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Meningeal cell-derived semaphorin 3A inhibits neurite outgrowth

Simone P. Niclou,^{a,*} Elske H.P. Franssen,^a Erich M.E. Ehlert,^a Masahiko Taniguchi,^b
and Joost Verhaagen^a

^a Graduate School of Neurosciences Amsterdam, Netherlands Institute for Brain Research, Meibergdreef 33, 1105 AZ Amsterdam, The Netherlands

^b Department of Biochemistry and Molecular Biology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

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Abstract

The neural scar that forms after injury to the mammalian central nervous system is a barrier to sprouting and regenerating axons. In addition to reactive astrocytes that are present throughout the lesion site, leptomeningeal fibroblasts invade the lesion core. When isolated *in vitro*, these cells form a very poor substrate for growing neurites, even more so than reactive astrocytes. Nevertheless the molecular mechanisms involved in this growth inhibition are not well understood. Semaphorins have been reported to be upregulated in meningeal cells (MCs) on mechanical injury to the brain and spinal cord. In the present study, we show that *Sema3A* mRNA and active protein are produced by cultured meningeal cells. A protein extract from these cells induces the collapse of embryonic dorsal root ganglion (DRG) growth cones. This collapsing activity is partially blocked by neuropilin-1 antibodies and is absent in meningeal cells derived from *Sema3A*-knockout mice. In addition to growth cone collapse, recombinant *Sema3A* but not *Sema3C* inhibits neurite outgrowth of embryonic DRGs. Consistent with this result we find that the inhibitory effect of meningeal cells on neurite outgrowth is partially overcome on *Sema3A*-deficient MCs. Furthermore we show that the inhibitory effect of MC-derived *Sema3A* on neurite outgrowth is modulated by nerve growth factor. Our results show that *Sema3A*, a chemorepellent during nervous system development, is a major neurite growth-inhibitory molecule in meningeal fibroblasts and is therefore likely to contribute to the inhibitory properties of the neural scar.

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Introduction

Regenerating neurons are unable to cross the glial/fibrotic scar that forms after penetrating injuries to the adult mammalian central nervous system (CNS), yet the molecular and cellular culprits responsible for this potent inhibition of axon growth are largely unknown. Although potent inhibitory proteins such as *NogoA*, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp) are released from injured myelin (Spencer et al., 2003), these are largely excluded from the scar proper (Wang et al., 2002) and therefore unlikely to contribute significantly to the barrier imposed by the scar.

The scar is made up primarily of reactive astrocytes characterized by enhanced glial fibrillary acidic protein (GFAP) expression and a variable number of other cell

types including microglia/macrophages, oligodendrocyte precursors, and leptomeningeal fibroblasts. The latter migrate in from injured meninges and disrupted blood vessels to form the fibrotic scar (Ross, 1968; Berry et al., 1983; Carbonell and Boya, 1988). At the interface between glial and meningeal cells, an extensive basal lamina is deposited and astrocytes undergo morphological changes leading to the formation of a new glia limitans (Shearer and Fawcett, 2001). This process, which seals off the CNS tissue from the non-CNS environment, is thought to require the presence of leptomeningeal cells (Abnet et al., 1991; Sievers et al., 1994).

In vitro astrocytes are normally considered to be a permissive substrate for neurite growth similar to Schwann cell cultures (Noble et al., 1984) and astrocyte conditioned medium is often used for the maintenance of cultured neurons. Although their role in regeneration is debated, astrocytes also display growth-inhibitory properties depending on culture type, time in culture, and source and age of the astrocyte (reviewed in Reier et al., 1989; Fawcett and Asher,

* Corresponding author. Fax: +31-20-696-10-06.

E-mail address: s.niclou@nih.knaw.nl (S.P. Niclou).

1999). Based on a large number of studies it appears that the main class of inhibitory molecules produced by astrocytes are chondroitin sulfate proteoglycans (CSPGs). These molecules are upregulated around CNS injuries and many of them have been shown to inhibit axon growth in vitro (reviewed in Asher et al., 2001). In vivo the treatment of the lesion site with chondroitinase ABC, which removes the glycosaminoglycan chain from CSPGs, allows some axon regeneration through the glial scar in brain and spinal cord injuries (Moon et al., 2001; Bradbury et al., 2002).

