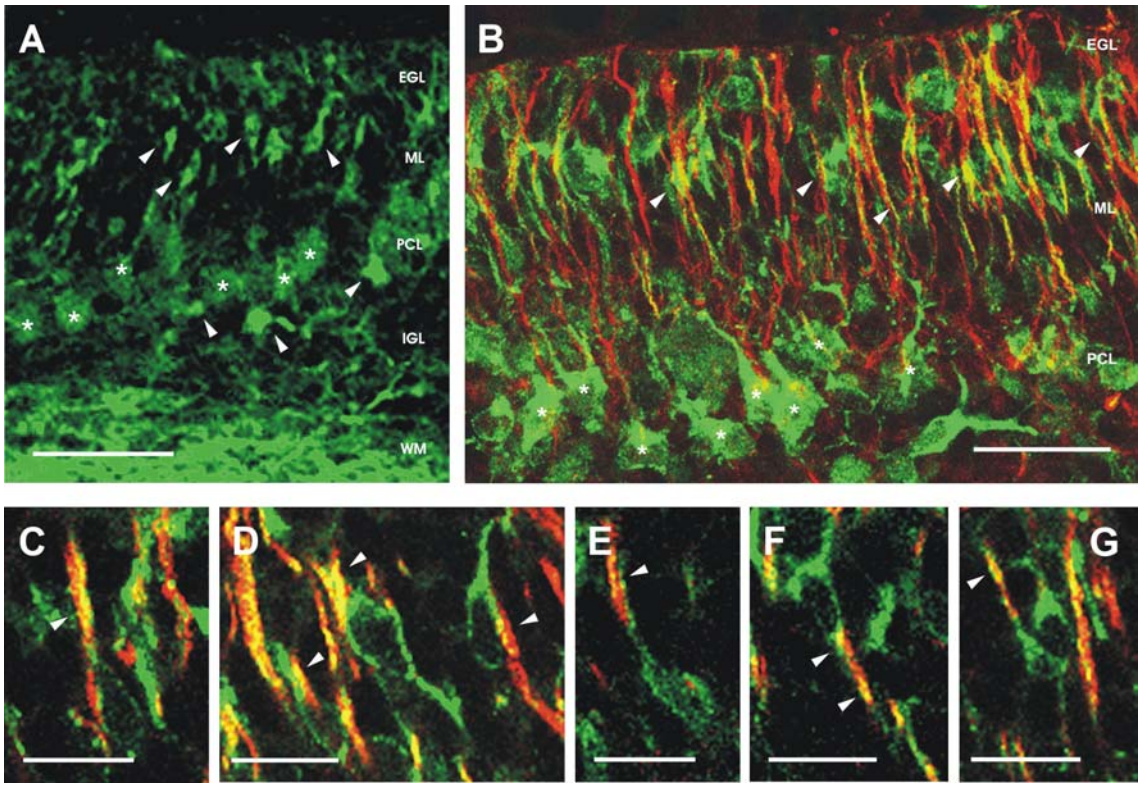

A
EGL
ML
PCL
IGL
WM
B
EGL
ML
PCL
C
D
E
F
G

Figure 5. Expression pattern of *Netrin1* and its receptors *Dcc*, *Unc5h2* and *Unc5h3* during postnatal development in cerebellum. Sagittal sections from P0 (A-D), P5 (E-H and M-P), P10 (I, J, L) and P15 (K) mouse brains were hybridized with *Unc5h2* (A-M), *Unc5h3* (B-N), *Dcc* (C-O) or *Netrin1* (D-P) riboprobes labelled with digoxigenin. (M-P) P5 cerebellar interneurons double-labelled with anti-Pax2 antibody (brown label) and digoxigenin riboprobes (blue label) against *Unc5h2* (M), *Unc5h3* (N), *Dcc* (O) and *Netrin1* (P); arrowheads point to cells with weak gene expression, whereas arrows show cells with evident gene expression. EGL: External Granular Layer; ML: Molecular Layer; PCL: Purkinje Cell Layer; IGL: Internal Granular Layer; WM: White Matter. Scale bar in A-L: 100 μm. Scale bar in M-P: 20 μm.

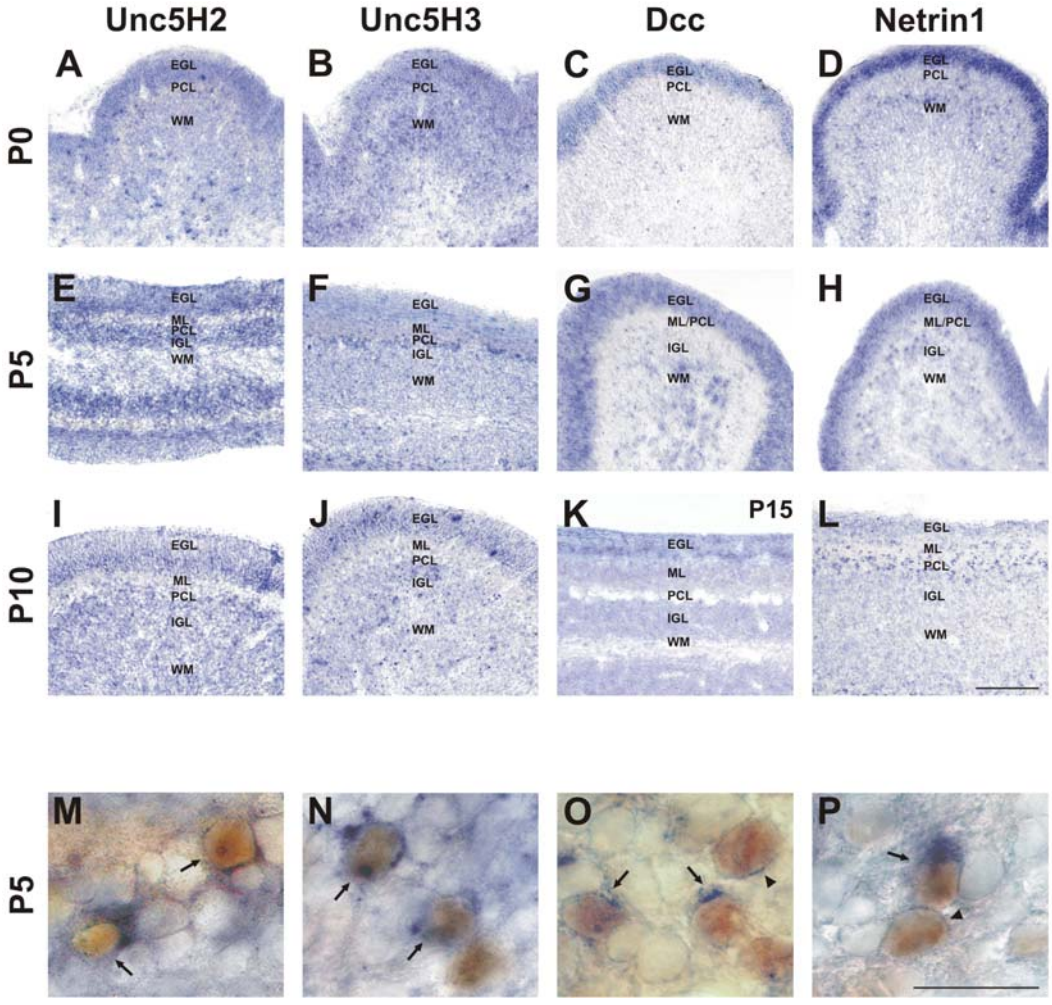

Unc5H2
Unc5H3
Dcc
Netrin1
P0
P5
P10
P5
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
P15
EGL
PCL
ML
ML/PCL
IGL
WM

Figure 6. Dcc does not mediate Netrin-1 chemorepulsive signal in cerebellar interneurons. P5 cerebellar explants (A-C) and E14 CA3 explants (D-F) were confronted to control cells (C,F) or cells expressing Netrin1 (A, B, D, E) and incubated with blocking anti-Dcc antibodies (B, C, E, F) or control anti-GST antibodies (A, D). Cerebellar interneurons were labelled with anti-GABA antibody and hippocampal axons with anti-III ßTubulin (TUJ1) antibody. Note that, whereas in CA3 explants the addition of blocking anti-Dcc antibody clearly diminishes the chemoattractive response on axons exerted by Netrin1 (compare D and E), the same blocking anti-Dcc antibody but five-fold more concentrated has no effect on migrating cerebellar interneurons (compare A and B), as they are still repelled by Netrin1-expressing cells. Quantification and statistical analysis is shown in Fig. 7. In all images, cell aggregate is to the left. Scale bar: 100 μm.

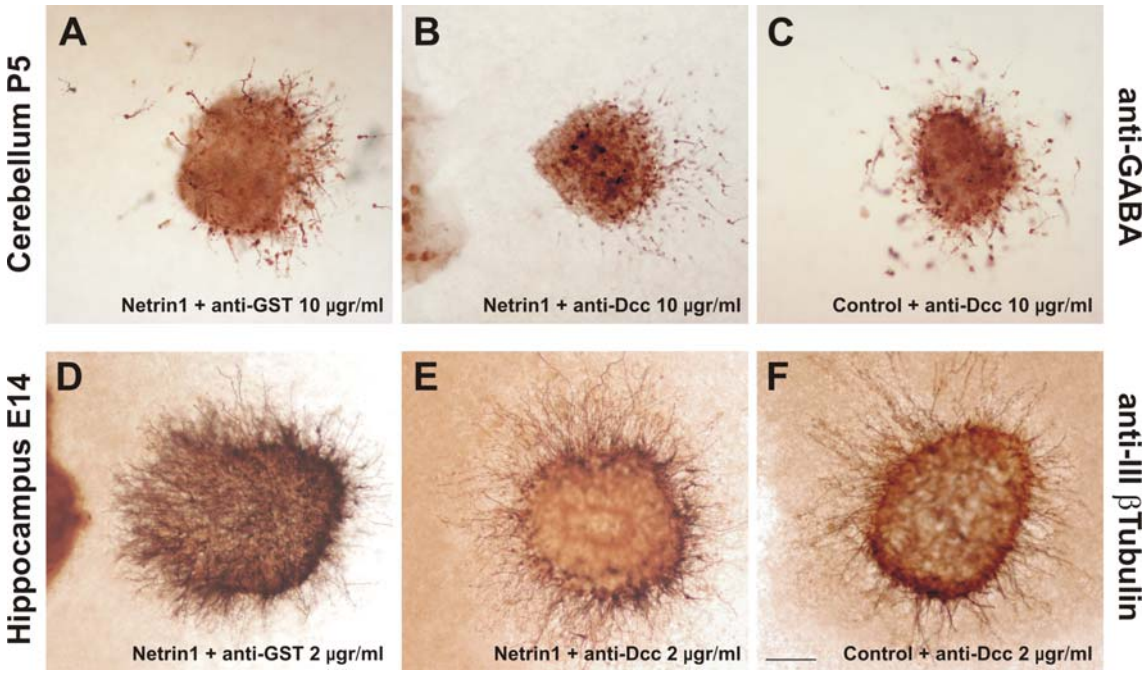

Cerebellum P5
Hippocampus E14
A
B
C
D
E
F
Netrin1 + anti-GST 10 µgr/ml
Netrin1 + anti-Dcc 10 µgr/ml
Control + anti-Dcc 10 µgr/ml
Netrin1 + anti-GST 2 µgr/ml
Netrin1 + anti-Dcc 2 µgr/ml
Control + anti-Dcc 2 µgr/ml
anti-GABA
anti-III βTubulin

Figure 7. Quantification and statistical analysis of the effect of blocking anti-Dcc antibody on P5 cerebellar explants (A) and E14 CA3 explants (B). The counting method used in A was the same as in Figs. 2 A, B. Compare Netrin1+GST (10 µgr/ml) and Netrin1+Dcc (10 µgr/ml) in (A), with Netrin1+GST (2 µgr/ml) and Netrin1+Dcc (2 µgr/ml) in (B). 1 asterisk: $p<0.05$. 2 asterisks: $p<0.03$. 3 asterisks: $p<0.01$. ns: not significant.