Meningeal cells (MCs) are clearly an unfavorable substrate for neurons in vitro (Noble et al., 1984; Hirsch and Bahr, 1999), but surprisingly little is known about the inhibitory molecules involved. The inhibition of neurite growth appears to result from cell surface interactions between neuronal and meningeal cells, as the effect was not apparent when neurons were cultured in conditioned medium of MCs (Noble et al., 1984). When neurons are seeded on a mixed substrate of astrocytes and MCs, neurite outgrowth decreases dramatically with increased numbers of fibroblasts, an effect that was attributed to the reduced availability of the permissive substrate (Rudge and Silver, 1990). MCs were also shown to negatively affect the growth-promoting characteristics of cultured astrocytes (Ness and David, 1997). In this case, the effect appears to be due to a soluble factor, as the changes in astrocyte morphology and growth promotion were also observed with MC-conditioned medium. MCs express keratan sulfates, versican, NG2, and possibly other proteoglycans but their contribution to the nonpermissiveness of MCs is poorly understood (Hirsch and Bahr, 1999; Asher et al., 2002; Morgenstern et al., 2002).

We have previously reported that MCs at the lesion site express the potent chemorepellent semaphorin 3A (Sema3A) in several models of brain and spinal cord injury (Pasterkamp et al., 1998, 1999). Other members of the class 3 semaphorins including Sema3B, Sema3C, Sema3F, and Sema3E transcripts are also upregulated in MCs in the neural scar (De Winter et al., 2002b). Neuropilins and plexinA proteins, which form a receptor complex for secreted semaphorins, are expressed in injured and noninjured neurons in the adult brain (Murakami et al., 2001; Reza et al., 1999; Gavazzi et al., 2000; De Winter et al., 2002b). Of note is that in neonatal rats, where axon regeneration takes place, infiltration of meningeal fibroblasts into the scar does not occur (Berry et al., 1983) and Sema3A expression is detected only in the intact meninges (Pasterkamp et al., 1999). Thus Sema3A and other class 3 semaphorins are prominent candidates to mediate growth inhibition by MCs in the glial/fibrotic scar.

To investigate this possibility, we used cultured MCs and showed that a protein extract from MC membranes induces the collapse of dorsal root ganglion (DRG) growth cones. This collapsing activity is inhibited by neuropilin-1 (NP1) antibodies and is absent in MCs from Sema3A-knockout mice. We further show that MCs are an inhibitory substrate for growing axons and that their inhibitory effect is partially

overcome on Sema3A-deficient MCs. Finally we show that the inhibitory effect of MC-derived Sema3A on neurite outgrowth is modulated by nerve growth factor (NGF).

Results

Characterization of cultured meningeal cells

Immunocytochemical markers expressed by cultured meningeal cells

MCs prepared from the leptomeninges of neonatal rat pups were used experimentally between passages 2 and 5. The cells were characterized by immunocytochemical staining for several marker proteins. As expected meningeal fibroblasts strongly express the extracellular matrix (ECM) molecule fibronectin and the cytoskeletal protein vimentin (Fig. 1A). They also stain positive with the CS-56 antibody recognizing chondroitin sulfates, although the staining intensity was variable from one cell to the other. In agreement with earlier studies (Avnur and Geiger, 1984), the CS-56 staining pattern was patchy, possibly representing agglomerates of CSPGs and other proteins (Fig. 1A). A novel marker for meningeal fibroblasts is the enzyme retinaldehyde dehydrogenase type 2 (Raldh2), an enzyme involved in the synthesis of retinoic acid (Zhao et al., 1996). This enzyme is widely expressed in the developing brain and spinal cord (Berggren et al., 1999), but is limited to the leptomeningeal sheet in the adult brain (J. Fawcett, personal communication). As shown in Fig. 1A, cultured MCs are also immunopositive for Raldh2. No staining for glial fibrillary acidic protein (GFAP) was detected in these cells (not shown). These data indicate that the MCs in culture are a pure cell population and express a very similar profile of marker proteins as described in vivo in the normal brain and the injured brain (Hirsch and Bahr, 1999; Pasterkamp et al., 1999).

Semaphorin expression in cultured meningeal cells

The lack of appropriate antibodies to Sema3A precludes a direct detection of the protein. We therefore investigated Sema3A expression in cultured MCs using in situ hybridization and reverse transcription (RT)-PCR. The Sema3A riboprobe gives a relatively weak signal with in situ hybridization, while a strong signal is obtained with the Sema3C probe (Fig. 1B). No in situ hybridization signal is seen with control sense probes (not shown). A Sema3A transcript is readily detected in MCs by RT-PCR (Fig. 1C). Again as seen with in situ hybridization, Sema3C expression is substantially higher compared with Sema3A (Fig. 1C). This is consistent with the in vivo observations after CNS injury, where MC-derived Sema3C expression is stronger than Sema3A expression (De Winter et al., 2002b). Expression of Sema3B, 3E, and 3F was detected at a very low level in cultured MCs using real-time quantitative PCR (not shown). Taken together, the data indicate that cultured MCs express a similar repertoire of semaphorins as they do in vivo and

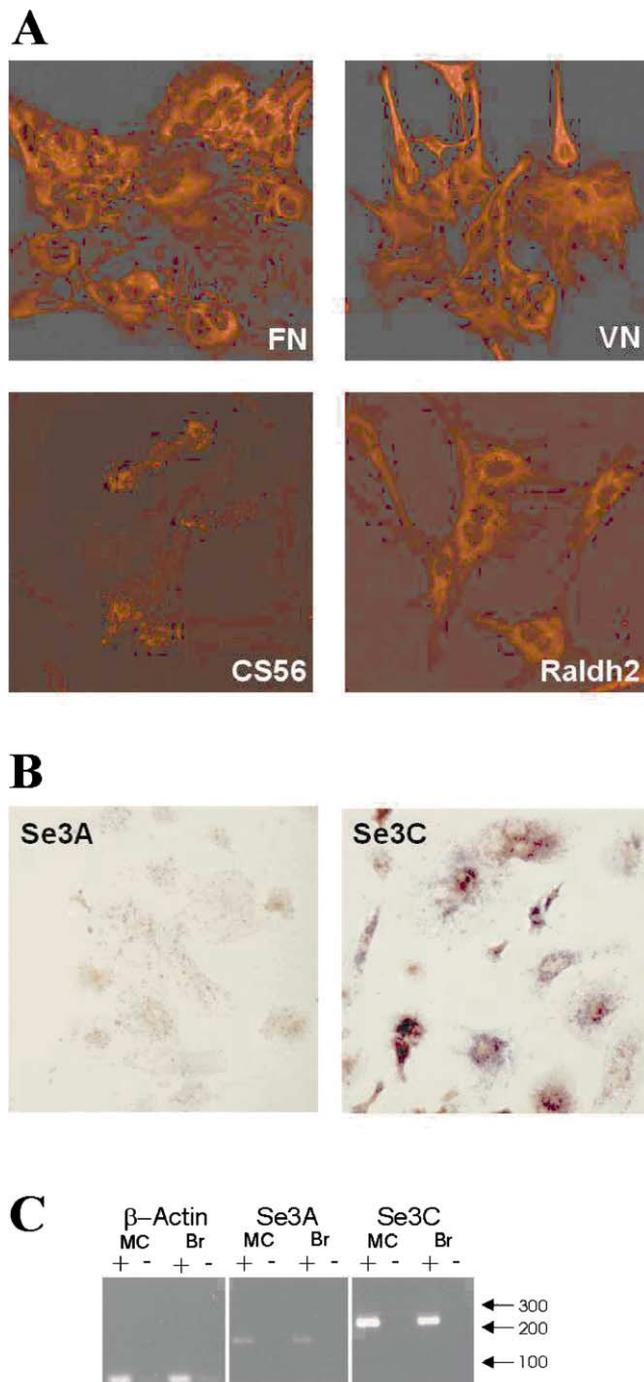


Fig. 1. Sema3A and Sema3C are expressed in cultured meningeal cells. (A) Meningeal cells (MCs) from neonatal rats are immunopositive for fibronectin (FN), vimentin (VN), chondroitin sulfates (CS-56), and retinaldehyde dehydrogenase type 2 (Raldh2). The staining is visualized with a Cy3-labeled secondary antibody. (B) In situ hybridization with digoxigenin-labeled riboprobes shows expression of Sema3A and Sema3C mRNA in cultured rat MCs. (C) RT-PCR on MC cultures reveals a transcript at the appropriate size for Sema3A and Sema3C. For comparison, the expression of these transcripts is shown in P9 brain (Br). β -Actin primers were used to control for a similar amount of starting material in MC and brain samples. RT-PCR was carried out with (+) or without (-) reverse transcriptase.

thus provide an adequate model to study the contribution of semaphorins to the inhibitory properties of these cells.