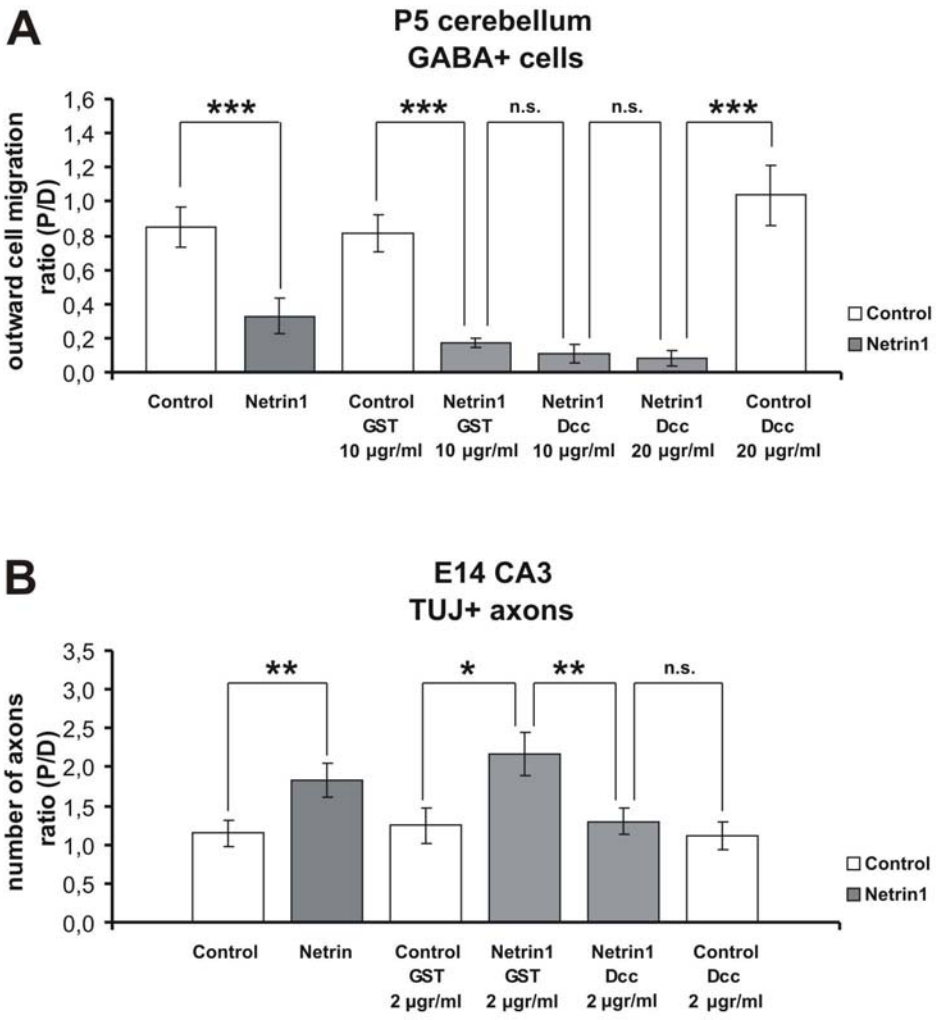
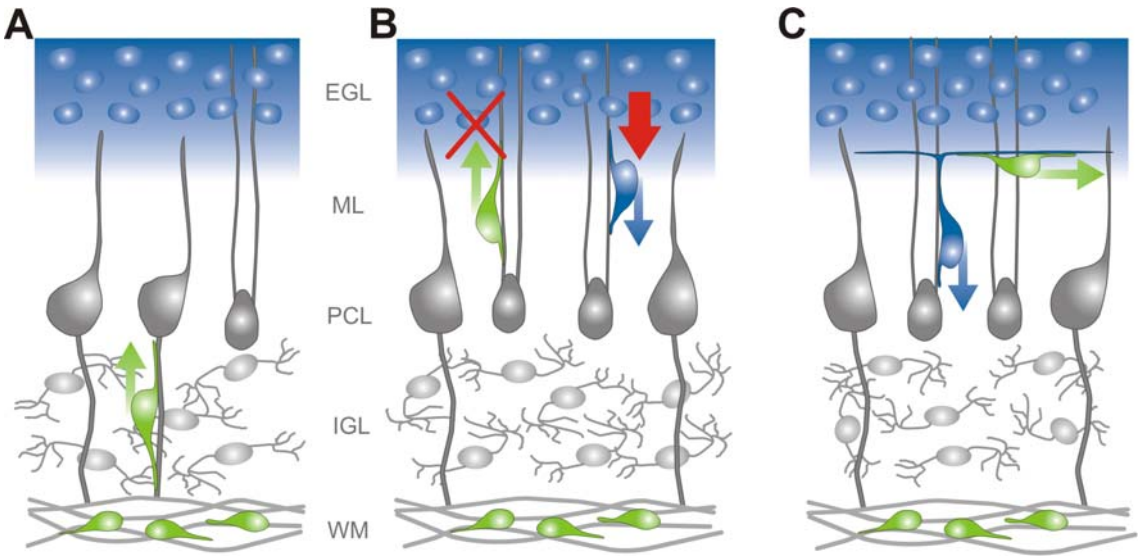

A
P5 cerebellum
GABA+ cells
outward cell migration ratio (P/D)
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
***
***
n.s.
n.s.
***
Control
Netrin1
Control GST 10 µgr/ml
Netrin1 GST 10 µgr/ml
Netrin1 Dcc 10 µgr/ml
Netrin1 Dcc 20 µgr/ml
Control Dcc 20 µgr/ml
Control
Netrin1
B
E14 CA3
TUJ+ axons
number of axons ratio (P/D)
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
**
*
**
n.s.
Control
Netrin
Control GST 2 µgr/ml
Netrin1 GST 2 µgr/ml
Netrin1 Dcc 2 µgr/ml
Control Dcc 2 µgr/ml
Control
Netrin1

Figure 8. Illustration proposing a migration pathway for interneurons moving from the white matter to the molecular layer of the cerebellar cortex. (A) Interneurons (shown in green) in the white matter may reach the Purkinje cell layer by attaching to the myelinated axons of Purkinje cells (Mathis et al., 2003). (B) From the Purkinje cell layer, interneurons may ascend the molecular layer using fibres of Bergmann glia as a scaffold, like precursors of granule cells (depicted in blue) travelling from the EGL in the opposite direction. In this scenario, the Netrin1 gradient generated by the EGL might have two actions (both based on a chemorepulsive mechanism): to prevent interneurons from erroneously entering the EGL, and to push granule cell precursors out of the EGL (see discussion). (C) Once inside the molecular layer, interneurons disperse along the coronal plane, perhaps following the parallel fibres of granule neurons (Zhang y Goldman, 1996b). EGL: External Granular Layer; ML: Molecular Layer; PCL: Purkinje Cell Layer; IGL: Internal Granular Layer; WM: White Matter.


A
B
C
EGL
ML
PCL
IGL
WM

CAPÍTULO II

Función de Sema3A y Sema3F en axones GABAérgicos hipocampales y entorrinales durante el desarrollo

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Resumen

Las semaforinas secretables pertenecientes a la clase 3 son conocidas por su función en guiar axones y células del SNC y periférico durante el desarrollo embrionario y postnatal. Sema3A y Sema3F participan en el establecimiento de las principales conexiones de la formación hipocampal, pero poco se sabe acerca de cómo los axones GABAérgicos de proyección local de esa misma región son dirigidos hacia su diana específica. En este estudio, nosotros demostramos que los axones GABAérgicos entorrinales e hipocampales son repelidos por Sema3A y Sema3F in vitro a edades embrionarias y postnatales. Ambas semaforinas y sus respectivos receptores específicos, Np1 y Np2 se expresan en el hipocampo durante el periodo en que aparecen las primeras sinapsis GABAérgicas, y las interneuronas hipocampales expresan Np1 y Np2. El conjunto de datos nos lleva a proponer, por una parte, que Sema3A, Sema3F y sus receptores Np1 y 2 podrían formar parte in vivo de las primeras etapas de la formación de la red de conexiones GABAérgicas en el hipocampo, y por otra parte, que podrían influir en la formación de la proyección entorrino-hipocampal, tanto de su componente glutamatérgico como del GABAérgico.

Effect of Sema3A and Sema3F on developing GABAergic axons of the hippocampus and the entorhinal cortex

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2.1. Abstract

Class 3 of vertebrate secreted semaphorins guide axons and cells from the peripheral and central nervous system during embryonic and postnatal development. Sema3A and Sema3F participate in the establishment of the main afferent and efferent connections in the hippocampal formation. However, little is known about how locally projecting, inhibitory fibres in this same region are directed to their specific target. Here we demonstrate that GABAergic entorhinal and hippocampal axons are repelled by Sema3A and Sema3F in vitro during embryonic and postnatal stages. These two semaphorins and their respective receptors, *Np1* and *Np2*, are expressed in the hippocampus during the period when the first GABAergic synapses are generated, and hippocampal interneurons express these two receptors. On the basis of these data, we propose that Sema3A, Sema3F and their receptors Np1 and Np2 participate in vivo in the first stages of the establishment of the GABAergic network in the hippocampus, and that they affect the formation of the glutamatergic and GABAergic components of the perforant pathway.

2.2. Introduction

Semaphorins form a family of soluble and membrane-associated proteins that are involved in several aspects of nervous system development and function (Fiore and Puschel, 2003). Class 3 of vertebrate soluble semaphorins was one of the first in the family to be discovered (Kolodkin et al., 1993; Luo et al., 1993), and by far the most extensively studied. This class displays chemotactic activity on developing axons in several regions of the central and peripheral nervous system in vitro, showing mainly a repulsive effect on axons although they can also exert attraction (Fiore and Puschel, 2003). In addition, they influence the migration of forebrain interneurons (Marin and Rubenstein, 2003), neural crest cells (Osborne et al., 2005), and glial cells (Spassky et al., 2002). Class 3 semaphorins signal through a receptor complex that contains neuropilins as the binding component and plexins, members of the CAM family, or VEGFR as transducing elements (Tamagnone and Comoglio, 2004).

In the hippocampal formation, soluble semaphorins and neuropilins are involved in axonal guidance during embryonic and postnatal development, and mainly affect principal projecting fibres; thus, it has been suggested that Semaphorin 3A (Sema3A) and Semaphorin 3F (Sema3F) participate in the establishment of the entorhinal and mossy fibre projection (Steup et al., 2000; Steup et al., 1999), and that the cooperative action of these two semaphorins might help septal fibres to leave the septum on their way to the hippocampus and other targets (Pascual et al., 2004; Pascual et al., 2005). Moreover, Sema3A may be the repulsive factor secreted by the entorhinal cortex and neocortex that push hippocampal axons to the fimbria, thereby making them initiate the projection to their final destinations (Chedotal et al., 1998). However, to our knowledge, the guidance of axons from locally projecting interneurons in the hippocampal formation remains to be elucidated.

Recently, it has been shown that Sema3A, Sema3F and their respective receptors, Neuropilin 1 (Np1) and Neuropilin 2 (Np2), also participate in the developmental program of forebrain interneurons, which are generated in the subpallium and move along a tangential trajectory until reaching the cerebral cortex and hippocampus (Marin and Rubenstein, 2001). Sema3A and Sema3F expression in the striatum directs subpalial GABAergic neurons expressing Np1 and Np2 (Marin et al., 2001) and posterior interactions between Sema3A/Sema3F and Np1/Np2 allow the distribution of cortical interneurons between the dorsal cortex and hippocampus, or lower cortical intermediate zone (Tamamaki et al., 2003). Once the tangential journey from the ganglionic eminences has ended, hippocampal interneurons initiate a developmental sequence of events that affect their morphologic and functional characteristics (Gozlan and Ben-Ari, 2003; Hennou et al., 2002). Starting with no synapses and a poorly developed appearance, interneurons begin to extend their processes and to generate excitatory $GABA_A$-type synapses; in posterior stages, they show longer and more developed axons and dendrites, and have $GABA_A$ as well as glutamatergic AMPA and NMDA synapses. The same developmental sequence is also observed in hippocampal pyramidal cells, but later than in interneurons (Tyzio et al., 1999); after reaching their position in the hippocampal plate by radial migration from the ventricular zone, pyramidal cells show only a primordial apical dendrite (which is generated before the basilar dendrite) and have no electrical activity. In a later stage, pyramidal cells elongate the apical dendrite across the stratum radiatum only, and have $GABA_A$ synapses. Finally, the apical dendrite reaches the stratum lacunosum-moleculare, and pyramidal cells show $GABA_A$ and glutamatergic synapses.

In this study, we found that hippocampal and entorhinal GABAergic axons during embryonic and postnatal stages in vitro are repelled by Sema3A and Sema3F. In the same developmental period, Sema3A and Sema3F are expressed in vivo in the hippocampus. Moreover, hippocampal interneurons express Np1 and Np2 in vivo. From these sets of data, we propose that Sema3A and Sema3F, through their receptors Np1 and Np2, guide the axons of interneurons in vivo and help them to establish local inhibitory circuitry. In addition, these semaphorins also affect the movement of both excitatory and inhibitory fibres of the perforant pathway.

2.3. Results

2.3.1. GABAergic hippocampal axons are repelled by Sema3A and Sema3F but GABAergic entorhinal axons are repelled only by Sema3F.

We focused on the axonal response of GABAergic neurons in the hippocampus and entorhinal cortex to Sema3A and Sema3F, two soluble semaphorins involved in the formation of the main connections in the hippocampus during embryonic development (Chedotal et al., 1998; Pascual et al., 2005; Pozas et al., 2001; Steup et al., 1999). For this purpose, we used the explant culture essay. Pieces of tissue dissected from mouse embryonic hippocampus and entorhinal cortex were placed in front of cell aggregates secreting Sema3A, Sema3F or control alkaline phosphatase, and embedded in a collagen matrix (Chedotal et al., 1998). After 3 days in culture, explants were fixed and immunostained against GABA.

Sema3A and Sema3F showed a clear chemorepulsive effect on GABAergic axons from hippocampal CA explants at E16, compared to controls (Fig. 1A, B, C). At P0, the repulsive action of Sema3A and Sema3F persisted, although it was attenuated (Fig. 1B, D, F). We measured the response of GABAergic axons in CA explants at E16 and P0 by counting the number of axons in the proximal quadrant (close to the cell aggregate) and in the distal quadrant (opposite to the proximal quadrant), and obtained a proximal/distal ratio of axonal growth (Fig. 3). Significant differences between controls and Sema3A or Sema3F ratios at E16 and P0 were observed (Fig. 3A). We did not perform the explant culture assay with hippocampal tissue at E14 because of the low number of axons per explant observed in preliminary experiments, which would have rendered visual and statistical analyses impossible. This low number of GABAergic axons detected in the hippocampus at E14 is consistent with a previous study reporting that the first interneurons tangentially migrating from the ganglionic eminences are seen in the hippocampal anlage at E15.5 (Pleasure et al., 2000). Thus, we conclude that, during embryonic and newborn stages of development, Sema3A and Sema3F repel GABAergic hippocampal axons in vitro, an effect that coincides with that observed previously for the overall axonal population in the hippocampus at the same ages (Chedotal et al., 1998; Pozas et al., 2001).

Regarding to the entorhinal cortex, we observed repulsion in GABA-immunostained explants in front of Sema3F cell aggregates at E14 and E16 (Fig. 2E, F). In contrast, at both ages, the growth of GABAergic axons in response to Sema3A was radial, similar to than shown by controls (Fig. 2C, D). The quantification of axonal response yielded a significant repulsive effect of Sema3F compared to controls, but there was no difference between Sema3A and control AP (Fig. 3B). Thus, in vitro, entorhinal GABAergic axons were repelled only by Sema3F at embryonic stages, while they were insensitive to Sema3A. This observation is surprising because previous studies on the action of Sema3A and Sema3F on the whole axonal population of entorhinal explants reported the opposite, that is to say, a repulsive effect only for Sema3A, and Sema3F having no effect during embryonic stages (Chedotal et al., 1998; Pozas et al., 2001).

2.3.2. *Sema3A*, *Sema3F* and their receptors *Np1* and *Np2* are expressed in vivo in the embryonic hippocampal formation

We examined the possible effect of Sema3A and Sema3F on hippocampal and entorhinal axons in vivo during embryonic and postnatal developmental stages, first by performing non-radioactive in situ hybridization on mouse sections at different ages. At E16, *Sema3A* was expressed mainly in the hippocampal plate and intermediate zone of the hippocampal anlage; at P0 *Sema3A* mRNA was detected in the hippocampal pyramidal cell layer and in scattered cells in the stratum radiatum of CA1 (Fig. 4A, C). The Sema3A receptor, *Np1*, was expressed at E16 in a similar pattern to that shown by *Sema3A*, that is, in the hippocampal plate and intermediate zone, and expression remained unchanged at P0 (Fig. 4E, G). *Sema3F* showed weak expression in the hippocampal plate at E16 and in the pyramidal cell layer and stratum oriens at P0 (Fig. 4B, D), while expression of the Sema3F receptor *Np2* was observed in the hippocampal plate and intermediate zone at E16, and showed a similar but more conspicuous pattern at P0 and with some additional sparse cells in the stratum radiatum (Fig. 4F, H). In summary, the expression pattern of *Sema3A*, *Sema3F*, and their receptors *Np1* and *Np2* share a common characteristic. At both stages tested, E16 and P0, the dendritic layers (stratum radiatum and lacunosum-moleculare) were devoid of hybridization signal, or this signal was low compared to the other hippocampal layers.

Thus, as reported in a previous study (Chedotal et al., 1998), *Sema3A*, *Sema3F*, *Np1* and *Np2* are expressed in the hippocampus at embryonic and postnatal stages. This expression pattern suggests that these secreted semaphorins may have an effect on axons of hippocampal interneurons in vivo.

2.3.3. Hippocampal interneurons express semaphorin receptors in vivo

Continuing in our effort to elucidate the effect of Sema3A and Sema3F on axons from hippocampal and entorhinal interneurons in vivo during development, we next examined whether interneurons in the hippocampus express *Np1* and *Np2* in vivo. By using a polyclonal antibody against Calbindin, we immunolabelled interneurons in the neocortex and hippocampus of embryonic mouse sections previously hybridized with *Np1* or *Np2* riboprobes. Calbindin antibody labelled many interneurons in the neocortex on their tangential journey from the ganglionic eminences, following two main streams through the marginal zone and the intermediate zone, as described in previous studies (Marin and Rubenstein, 2003). Most Calbindin-positive neurons coursing either of these two trajectories in the neocortex did not express *Np1* or *Np2* (Fig. 5A, F). The marginal and intermediate streams of cortical, Calbindin-positive neurons were in continuity with those in the inner marginal zone (prospective stratum radiatum) and subplate/intermediate zone, respectively, of the hippocampus, and there were also several labelled interneurons in the hippocampal plate (Fig. 5B, G). We detected many Calbindin-positive neurons in the hippocampal plate and in the intermediate zone/subplate which simultaneously expressed *Np1* or *Np2* (Fig. 5D, E. I, J). In the inner and outer marginal zone (prospective stratum radiatum and stratum lacunosum-moleculare, respectively), a dense population of interneurons was observed, as previously reported (Soriano et al., 1994), but no appreciable hybridization signal was detected in this layer, and hardly any/very few neurons colocalized Calbindin antigen and *Np1* or *Np2* riboprobe (Fig. 5C, H). Therefore, interneurons in the hippocampus expressed *Np1* and *Np2* in vivo during embryonic stages, thus suggesting that axons from hippocampal interneurons respond to Sema3A and Sema3F in vivo, probably by inhibiting their growth or by chemorepulsion.

2.4. Discussion

The function of class 3 semaphorins in the guidance of axons and cells during the development of the nervous system has been extensively reported (Fiore and Puschel, 2003). In the embryonic and postnatal hippocampal formation, Sema3A and Sema3F participate in the guidance of afferent and efferent connections by means of repulsive activity through their receptors Np1 and Np2 (Chedotal et al., 1998; Pascual et al., 2004; Pascual et al., 2005; Pozas et al., 2001; Steup et al., 2000; Steup et al., 1999). Moreover, the expression of neuropilins by tangentially migrating interneurons seems to specify the neocortex and, probably, also the hippocampus, as their final destination instead of the striatum (Marin et al., 2001; Tamamaki et al., 2003).

However, despite intense research effort in recent years to elucidate how axons from principal neurons travel along specified routes, no information is available on the guidance of interneuron fibres that project locally in a specific area. Moreover, neither have semaphorins been related to this process. Here we present data demonstrating that GABAergic interneurons in the developing hippocampal formation respond to secreted Sema3A and Sema3F in vitro. Their response is similar to that of the main neurons of the hippocampus, the pyramidal cells (Chedotal et al., 1998; Pozas et al., 2001). In addition, on the basis of our data we propose that, in vivo, Sema3A and Sema3F affect the guidance of locally projecting axons through their receptors Np1 and Np2. This hypothesis is supported by the observation that in a period when local connections by interneurons are developing, hippocampal tissue expresses semaphorins and neuropilins, and furthermore, hippocampal Calbindin-positive interneurons express *Np1* and *Np2*.

2.4.1. Sema3A and Sema3F may restrict initial GABAergic synaptic activity to the pyramidal apical dendrites in the stratum radiatum

It is currently assumed that the first electrical circuitry in the hippocampus is established during embryonic stages by excitatory GABAergic synapses (Ben-Ari et al., 2004). Thus, in embryonic rat slices, 65% of interneurons are already functional, whereas 88% of pyramidal cells are still silent (Gozlan and Ben-Ari, 2003; Hennou et al., 2002). During rat embryonic stages, GAD immunoreactivity is transiently concentrated in the stratum radiatum and lacunosum-moleculare, before changing to the pyramidal cell layer in the adult (Dupuy and Houser, 1996). This finding is consistent with the observation that in embryonic mouse hippocampus, GABA-positive cells are more densely distributed in the inner marginal zone/stratum radiatum (Soriano et al., 1994). Pyramidal neurons in their second developmental stage, with their apical dendrites restricted to the stratum radiatum, form functional GABAergic synapses, and only later, when apical dendrites reach the stratum lacunosum moleculare, do they have the capacity to generate glutamatergic synapses (Tyzio et al., 1999). Moreover, interneurons that synapse with pyramidal dendrites (peridendritic interneurons) mature earlier than interneurons contacting with the soma of pyramidal cells, and in the stratum radiatum of embryonic and newborn rats, interneurons are in a mid or advanced stage of maturation and tend to confine their axons to this same layer (Gozlan and Ben-Ari, 2003; Hennou et al., 2002). All these data suggest that, in the embryonic and newborn rodent hippocampus, GABAergic cells as well as GABA-mediated synaptic activity are localized mainly in the stratum radiatum, and are most likely related to the immature apical dendrites of pyramidal neurons. The expression pattern of *Sema3A* and *Sema3F*, and that of their receptors *Np1* and *Np2*, showed a low or no signal in the stratum radiatum and lacunosum-moleculare. Moreover, Calbindin-positive interneurons colocalized only with neuropilins in the hippocampal plate and subplate/intermediate zone. On the basis of these observations, we propose that the repulsive effect of Sema3A and Sema3F on GABAergic axons during embryonic and newborn stages observed in vitro serves to inhibit the axonal elongation of interneurons in the hippocampal plate and intermediate zone, thus impeding the formation of synapses in the soma and in the primordial basilar dendrite of immature pyramidal cells (Hennou et al., 2002). Furthermore, the lack of *Sema3A* and *Sema3F* expression in the stratum radiatum, and the observation that the interneurons in this layer do not express neuropilins and hence are not sensitive to semaphorins, would facilitate their developmental progress, extending their axons and making $GABA_A$-type synaptic contacts with the immature pyramidal apical dendrite, which, during these stages, is restricted mostly to the stratum radiatum (Tyzio et al., 1999). Given that interneurons in the stratum radiatum do not express neuropilins, other inhibitory signals instead of secreted semaphorins work on interneurons in the stratum radiatum in order to retain them in that layer and prevent invasion of the hippocampal plate and subplate/intermediate zone.

2.4.2. Opposite action of Sema3A and Sema3F on principal neurons and interneurons of the entorhinal cortex

Sema3A, but not Sema3F, exerts a chemorepulsive action on entorhinal axons in vitro during embryonic stages (Chedotal et al., 1998; Pozas et al., 2001; Steup et al., 2000; Steup et al., 1999). The expression pattern of Sema3A described in previous studies shows mRNA in the dentate gyrus and entorhinal cortex, and a sudden change in the intensity of hybridization signal in the border between entorhinal cortex and subiculum (Chedotal et al., 1998; Skaliora et al., 1998; Steup et al., 2000). These data led to the proposal that Sema3A participates in vivo in the formation of the perforant pathway, by steering entorhinal axons through the subiculum and preventing them from entering the dentate granule cell layer (Steup et al., 2000). Our data in the embryonic hippocampus showed robust expression of *Sema3A* also in hippocampal plate and intermediate zone, while the stratum radiatum and lacunosum-moleculare were almost devoid of signal. Moreover, in the embryonic entorhinal cortex, both *Sema3A* and *Np1* were expressed in superficial layers, which are the source of perforant pathway fibres (Chedotal et al., 1998; Skaliora et al., 1998). Thus, we believe that our expression data reinforce the previous notion that Sema3A directs entorhinal fibres across the hippocampus and helps them to reach the stratum lacunosum-moleculare, where barely any *Sema3A* mRNA was detected. Moreover, we also propose that the expression of *Sema3A* and *Np1* in superficial entorhinal layers help axons of the perforant pathway to initiate their journey to the hippocampus.