Meningeal cells produce functional Sema3A protein

Although Sema3A is a secreted protein, we did not detect significant collapsing activity in MC-conditioned medium on E15 rat DRGs. Since collapsin-1 (chicken Sema3A) was originally purified from brain membranes (Luo et al., 1993), we turned to a protein extract prepared from MC membranes and tested it for Sema3A collapsing activity. As shown in Fig. 2A, extracts from cultured rat MCs cause growth cone collapse in DRG neurons in a dose-dependent manner. Protein extracts from adult MCs contained a similar collapsing activity (not shown); however, since these cells are more difficult to grow, the remaining experiments were carried out with MCs from newborn pups. To investigate whether the collapsing activity was due to Sema3A, we applied blocking antibodies against the Sema3A receptor component neuropilin-1 (NP1) concomitant with the protein extract. The collapsing activity is partially blocked on addition of anti-NP1 antibodies (Fig. 2B), indicating that at least part of the collapsing activity requires a functional NP1 receptor. The blocking effect of NP1 antibodies is dose-dependent (Fig. 2B); however, due to limited amounts of NP1 antibody available, 200 μ g per well (400 μ g/ml) was the highest dose tested, and did not result in full inhibition of growth cone collapse. Although the trend of the dose-response curve suggests that the blockade may be complete with a high enough antibody concentration or with earlier administration of the antibody, we cannot exclude that additional chemorepellents independent of NP1 receptor contribute to the collapsing activity detected in MCs.

To analyze this question further, we turned to MC cultures prepared from Sema3A-deficient MCs, derived from neonatal mouse pups, that were homozygous for a targeted mutation in the Sema3A gene (Taniguchi et al., 1997). MC extracts from wild-type littermates had a collapsing activity similar to that of extracts from rat MCs (compare Figs. 2A and C), while no collapsing activity could be detected in MC extracts prepared from Sema3A-knockout (KO) pups (Fig. 2C). Interestingly, the collapsing effect was completely abolished even after administering a high protein concentration, indicating that Sema3A is the sole responsible MC-derived collapsing agent measured in this assay. Sema3E has also been reported to collapse embryonic DRGs (Miyazaki et al., 1999a; Miyazaki et al., 1999b); however, our results suggest that its expression in MCs is not sufficient to contribute to the collapsing activity detected in these cells. It is noteworthy in this context that E15 DRGs are not responsive to Sema3C (Koppel et al., 1997; Chen et al., 1998) (see Fig. 4 below), presumably because at this stage of development they do not express detectable amounts of NP2 receptor (Chen et al., 1997; Kolodkin et al., 1997). Taken together, our results demonstrate that wild-type MCs produce functional Sema3A protein that is able to negatively affect growth cone behavior.