Here we show that the effect of Sema3A and Sema3F on axons from the GABAergic population of the entorhinal cortex is the opposite: namely, they are repelled by Sema3F but not by Sema3A. This result is difficult to explain. Indeed, the perforant pathway has a small GABAergic component (Germroth et al., 1989). In addition, the expression of *Sema3F* in the entorhinal cortex and hippocampus during embryonic stages is roughly similar to that of *Sema3A*, that is to say, expression is basically located in the superficial half of the entorhinal cortex and in the hippocampal plate, and *Np2* is also expressed in embryonic entorhinal superficial layers (Chedotal et al., 1998). In the light of this set of data, we hypothesized that, at E14-E16, Sema3A and Sema3F influence the formation of the perforant pathway in vivo in a similar way, acting in distinct entorhinal neuron populations: principal, glutamatergic entorhinal neurons (which comprise most of the population immunostained by an anti-tubulin antibody) would respond to Sema3A and not to Sema3F (Chedotal et al., 1998), and, on the contrary, GABAergic entorhinal neurons also projecting to the hippocampus would be sensitive to Sema3F but not to Sema3A.

2.5. Material and Methods

2.5.1. Animals and histology

OF1 embryos (E14, E16) and newborn mice (Charles River, Lyon, France) were used for the explant cultures, immunohistochemical staining and non-radioactive in situ hybridizations. The mating day was considered as E0, and the day of birth as P0. OF1 mice were kept under controlled temperature (22 ± 2°C), humidity (40-60%), and light (12 hr cycles) and treated in accordance with the European Community Council Directive (86/609/EEC). For immunohistochemical and in situ hybridization processing, animals were anaesthetized with ketamine-xylazine injections (150 and 6 µgr/gr, respectively) and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.3. Brains were removed, post-fixed in the above fixative at 4ºC and cryoprotected in 30% sucrose in 0.1 M phosphate-buffered saline (PBS), pH 7.3. After freezing on dry ice, sections with a thickness of 30-50 µm were collected in a cryoprotectant solution (30% glycerol, 30% ethylene glycol, 40% 0.1M PBS) and stored at –30ºC until use.

2.5.2. Explant cultures

For hippocampal and entorhinal explants, pregnant OF-1 females or postnatal pups were anaesthetized with ketamine-xylazine injections (150 and 6 µgr/gr, respectively), and embryos were removed. After removing the brain from the skull, we dissected out the hippocampus, entorhinal cortex and parietal cortex of E14-P0 animals in a single piece, and 250-350 µm-thick horizontal slices were obtained using a tissue chopper. Selected slices containing the hippocampus and entorhinal cortex were further dissected using fine tungsten needles to obtain small tissue explants (approximately 300 µm-thick) (Borrell et al., 1999; Chedotal et al., 1998). Dissection was performed in sterile conditions using 0.6% glucose in 0.1M PBS and DMEM supplemented with L-glutamine, D-glucose, Hepes buffer (Invitrogen).

Explants were then co-cultured at 100-300 µm with aggregates of COS cells transiently transfected with a construct encoding human Sema3A-myc (Messersmith et al., 1995), human AP-Sema3F (Chen et al., 1997) or control secreted alkaline phosphatase (AP). Explants and cell aggregates were embedded in a matrix composed of rat tail collagen, as previously described (Lumsden and Davies, 1986), and were cultured in Neurobasal medium supplemented with L-glutamine 2mM, D-glucose 0.55%, Hepes buffer 25 mM, penicillin-streptomycin and supplement B27. All explant cultures were kept in a 5% CO2, 95% humidity incubator at 37 ºC for 72 hours.

2.5.3. Immunohistochemistry

For single and double labelling immunostaining procedures, polyclonal rabbit antibodies against Calbindin (Swant, 1:2500), and guinea pig antibody against GABA (Biogenesis, 1:1000) were used. All solutions applied were made with 0.1M PBS, 0.2% gelatin, 5% serum and 0.5% triton. Washes between incubations were made in 0.1M PBS, 0.5% triton. Collagen matrices containing explants and cell aggregates were fixed for 30 min at 4ºC in 4% PFA 0.1% Glutaraldehyde in 0.1M phosphate buffer for

GABA immunostaining. For Calbindin immunostaining, brain sections previously hybridized with *Np1* or *Np2* riboprobes were used (see below). After 3 washes in 0.1M PBS, cultures/sections were blocked in a solution containing 10% serum and 0.5% triton for 2 h at room temperature (RT). After blocking, cultures/sections were incubated O/N at 4ºC in primary antibody at proper dilution, and for 2 h at RT in secondary biotinylated antibody (Vector, 1:200). Streptavidin-peroxidase complex (Amersham, 1:400) was applied for 2 h at RT, and after 3 washes in 0.1M PBS, peroxidase reaction was performed using diaminobenzidine (DAB, 0.06%) and 0.01% H_2O_2. Finally, explant cultures and sections were mounted on gelatinised slides and coverslipped with Mowiol[tm].

2.5.4. In situ hybridisation

In situ hybridisation was performed on free-floating sections, essentially as described (Alcantara et al., 1998). Sections were permeabilized in 0.2% Triton X-100 (15 min), treated with 2% H_2O_2 (15 min), deproteinized with 0.2 N HCl (10 min), fixed in 4% PFA (10 min) and blocked in 0.2% glycine (5 min). Thereafter, sections were pre-hybridised at 60°C for 3 h in a solution containing 50% formamide, 10% dextran sulphate, 5X Denhardt's solution, 0.62 M NaCl, 10 mM EDTA, 20 mM Pipes (pH6.8), 50 mM DTT, 250 µgr/ml yeast t-RNA and 250 µgr/ml denatured salmon sperm DNA. *Sema3A, Sema3F, Np1* and *Np2* riboprobes were labelled with digoxigenin-dUTP (Boehringer-Mannheim) by in vitro transcription. Labelled antisense cRNA was added to the pre-hybridisation solution (250-500 ngr/ml) and hybridisation was performed at 60 ºC overnight. Sections were then washed in 2XSSC (30 min, RT), digested with 20 mgr/ml RNAse A (37°C, 1 h), washed in 0.5XSSC/50% formamide (4 h, 55°C) and in 0.1XSSC/0.1% sarcosyl (1 h, 60°C). After rinsing in Trisbuffered saline (TBS)/0.1% Tween 20 (15 min), sections were blocked in 10% normal goat serum (2 h) and incubated overnight with an alkaline.phosphatase-conjugated antibody to digoxigenin (Boehringer-Mannheim, 1:2000). After washing, sections were developed with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Life Technologies), mounted on gelatinised slides and coverslipped with Mowiol[tm].

Control hybridisations, including hybridisation with sense digoxigenin-labelled riboprobes or RNAse A digestion prior to the hybridisation, prevented alkaline phosphatase staining above background levels.

2.5.5. Analysis and quantification

Hybridised and double hybridised/immunostained sections and explant cultures were examined under a NIKON vertical microscope (Nikon GmbH, Germany). The effect of Sema3A and Sema3F on explants was studied using the AnalySIS[TM] software (Soft Imaging System GmbH, Germany). Each explant was divided into four quadrants, and all axons that crossed a line placed at a distance of 100 µm from the limit of the explant were counted for the proximal and distal quadrants. The ratio of axonal growth was obtained by dividing the number of axons from the proximal quadrant by that of the distal quadrant. The proximal/distal (P/D) ratio for axonal growth yields 1 for radial growth, more than 1 for attractive effect, and less than 1 for repulsive effect. Data were statistically analysed using the Student's t test and the F test for variance.

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2.7. Figures

Figure 1. Mouse hippocampal explants (CA) from E16 (A, C, E) and P0 (B, D, F) stages were incubated with COS cell aggregate expressing control secreted AP (SeAP) (A, B), Sema3A (C, D) and Sema3F (E, F) for 72 hours. They were then fixed and immunostained against GABA. While control explants exhibit radial axonal outgrowth, explants show clear repulsion in response to sources of Sema3A and Sema3F. Scale bar: 200 μm.

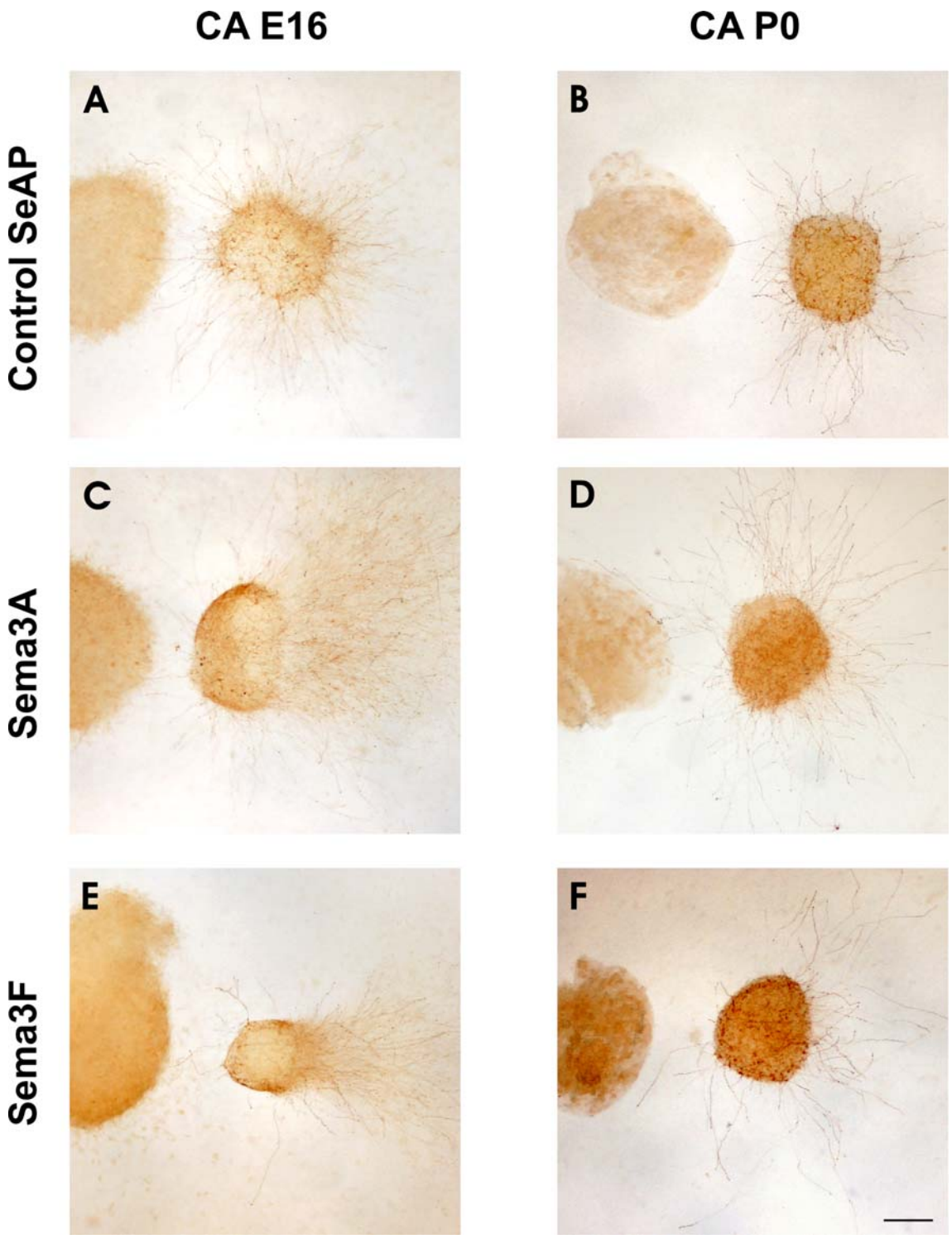

CA E16
CA P0
Control SeAP
Sema3A
Sema3F
A
B
C
D
E
F

Figure 2. Mouse entorhinal explants (EC) from E14 (A, C, E) and E16 (B, D, F) stages were incubated with COS cell aggregate expressing control secreted AP (SeAP) (A, B), Sema3A (C, D) and Sema3F (E, F) for72 hours. They were then fixed and immunostained against GABA. Surprisingly, in vitro, GABAergic axons show the opposite behaviour to axons immunostained against β-tubulin, most of which are considered to belong to pyramidal cells (Chedotal et al., 1998): Sema3F repel GABAergic axons (E, F), while Sema3A and control secreted AP have noeffect (A-D). Scale bar: 200 μm.

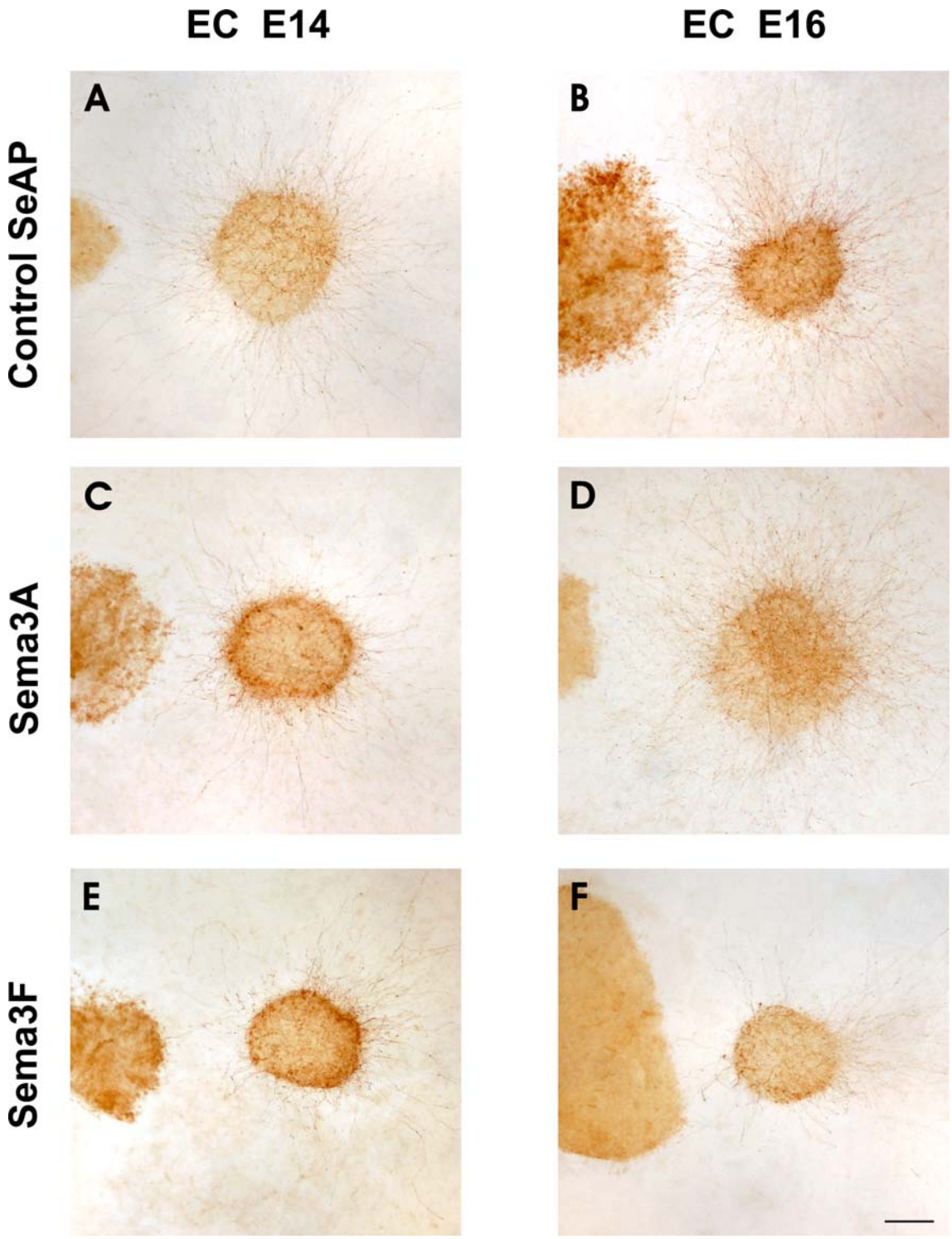
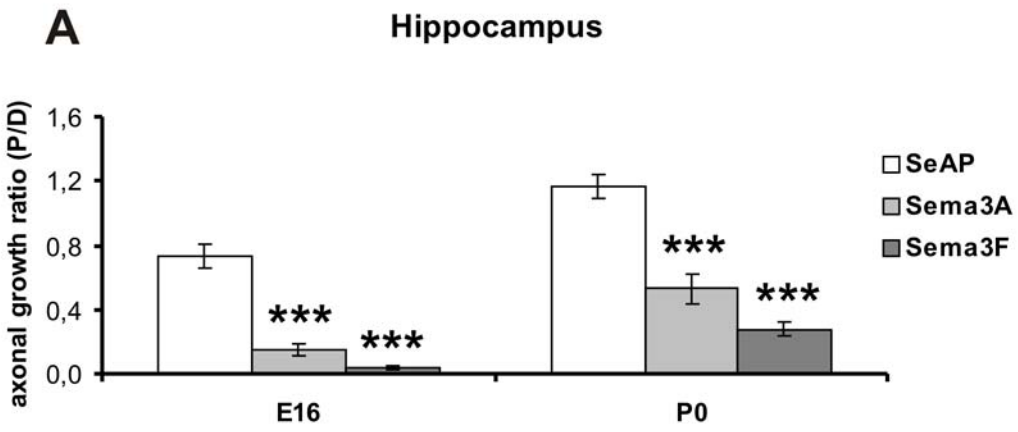

EC E14
EC E16
Control SeAP
Sema3A
Sema3F
A
B
C
D
E
F

Figure 3. Quantification and statistical analysis of the action of Sema3A and Sema3F on hippocampal (A) and entorhinal (B) GABAergic axons in vitro. Axons in the proximal and distal quadrants were counted and a P/D ratio of axonal growth was obtained. 3 asterisks: $p<0.01$.


A
Hippocampus
axonal growth ratio (P/D)
1,6
1,2
0,8
0,4
0,0
***
***
***
***
E16
P0
SeAP
Sema3A
Sema3F

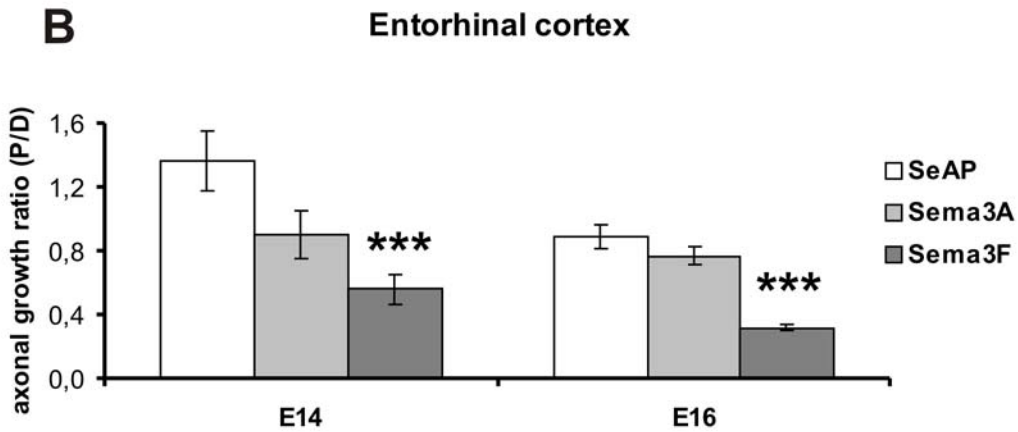

B
Entorhinal cortex
axonal growth ratio (P/D)
1,6
1,2
0,8
0,4
0,0
***
***
E14
E16
SeAP
Sema3A
Sema3F

Figure 4. Expression pattern of *Sema3A* (A, C), *Sema3F* (B, D), *Np1* (E, G) and *Np2* (F, H) at E16 and P0. Note that in all cases, the marginal zone at E16 (which, at later stages, corresponds to the stratum radiatum and lacunosum-moleculare) is the region with less hybridization signal. Scale bar: 100 μm. alv: alveus; hp: hippocampal plate; iz: intermediate zone; mz: marginal zone; pl: pyramidal layer; slm: stratum lacunosum-moleculare; so: stratum oriens; sr: stratum radiatum.

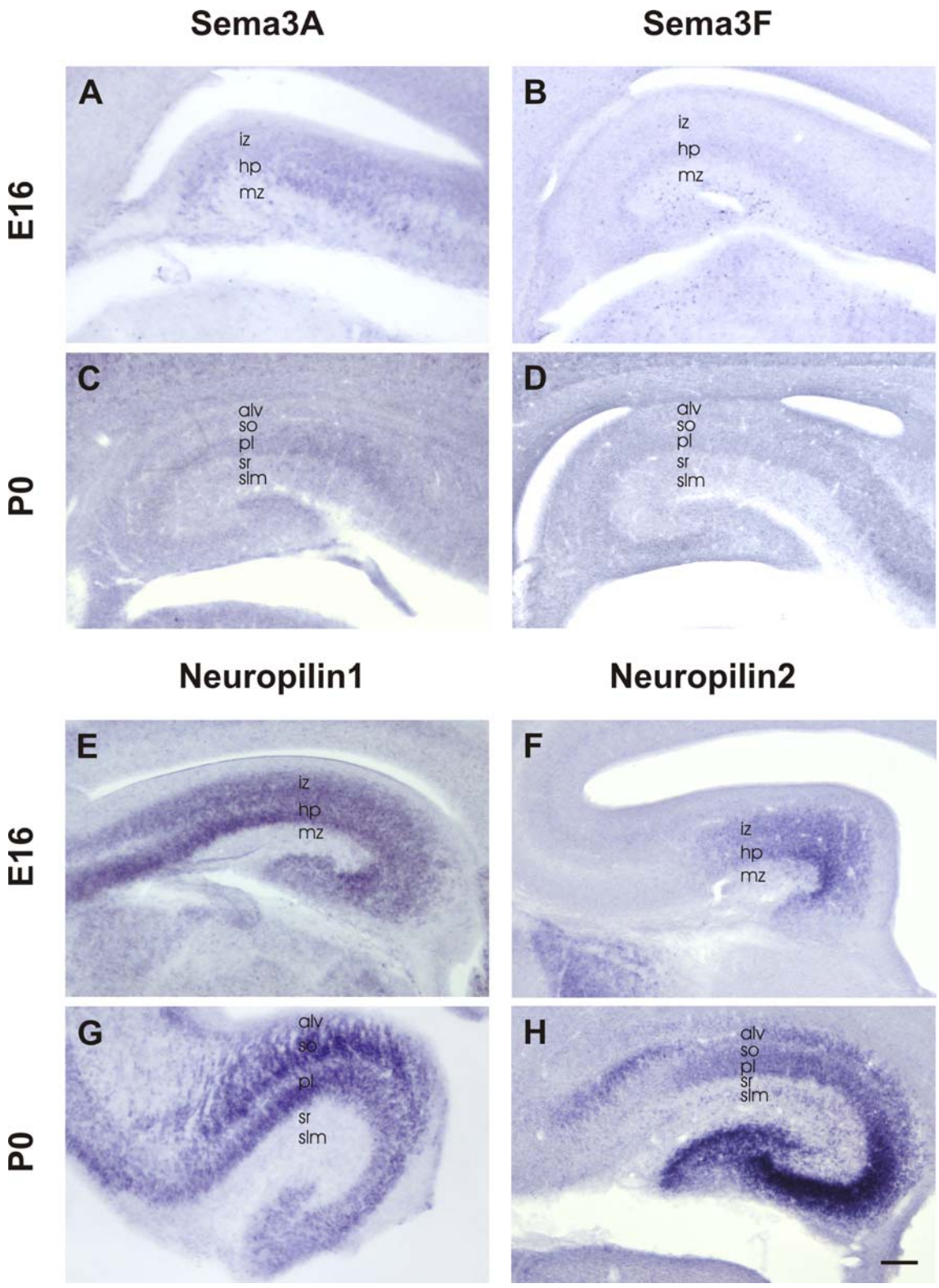
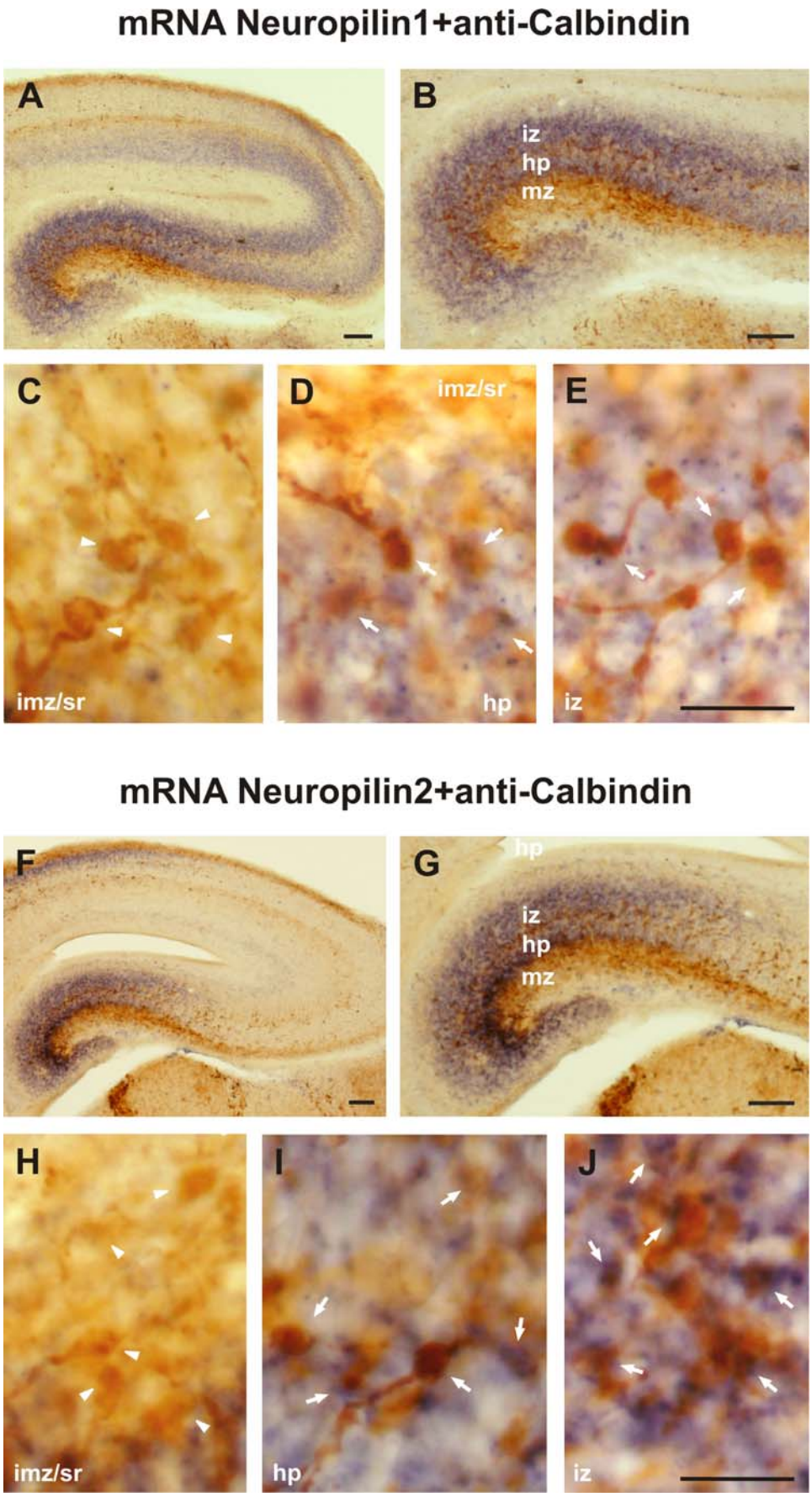