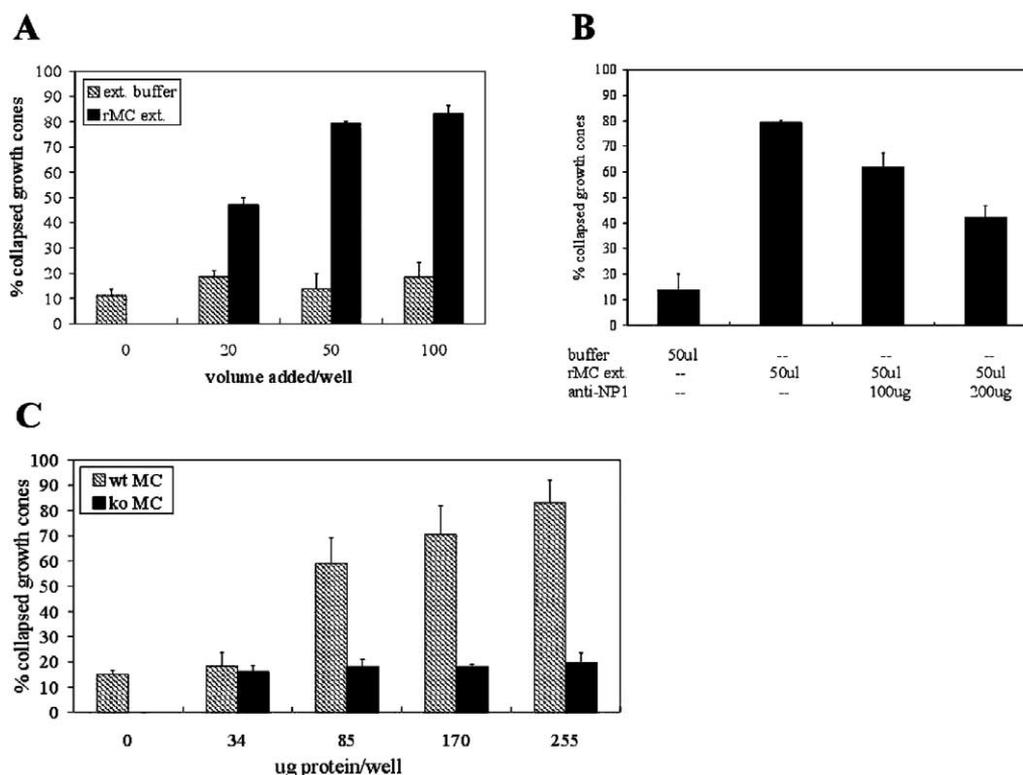


Fig. 2. Meningeal cell-derived Sema3A induces DRG growth cone collapse. Rat E15 DRGs were incubated with protein extract from meningeal cell (MC) membranes of rat (A, B) or mouse (C) origin. (A) Rat MC extract (rMC ext.) induces growth cone collapse of DRG neurons in a dose-dependent manner. No collapse is observed with equal amounts of extraction buffer (ext. buffer). The indicated volume is added to 500 μ l culture medium, with 50 μ l extract corresponding to approximately 250 μ g total protein. (B) Neuropilin-1 antibodies (anti-NP1) were added simultaneously with the MC extract at the indicated amount. All volumes are per 500 μ l culture medium. The collapsing activity of the MC extract is inhibited with increasing amounts of NP1 antibodies. (C) Collapsing activity is absent in MC extract derived from Sema3A-knockout mice (ko MC), while MC extract from wild-type mice shows a dose-response curve similar to that of rMC extract (compare C with A). Thus Sema3A is the responsible growth cone collapsing agent in MC extracts.

Comparison of neurite growth on a laminin, a meningeal cell, and an astrocyte substrate

It has long been established that MCs are an unfavorable substrate for growing neurites in culture, while astrocytes are permissive for such growth (Noble et al., 1984; Rudge and Silver, 1990). Yet the reason for this nonpermissive nature of MCs is poorly understood. Here we address the question of (i) how inhibitory an MC substrate is compared with more permissive substrates and (ii) whether Sema3A is involved in this neurite growth inhibition. To this end DRG explants were grown for 24 h on either laminin-coated glass coverslips or a monolayer of rat MCs or astrocytes.

Inhibition of neurite outgrowth on a meningeal cell substrate

As expected, neurite outgrowth was drastically reduced on a MC substrate compared with either laminin or astrocytes (Fig. 3A). Typically neurites on laminin grew out regularly, forming a thin homogenous sheet of individual neurites. Conversely, outgrowth on MCs was very

sparse and much more variable, with neurites extending from the explant in an irregular pattern and often in thick fascicles. Fasciculation is often thought to be the result of a nonpermissive environment where neurites prefer to grow on each other rather than on the substrate (Kapfhammer et al., 1986). Moreover the number of neurites that grew out on MCs and the average neurite length were dramatically reduced. Interestingly, the morphology of neurites on an astrocyte monolayer was also significantly different from that of either laminin or MCs. Although the density of neurites was high on astrocytes, they were also much fasciculated and the longest neurites never reached the length obtained on laminin. For quantification of neurite growth the surface area outside the ganglion that was covered with neurites was determined. This showed that neurite growth on a MC substrate was 32.9% of the growth obtained on laminin ($P < 0.001$) and about half the growth observed on an astrocyte monolayer ($P < 0.05$) (Fig. 3B). Although the growth pattern on astrocytes compared with laminin was qualitatively different and measured to be 62.3% of control, this difference was statistically not significant ($0.05 < P < 0.1$).

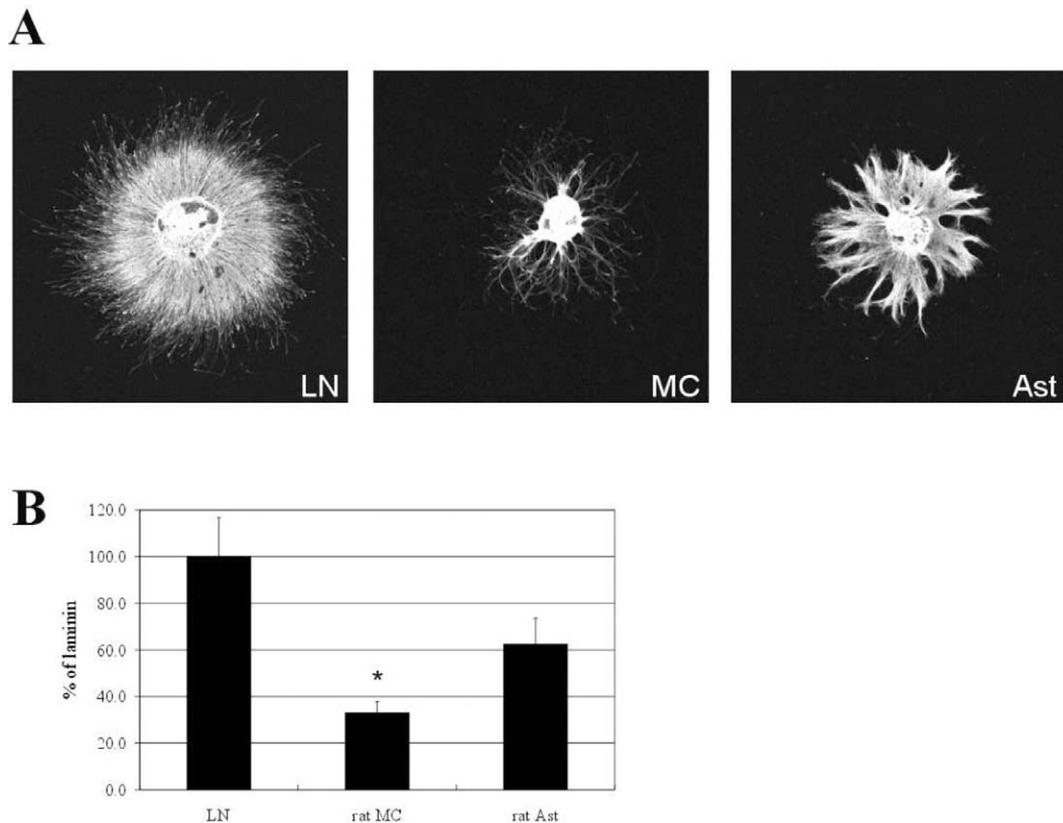


Fig. 3. Neurite growth inhibition on a meningeal cell substrate. (A) E15 DRGs were grown for 24 h on a laminin substrate (LN) or on a monolayer of rat meningeal cells (MC) or rat astrocytes (Ast). Neurofilament staining (2H3 antibody) was used to visualize neurites. (B) Quantification of neurite outgrowth on different substrates as shown in (A). Outgrowth on LN is set at 100% and the experimental conditions are expressed as a percentage thereof (y axis: % of laminin). Outgrowth on MCs was 32.9% of LN control ($P < 0.001$), while outgrowth on astrocytes was 62.3% of LN control (with $0.05 < P < 0.1$).

*Enhanced neurite growth on meningeal cells from *Sema3A*-knockout mice*

Sema3A inhibits neurite outgrowth

To determine whether *Sema3A* added to the medium of cultured DRGs could affect their outgrowth pattern, DRG explants were grown on a laminin substrate in the presence of *Sema3A*-containing conditioned medium (*Sema3A*-CM). Conditioned medium of GFP-transfected cells (GFP-CM) was used as a control. As seen in Fig. 4A, *Sema3A*-CM reduces neurite outgrowth from DRG neurons to about 40% of control. Although *Sema3C* does not induce growth cone collapse in E15 DRGs (Koppel et al., 1997; and data not shown), it was not clear whether it could affect neurite outgrowth of these cells. Since we found that MCs express high levels of *Sema3C* (Fig. 1B), we tested its effect on neurite outgrowth by adding *Sema3C*-CM to cultured DRGs. As expected from the result on growth cone collapse, *Sema3C* did not reduce neurite outgrowth of E15 DRG neurons; if anything it tended to increase growth (134% of control, $0.05 < P < 0.1$). These data show that *Sema3A*, besides being a chemorepellent that deviates axon growth, is a bonafide inhibitor of axon outgrowth of E15

DRG neurons and this outgrowth is not inhibited by *Sema3C*.