Sema3A
Sema3F
E16
P0
A
iz
hp
mz
B
iz
hp
mz
C
alv
so
pl
sr
slm
D
alv
so
pl
sr
slm
Neuropilin1
Neuropilin2
E
iz
hp
mz
F
iz
hp
mz
G
alv
so
pl
sr
slm
H
alv
so
pl
sr
slm

Figure 5. Double immunohistochemistry against Calbindin (brown label) and in situ hybridization (blue label) using *Np1* (A-E) and *Np2* riboprobes (F-J). White arrowheads point to cells labelled with Calbindin only, most of which are located in the inner marginal zone/prospective stratum radiatum. White arrows show Calbindin-positive cells also labelled with *Np1* or *Np2* riboprobe, situated mainly in the hippocampal plate and subplate/intermediate zone. Scale bar: 100 μm in A, B, F, G / 25 μm in C, D, E, H, I, J. hp: hippocampal plate; iz: intermediate zone; mz: marginal zone; imz/sr: inner marginal zone/prospective stratum radiatum.


mRNA Neuropilin1+anti-Calbindin
A
B
iz
hp
mz
C
imz/sr
D
imz/sr
hp
E
iz
mRNA Neuropilin2+anti-Calbindin
F
G
hp
iz
hp
mz
H
imz/sr
I
hp
J
iz

CAPÍTULO III

La falta de Sema3A produce un aumento de la regeneración de axones entorrinales axotomizados en cultivos organotípicos entorrino-hipocampales

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Resumen

Las semaforinas forman una extensa familia de proteínas secretables y asociadas a membrana, cuya actividad quimiotáctica juega un papel esencial en el desarrollo del sistema nervioso, al participar en varios procesos entre los que se encuentran la guía de axones y células, y la fasciculación axonal. Sin embargo, su función en la edad adulta ha sido poco estudiada. Recientemente, un miembro de la clase 3 de la familia de las semaforinas, Sema3A, ha sido implicado en la inhibición de axones regenerantes tras lesión del SNC y SNP. En este estudio mostramos que, tras practicar la axotomía en la proyección entorrino-hipocampal in vitro, la expresión de Sema3A resulta potenciada en la corteza entorrinal y en el hipocampo, mientras que la expresión de su receptor Np1 aumenta sobretodo en la corteza entorrinal. Además, la supresión de la función de Sema3A da lugar a la recuperación parcial de la capacidad regenerativa en axones entorrinales previamente axotomizados in vitro. Por lo tanto, nuestros resultados apoyan la hipótesis formulada previamente de que Sema3A puede actuar como un factor inhibidor para la regeneración de axones lesionados.

The lack of Sema3A increases the regeneration of entorhino-hippocampal axons in organotypic slice cultures

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3.1. Abstract

Semaphorins comprise a wide family of secreted and membrane-associated proteins with chemotactic activity. They play crucial roles in the development of nervous system, participating in multiple processes including axon guidance, axonal fasciculation and cell migration. However, little attention has been given to the function of semaphorins in adulthood. Recently, a member of class 3 vertebrate secreted semaphorins, Sema3A, has been reported to be involved in inhibitory actions on regenerating axons after injury of both the central and peripheral nervous system. Here we show that, after axotomy of the entorhino-hippocampal projection in vitro, Sema3A expression is enhanced in the entorhinal cortex and hippocampus, while expression of the Sema3A receptor Neuropilin 1 increases mostly in the former. Moreover, genetic suppression of Sema3A function led to a partial recovery of the regenerative capacity of axotomized entorhinal axons in vitro. Thus, our data support the hypothesis that Sema3A inhibits the regeneration of injured axons.

3.2. Introduction

Since its discovery in the nineties, the semaphorin family has been closely linked to several aspects of the development of the nervous system, such as axon and dendritic guidance, neuronal migration, axonal fasciculation and branching, synaptogenesis and apoptosis (reviewed in Fiore and Puschel, 2003). Semaphorins are secreted and membrane-associated proteins that show generally chemorepulsive activity on developing neuronal processes and cells in the nervous system, although they can also act as attractive cues (Fiore and Puschel, 2003). They have been classified in eight groups, in function of common structure and vertebrate or invertebrate origin (de Wit and Verhaagen, 2003). Amongst the various proteins that can act as semaphorin receptors, neuropilins and plexins are considered the most important: plexins bind directly to class 1, 4 and 7 semaphorins and trigger their intracellular signalling, while secreted class 3 semaphorins required a receptor complex composed of neuropilins as binding units, and plexins as signal transducers (de Wit and Verhaagen, 2003; Pasterkamp and Kolodkin, 2003). In particular, Sema3A receptor complex is formed by Neuropilin 1 (Np1) homodimers and plexins mainly from class A (Kolodkin et al., 1997; Takahashi et al., 1998; Tamagnone et al., 1999; Yaron et al., 2005).

In addition to a wide distribution during embryonic and postnatal development, several semaphorins are also expressed in specific neuronal populations of the adult brain, including the hippocampal formation. The most extensively studied semaphorin in adulthood is Sema3A, which is involved in two phenomena. First, as *Sema3A* is expressed in adult layer II of the entorhinal cortex and may be secreted by entorhinal terminals into the molecular layer of the dentate gyrus, it may guide dendrites and axons of newly generated granule cells (de Wit and Verhaagen, 2003). Furthermore, a decrease in the expression of this semaphorin might explain the reactive sprouting following entorhinal axotomy or electrical induction of epilepsy (Giger et al., 1998; Holtmaat et al., 2003). Second, Sema3A has been proposed as an inhibitor of axonal regeneration in the peripheral (PNS) and central nervous system (CNS). The down-regulation of *Sema3A* mRNA in motoneurons of spinal cord after peripheral injury may facilitate the regeneration of the tract by impeding accumulation of a repulsive factor in the lesion zone (Pasterkamp et al., 1998a), and the expression of *Sema3A* and other secreted semaphorins by the fibroblastic component of the glial scar may impair axonal regrowth after injury to the CNS (De Winter et al., 2002; Lindholm et al., 2004; Pasterkamp et al., 1999; Pasterkamp et al., 2001). In line with this, the continuous infusion of Xantofulvin, a specific inhibitor of Sema3A, strongly stimulates the regeneration of olfactory fibres after sectioning the olfactory nerve (Kikuchi et al., 2003).

Using the entorhino-hippocampal slice culture as an in vitro model of axotomy, we further assessed the inhibitory role of Sema3A on regenerating axons. We performed in situ hybridization to examine the expression pattern of *Sema3A* and *Np1* in slice cultures. After axotomy of the entorhino-hippocampal connection (also called the entorhino-hippocampal pathway), *Sema3A* mRNA was upregulated throughout the culture, while *Np1* expression was increased specifically in the entorhinal cortex. These observations suggest that the expression of *Sema3A* in the entorhinal cortex and hippocampus may inhibit the growth of sectioned *Np1*-expressing entorhinal axons. Moreover, we confirm this preliminary hypothesis by showing that homozygous *Sema3A*-deficient slice cultures show a significant increase in the number of regenerating entorhinal axons after injury to the entorhino-hippocampal pathway, which indicate the participation of Sema3A and Np1 in preventing the regeneration of entorhino-hippocampal axons after injury.

3.3. Results

3.3.1. Axotomy of the perforant pathway in vitro enhances the expression of *Sema3A* and *Np1*

As a first step to ascertain whether Sema3A affects the regenerating capacity of injured fibers, we studied the expression of *Sema3A* and its receptor *Np1* in organotypic slice cultures of the entorhino-hippocampal pathway obtained from P0-P1 mouse pups, a system which has been previously used as a model of injury in vitro (del Rio et al., 2002; Mingorance et al., 2004; Mingorance et al., 2005; Mingorance et al., 2006). For this purpose, we performed enzymatic in situ hybridizations on organotypic cultures in which the entorhino-hippocampal pathway was either intact (Control, Fig. 1A, D) or was surgically injured at the level of the subicular complex (Axotomy, Fig. 1B, C, E, F) as described in previous studies (del Rio et al., 2002; Li et al., 1994). Two-week-old cultures were chosen for these

experiments since the axotomy of the entorhino-hippocampal pathway at this age yields no significant regrowth of entorhinal fibers into the hippocampal tissue (see below) (del Rio et al., 2002).

In control cultures, *Np1* was expressed mainly in all projection layers of the hippocampus: in the CA1-CA3 pyramidal layer, in the granule layer and hilus of the dentate gyrus, and also in the subicular complex. *Np1* hybridization signal was also detected in the entorhinal cortex, although it was less intense than in the hippocampus. Three days after axotomy of the entorhinal fibers at 15 DIV, *Np1* expression was maintained at roughly the same level in the hippocampus, but was enhanced in the entorhinal cortex (Fig. 1B). This increase in *Np1* mRNA became progressively lower with time, and at 10 days after axotomy, expression of this receptor resembled that observed in control cultures (Fig. 1C). *Sema3A* signal was low but appreciable in the entorhinal cortex and the hippocampus of control cultures (Fig. 1D). However, this low level of *Sema3A* expression increased after axotomy (Fig. 1E), especially in the hippocampus and to a lesser extent in the entorhinal cortex, and high expression was maintained several days after injury (Fig. 1F). On the basis of these observations, we propose that Sema3A, abundantly expressed in the hippocampal tissue after axotomy, has an inhibitory effect on sectioned entorhinal fibers, which are more sensitive to this semaphorin because of enhanced Np1 expression after axotomy. Thus, the expression patterns of *Sema3A* and *Np1* shown here correlate well with these molecules having a negative role on the regeneration of the entorhino-hippocampal pathway after lesion.