*Neurite outgrowth is enhanced on *Sema3A*-deficient MCs*

The data presented so far show that MCs poorly support neurite growth and suggest that *Sema3A* contributes to their inhibitory properties. To further address this, we performed the neurite outgrowth assay on *Sema3A*-deficient MCs. As seen in Fig. 4B, outgrowth on wild-type mouse MCs was strongly inhibited; the outgrowth measured was even less than that obtained on rat MCs (13% on mouse MCs and 33% on rat MCs as compared with laminin) (compare Figs. 3B and 4C). Instead, neurite outgrowth improved significantly (21%) when DRGs were seeded on *Sema3A*-deficient MCs (Fig. 4C). The increase was measured to be 160% under regular assay conditions (1.6-fold increase; $P < 0.05$), demonstrating that *Sema3A* is a growth-inhibitory molecule produced by MCs.

*The response to MC-derived *Sema3A* is modulated by NGF*

A recent study reported that NGF treatment increases resistance of sensory growth cones to *Sema3A*-induced col-

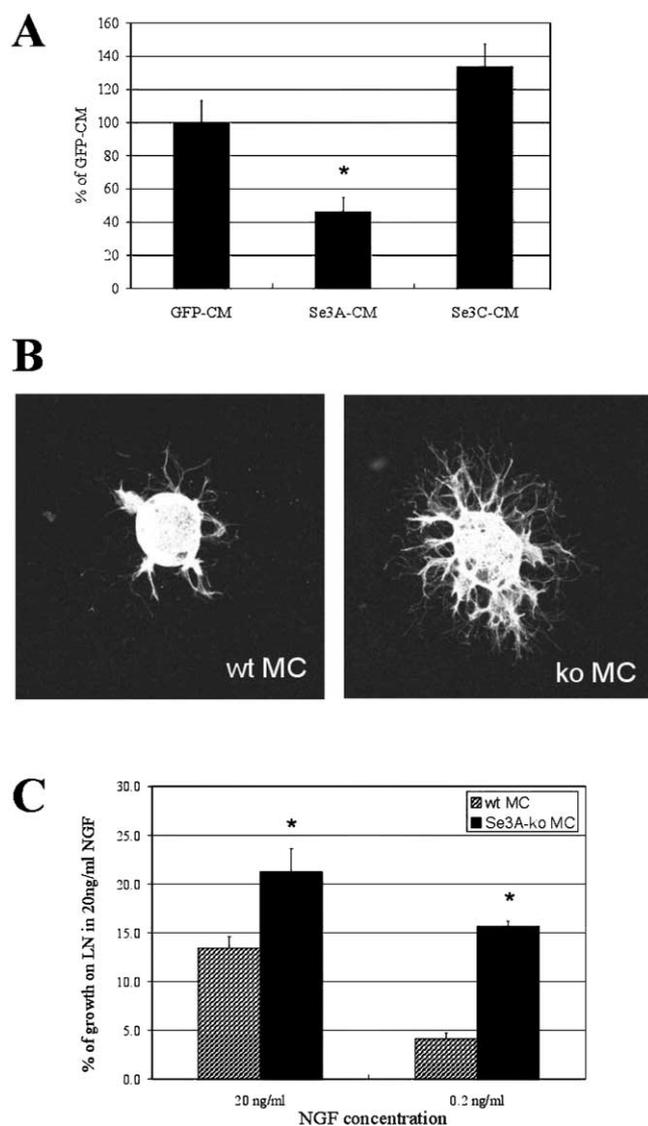


Fig. 4. MC-derived Sema3A inhibits neurite outgrowth, an effect that is modulated by NGF. (A) E15 DRGs were grown on laminin-coated coverslips in the presence of conditioned medium of 293T cells transfected with GFP (GFP-CM), Sema3A (Se3A-CM), or Sema3C (Se3C-CM). Outgrowth in GFP-CM was set to 100% (y axis). In the presence of Sema3A, outgrowth was reduced to 46.5% of control GFP-CM ($P < 0.05$), while in the presence of Sema3C outgrowth was not significantly different from control (134% with $0.05 < P < 0.1$). (B) E15 DRGs were grown for 24 h on a monolayer of MCs derived from wild-type (wt MC) or Sema3A-knockout mice (ko MC). Growth was in the presence of 20 ng/ml NGF. Neurofilament staining (2H3 antibody) was used to visualize neurites. (C) Quantification of neurite outgrowth on wt MC and Sema3A ko MC in the presence of either 20 or 0.2 ng/ml NGF. Outgrowth is expressed as a percentage of growth on LN control in 20 ng/ml NGF (set at 100%). Outgrowth on LN in 0.2 ng/ml NGF was about 60% of LN control (not shown). Note that in 20 ng/ml NGF a 1.6-fold increase in outgrowth is observed on Sema3A-ko MC ($P < 0.05$), while this increase amounts to 3.8-fold in 0.2 ng/ml NGF ($P < 0.001$).