3.3.2. Increased regrowth of entorhinal fibers in organotypic cultures from *Sema3A* knock-out mice

To further study the possible effect of Sema3A in inhibiting the regeneration of entorhinal axons, organotypic entorhino-hippocampal slices were obtained from a *Sema3A* knock-out mouse strain (Behar et al., 1996). As described previously (del Rio et al., 2002), tracing of the entorhino-hippocampal pathway in non-axotomized wild-type cultures at 7 and 15 DIV labels many entorhinal fibers that innervate the stratum lacunosum-moleculare and the dentate molecular layer, which are the termination fields of the entorhino-hippocampal pathway in vivo; this pattern of innervation was similar in *Sema3A* knock-out heterozygous and homozygous cultures (data not shown). Thus, the deficiency in Sema3A did not significantly affect the formation of the entorhino-hippocampal pathway in vitro.

After 15 days in culture, axotomy of the entorhino-hippocampal tract was performed at the level of the subicular complex, as described above, and at 10 days post-axotomy the anterograde tracer biocytin was injected in the entorhinal cortex of wild-type and *Sema3A* knock-out heterozygous and homozygous organotypic cultures in order to label and quantify the regrowth of entorhinal fibers. Corroborating previous results (del Rio et al., 2002), few regenerating entorhinal fibers invaded the hippocampus in wild-type cultures (Fig. 2C, D and E). In contrast, the number of axons labelled by biocytin in *Sema3A* homozygous mutant cultures was increased two-fold compared with wild-type cultures (Fig.2A, B and E). In those cases where the ingrowing entorhinal axons extended further across the hippocampal tissue, most terminated in the stratum lacunosum moleculare of the hippocampus proper and in the molecular layer of the dentate gyrus (Fig. 2A, B), thus maintaining the innervation specificity of the entorhino-hippocampal pathway in vivo (del Rio et al., 2002; Frotscher and Heimrich, 1993). Unlike the entorhino-hippocampal projection, fibers starting from the subiculum and CA1 of the hippocampus and innervating deep layers or the entorhinal cortex regenerate after the axotomy of the entorhino-hippocampal pathway (del Rio et al., 2002). We found no significant difference in retrogradely labelled neurons in subiculum and CA1 between *Sema3A*-deficient homozygous mutant cultures and those obtained from heterozygous and wild-type mice (data not shown), indicating that the inhibitory effect of Sema3A is specific for axotomized entorhinal axons. Taken together, these data point to Sema3A as a possible inhibitor of axonal regrowth after lesion.

3.4. Discussion

Several studies have focused on the role of semaphorins as chemorepulsive factors for axons and cells in the developing nervous system (Fiore and Puschel, 2003). However, only recently has effort to uncover the function of semaphorins been extended to adulthood, and it has been proposed that, during the adult period, semaphorins not only participate in the stabilization and maintenance of neural connections, but also contribute to the failure of axonal regeneration in the adult nervous system (de Wit and Verhaagen, 2003). Using an in vitro model of injury, here we show that the expression of *Sema3A*

and its receptor *Np1* is increased in entorhino-hippocampal slice cultures after axotomy of the entorhino-hippocampal pathway. This observation indicates that Sema3A expressed in the hippocampus may have an inhibitory effect on sectioned *Np1*-expressing entorhinal fibers. Moreover, in support of this initial assumption, we demonstrate that, in organotypic slices from *Sema3A*-deficient animals, the regenerating capacity of sectioned entorhinal axons is partially recovered. Thus, Sema3A acts as an inhibitory cue for injured axons in vitro.

3.4.1. The absence of *Sema3A* increases the regrowth of *Np1*-expressing entorhinal axons after lesion.

In this study, we used in situ hybridization techniques to analyze the distribution of *Sema3A* and *Np1* mRNA in entorhino-hippocampal organotypic slice cultures with or without injury in the entorhino-hippocampal pathway. In this in vitro model, expression of *Sema3A* and *Np1* was detected in the hippocampus and entorhinal cortex: a low signal for *Sema3A*, and a robust signal for *Np1*. After axotomy of the entorhinal fibers, these two genes were clearly upregulated, *Np1* mRNA level increasing only in the entorhinal area, and *Sema3A* mRNA level increasing in the hippocampus and entorhinal cortex. This expression pattern of *Sema3A* and *Np1* in an in vitro model of the entorhino-hippocampal pathway differs considerably from the homologous situation in vivo. The hippocampal formation of the adult rat shows robust expression of *Sema3A* only in layers II and III of the entorhinal cortex, the hippocampus proper and dentate gyrus being devoid of specific hybridization label (Giger et al., 1998). In embryonic stages of the mouse, the expression of Np1 mRNA is strong in the hippocampal pyramidal layer, dentate granule layer and entorhinal cortex, but declines one week after birth and is reduced to low levels in the adult (Kawakami et al., 1996; Steup et al., 1999, and data not shown). After a penetrant injury to the entorhino-hippocampal pathway, a glial scar showing clear *Sema3A* expression forms in the lesion zone (Pasterkamp et al., 1999). We have obtained preliminary data about the in vivo expression of *Sema3A* and *Np1* in the mouse hippocampal formation (data not shown). These data indicate that, after in vivo axotomy of the entorhino-hippocampal pathway, there is no change in the specific hybridization signal of *Sema3A* and *Np1*, which differs from the data obtained in vitro in this study.

Nevertheless, and in spite of differences in *Sema3A* and *Np1* expression between the in vitro and the in vivo models, both models indicate that an inhibitory action of Sema3A may be partially responsible for the lack of axonal regeneration observed in the entorhino-hippocampal pathway: after injury in vivo, adult entorhinal axons with low *Np1* expression encounter the glial scar formed in the damaged area, where considerable amounts of *Sema3A* mRNA are present (Kawakami et al., 1996; Pasterkamp et al., 1999; Steup et al., 1999); besides, axotomy of the entorhino-hippocampal pathway in organotypic slice cultures increases the already high expression of *Np1* shown by entorhinal axons, which respond to the enhanced level of Sema3A mRNA in the entorhinal area and hippocampus.

3.4.2. Sema3A together with other inhibitory cues blocks the in vitro regrowth of injured entorhinal axons**.**

After axotomy of the entorhino-hippocampal pathway, a significant increase in the amount of regenerating entorhinal axons was achieved when organotypic cultures were obtained from homozygous mice deficient in *Sema3A*. This result demonstrates the inhibitory action of Sema3A on sectioned fibers of the entorhino-hippocampal pathway in vitro, thus confirming previous studies that report a negative effect of this semaphorin on axonal regeneration in the central and PNS (Pasterkamp et al., 1998a; Pasterkamp et al., 1998b; Pasterkamp et al., 1999; Pasterkamp et al., 2000; Pasterkamp et al., 2001); it's likely that Np1 in entorhinal axons probably participates in the transduction of the Sema3A inhibitory signal, as inferred from the expression pattern of *Sema3A* and *Np1* in non-damaged and injured entorhino-hippocampal cultures.

However, the degree of regeneration obtained by abolishing the inhibitory action of Sema3A was clearly inferior to the entorhinal innervation of hippocampus observed in control non-axotomized organotypic cultures. Similar partial regrowth of the entorhino-hippocampal pathway is also obtained when functional blockade of other inhibitory factors for axonal growth, like myelin-associated glycoprotein (MAG), Nogo66 or chondroitin sulfate proteoglycans (CSPG), is performed on entorhino-hippocampal organotypic cultures (Mingorance et al., 2004; Mingorance et al., 2005; Mingorance et al., 2006). Nor does the functional suppression of myelin inhibitors or Sema3A lead to such a massive

regeneration of the entorhino-hippocampal pathway in vitro as that obtained when a piece of young hippocampal tissue that contains Cajal-Retzius cells is transplanted into an axotomized entorhino-hippocampal organotypic culture (del Rio et al., 2002). Thus, it is plausible that several factors and not a single one contribute to create an inhibitory environment for regenerating axons of the entorhino-hippocampal pathway. Indeed, it has been proposed that neutralization of the inhibitory effects present in the damaged tissue, along with enhancement of axonal growth might improve the regrowth of injured axons (He and Koprivica, 2004). On the basis of this idea of multi-factorial inhibition of axonal regeneration, Mingorance and colleagues applied a combined treatment to axotomized entorhino-hippocampal cultures, which consisted in degradation of CSPG by the application of chondroitinase ABC (chABC) and blockade of Nogo66 by the application of a synthetic peptide (NEP1-40) that interferes with Nogo66/NgR interaction (Mingorance et al., 2006). Surprisingly, no significant improvement in the regeneration of entorhinal axons was observed, when compared to the treatment with chABC alone. This lack of synergistic effect in the combined functional suppression of the two inhibitors was interpreted as caused by an intracellular convergence of CSPG and myelin component signalling, since these two kinds of inhibitors activate PKC, and the inhibition of PKC in vivo promotes the regeneration of specific tracts after sectioning the spinal cord (Hasegawa et al., 2004; Sivasankaran et al., 2004). In addition, blocking the activity of PKC in entorhino-hippocampal slice cultures after axotomy enhances entorhinal regeneration (Prang et al., 2001).

In line with this, the intracellular signalling cascade of Sema3A differs from that of the above inhibitors. Sema3A belongs to the group of chemotactic factors that increase the cytosolic level of cGMP and subsequently activates PKG, without altering cytosolic levels of cAMP and Ca+2 (Song and Poo, 1999). In contrast, MAG inhibits neuritic growth by decreasing intracellular cAMP levels and increases cytosolic Ca+2 in cultured neurons (Cai et al., 1999; Hasegawa et al., 2004; Song et al., 1998). Moreover, inhibition of PKG by adding antagonists of cGMP cannot overcome the inhibitory effect of myelin on neuronal cultures (Sivasankaran et al., 2004). Thus, Sema3A triggers a signalling cascade that differs from that of myelin and proteoglycans. Therefore, it is plausible that simultaneously blocking of Sema3A and myelin/proteoglycan intracellular pathways would result in a synergistic enhancement of axonal growth after injury.

3.5. Materials and Methods

3.5.1. Entorhino-hippocampal organotypic slice co-cultures and axotomy of the perforant pathway in vitro

Entorhino-hippocampal slice co-cultures were prepared as described (Del Rio et al., 1997; Stoppini et al., 1991). Slice co-cultures were obtained from a mouse strain deficient in *Sema3A* (Behar et al., 1996), and homozygous, heterozygous and wild-type animals were used. To identify the genotype, PCR was performed using two set of primers that bind wild-type *Sema3A*, and the neomycin gene introduced by homologous recombination (Behar et al., 1996). P0-P1 animals were anaesthetized by hypothermia, and the hippocampus and entorhinal cortex were dissected out. Horizontal sections (300-350 μm thick) were obtained in a McIlwain tissue chopper (Mickle Laboratory). Slices were maintained in Minimum Essential Medium (MEM) supplemented with glutamine (2mM) for 45 min at 4ºC. Slices were then placed on a porous Millicell CM-membrane (Millipore) and incubated using the interface culture technique (Stoppini et al., 1991). Incubation medium was 50% MEM, 25% horse serum, 25% Hank's balanced salts, supplemented with L-glutamine (2mM) (all reagents were purchased from GIBCO Life Techn.). The medium was replaced/renewed after 24 h and thereafter every 48 h until the tissue was examined. After 15 days in vitro (DIV), the entorhino-hippocampal projection was axotomized by cutting the organotypic cultures from the rhinal fissure to the ventricular side along the entire entorhino-hippocampal interface using a tungsten knife.

3.5.2. In situ hybridization

Slice cultures destined to be processed by in situ hybridization were fixed in a phosphate-buffered (PB) 0.1M solution containing 4% paraformaldehyde for 1 h at room temperature. After rinsing in phosphate-buffered saline (PBS) 0.1M, slices were carefully detached from Millicell membranes, placed on the flat surface of square platforms (made of gelatine and BSA thoroughly mixed in PBS and

clotted with glutaraldehyde) and allowed to dry. Cultures fixed on the platforms were then embedded in a second mixture of gelatine and BSA in PBS 0.1M. The mixture covering the cultures were allowed to coagulate by the action of glutaraldehyde, and sections of 50 μm in thickness were obtained using a vibratome. Free-floating sections were rinsed in PBS 0.1M, and permeabilized in 0.2% Triton X-100 (15 min). They were then treated with 2% H_2O_2 (15 min), deproteinized with 0.2 N HCl (10 min), fixed in 4% PFA (10 min) and blocked in 0.2% glycine (5 min). Thereafter, sections were pre-hybridised at 60°C for 3 h in a solution containing 50% formamide, 10% dextran sulphate, 5X Denhardt's solution, 0.62 M NaCl, 10 mM EDTA, 20 mM Pipes (pH 6.8), 50 mM DTT, 250 μgr/ml yeast t-RNA and 250 μgr/ml denatured salmon sperm DNA. Sema3A and Np1 riboprobes were labelled with digoxigenin-dUTP (Boehringer-Mannheim) by in vitro transcription. A 2-kb fragment of *Sema3A* cDNA (Messersmith et al., 1995) and a 1.1-kb fragment of *Np1* cDNA (He and Tessier-Lavigne, 1997) were transcribed using T3 polymerase (Ambion) for antisense probes, and T7 polymerase (Ambion) for sense probes. Labelled antisense cRNA was added to the pre-hybridisation solution (500-1000 ngr/ml) and hybridisation was performed at 60 ºC overnight. Sections were then washed in 2XSSC (30 min, room temperature), digested with 20 mgr/ml RNAse A (37°C, 1 h), washed in 0.5XSSC/50% formamide (4 h, 55°C) and in 0.1XSSC/0.1% sarcosyl (1 h, 60°C). After rinsing in tris-buffered saline (TBS)/0.1% Tween 20 (15 minutes), sections were blocked in 10% normal goat serum (2 h) and incubated overnight in an alkaline phosphatase-conjugated antibody to digoxigenin (Boehringer-Mannheim, 1:2000). Sections were washed and then developed with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Life Technologies), mounted on gelatinised slides and coverslipped with Mowiol[tm].

3.5.3. Biocytin tracing, immunohistochemical method and quantification of entorhinal axons.

9-10 days after the axotomy of the entorhino-hippocampal pathway, a small crystal of biocytin (Sigma-Aldrich) was placed in the entorhinal cortex in order to trace the entorhino-hippocampal projection. Twenty-four hours later, slice cultures were fixed in a PB 0.1M solution containing 4% paraformaldehyde for 1 h at room temperature. After rinsing the slices in PBS, sections of 50 μm in thickness were obtained using a vibratome, as described above. Free-floating sections were rinsed in PBS 0.1M, permeabilized by increasing concentrations of DMSO (10%, 20%, 40%) diluted in PB 0.1M, rinsed again in PBS 0.1M, blocked with 10% horse serum and incubated overnight with the avidin-biotin-peroxidase complex (ABC-elite[tm] Vector Laboratories) diluted 1:100. After rinsing sections in PBS 0.1M, peroxidase activity was visualized using a nickel-enhanced diaminobenzidine (DAB) reaction (Del Rio et al., 1997). Sections were then mounted onto gelatinized slides, Nissl-stained and coverslipped with Eukitt[tm] (Merck). For quantification, a segment of 400 μm was traced parallel to the lesion interphase and from there, at a distance of 75-80 μm, the number of axons crossing the segment in consecutive sections from each culture was calculated using a 40X oil-immersion objective.

3.6. References

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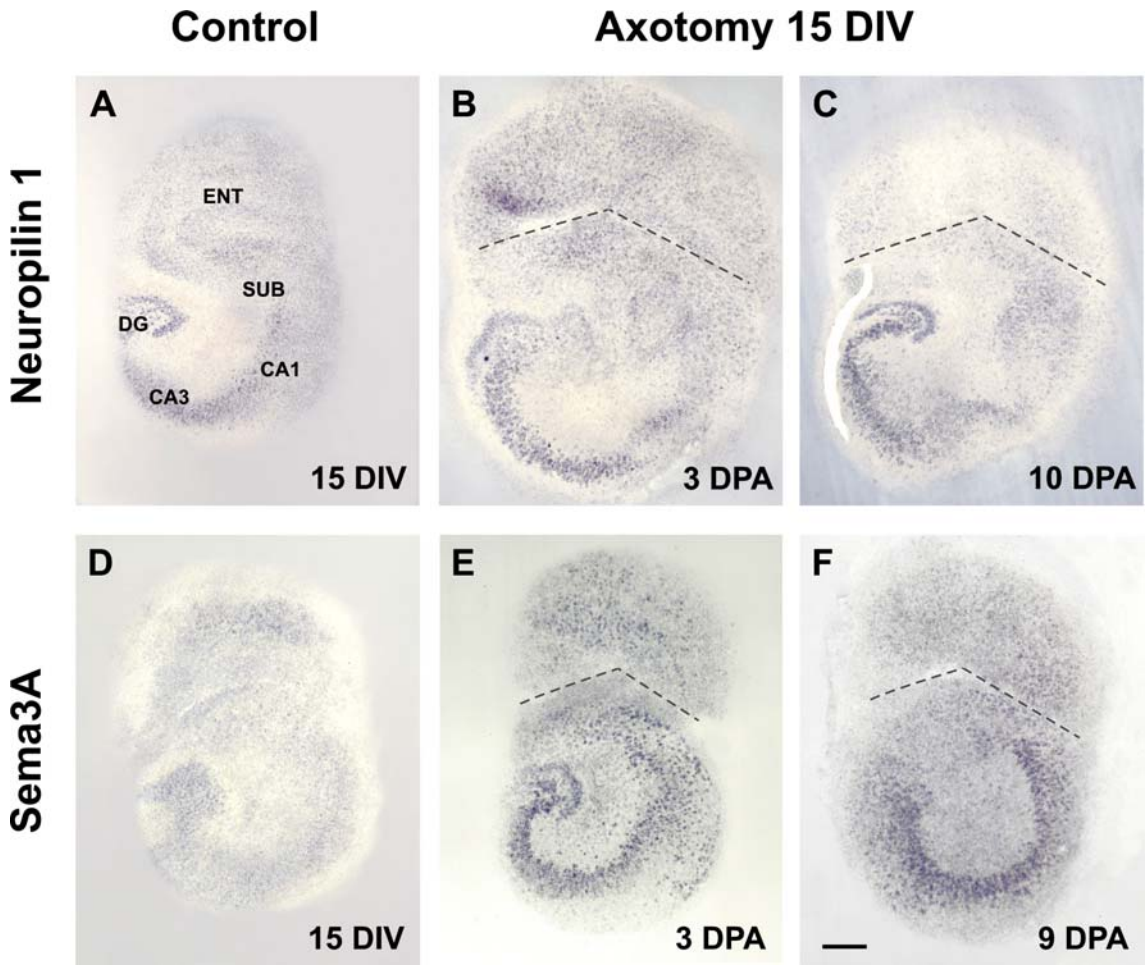
3.7. Figures

Figure 1. Expression of *Sema3A* and its receptor *Np1* in non-injured entorhino-hippocampal organotypic cultures (A, D) and cultures with a lesion of the entorhino-hippocampal pathway at 15 DIV (B, C, E, F). Dashed lines indicate the site of the axotomy. *Np1* expression increases in the entorhinal cortex after 3 days post-axotomy (DPA) (B), and progressively increases to recover the expression level observed in control cultures (C). An increase in *Sema3A* expression is induced after injury of the entorhino-hippocampal pathway in the entorhinal cortex and the hippocampus (E), and unlike *Np1*, this enhancement persists several days after axotomy (F). CA: hippocampal CA. DG: dentate gyrus. ENT: entorhinal cortex. SUB: subiculum. Scale bar: 200 μm.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Neuropilin 1
Sema3A
A
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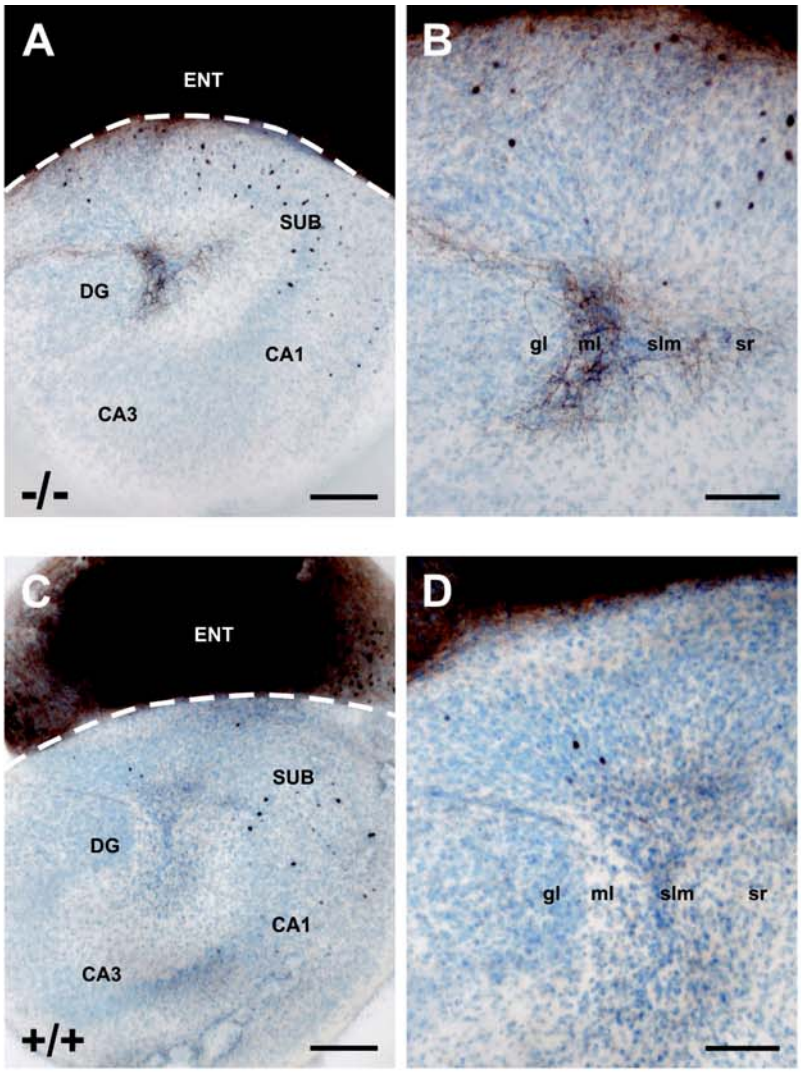
DG
CA1
CA3
15 DIV
3 DPA
10 DPA
15 DIV
3 DPA
9 DPA

Figure 2. Regeneration of entorhino-hippocampal fibers in organotypic cultures of *Sema3A* mutant (A, B) and wild-type mice (C, D). Dashed lines indicate the site of the axotomy. Note that in homozygous mutant slices, ingrowing entorhinal axons specifically innervate the stratum lacunosum-moleculare (SLM) and molecular layer (ML) in the hippocampus and dentate gyrus respectively. (E) Quantification and statistical analysis of the regenerating entorhinal fibers; $p<0.05$ (ANOVA analysis). CA: hippocampal CA. DG: dentate gyrus. ENT: entorhinal cortex. gl: granular layer of the dentate gyrus. ml: molecular layer of de dentate gyrus. slm: stratum lacunosum-moleculare of hippocampal CA. sr: stratum radiatum of hippocampal CA. SUB: subiculum. Scale bar: 200 μm in A, C / 100 μm in B, D.


A
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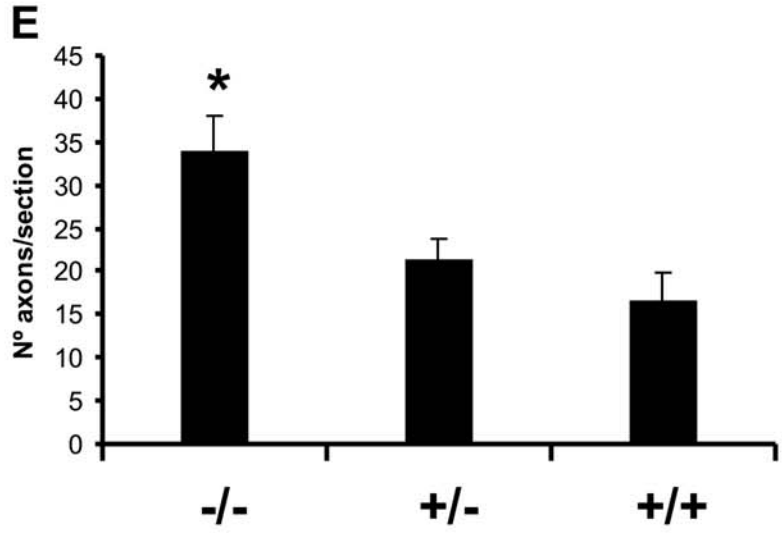
E
Nº axons/section
45
40
35
30
25
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5
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