lapse (Dontchev and Letourneau, 2002). Since in our assays neurons were routinely cultured in 20 ng/ml NGF (corresponding to 10^{-9} M NGF in their study), we wondered

whether changing the NGF concentration would also modulate the effect of MC-derived Sema3A on neurite outgrowth. When the assay was performed under 100-fold lower NGF concentration (0.2 ng/ml), outgrowth on laminin was reduced to about 60% of that seen under high NGF (not shown). Growth on wild-type mouse MCs was almost completely abolished in the low NGF condition (4.1% of laminin control; Fig. 4C). Interestingly, on Sema3A-deficient MCs, neurite growth increased to 15.6% of laminin control, meaning an increase of 380% compared with growth on wild-type MCs (3.8-fold increase, $P < 0.001$). Thus the effect of Sema3A on neurite growth is more potent in a low NGF concentration, which can be seen by the increased release of the blockade on neurite growth in the absence of Sema3A. This result supports the notion that Sema3A activity is regulated by neurotrophin signaling and shows that this regulation occurs not only in short term responses, i.e., collapse response, but also in a long-term process such as neurite outgrowth.

Discussion

Class 3 (secreted) semaphorins were found to be upregulated in meningeal fibroblasts in a neural scar formed after mechanical injury to brain and spinal cord (Pasterkamp et al., 1999; De Winter et al., 2002b). Semaphorins and, in particular, Sema3A are potent chemorepellents for growing axons during nervous system development (Kolodkin, 1998; Raper, 2000); they are therefore likely candidates to mediate neurite growth inhibition in the neural scar. In the present study, we show that Sema3A mRNA and active protein are produced by cultured MCs. Sema3A derived from MCs induces growth cone collapse in DRG neurons. We further show that Sema3A contributes to the inhibitory properties of MCs, since neurite outgrowth is increased on MCs that lack Sema3A expression. Furthermore, we find that the inhibitory effect of MC-associated Sema3A on axon growth is modulated by NGF.

Meningeal cells express functional Sema3A

Protein extracts from MCs contain a collapsing activity on DRG growth cones that is blocked by NP1 antibodies and is absent in Sema3A-deficient MCs. This demonstrates for the first time that Sema3A protein is present in MCs in sufficient amounts to functionally affect growth cone behavior. Interestingly, since no residual collapsing activity could be measured in Sema3A-KO MCs using E15 DRGs as an assay system, our results indicate that this activity can be attributed solely to Sema3A. This indicates that Sema3A is a major MC-derived chemorepellent for sensory growth cones. Nevertheless we cannot formally rule out that the improved outgrowth observed on Sema3A-deficient MCs is an indirect consequence of the loss of Sema3A expression. In such a scenario, Sema3A deficiency would induce

changes in the gene expression profile of MCs including, e.g., the downregulation of other repellent guidance factors.

Taking into account that our assay only reveals the activity of proteins that have their receptors expressed on DRG neurons, other semaphorins like Sema3C and additional chemorepellents expressed in MCs may exert their inhibitory effect on different neuronal populations. In an accompanying study by Shearer et al. (submitted), it was found that an antibody to NP2 facilitates axon crossing of an astrocyte/meningeal cell border, suggesting that an NP2-dependent semaphorin such as Sema3C or Sema3F contributes to the repulsive nature of MCs. In their study DRGs from neonatal rats were used, which, in contrast to E15 DRGs (Chen et al., 1997; Kolodkin et al., 1997), do express the NP2 receptor (F. De Winter and J. Verhaagen, personal communication). Thus the sensitivity of neurons to different semaphorins is determined by the receptor repertoire expressed on their growth cone. Since neuropilin and plexin expression is still present in intact and injured neurons in the adult (Giger et al., 1998; Reza et al., 1999; De Winter et al., 2002b; Gavazzi et al., 2000) and adult sensory neurons have been shown to remain sensitive to Sema3A (Tanelian et al., 1997; Owesson et al., 2000), it is reasonable to assume that injured neurons are repelled by MC-derived semaphorins in the neural scar. The response to neural trauma has been reported to be different in mice and rats, the most prevalent differences being the absence of cyst formation and the presence of a dense connective tissue matrix at the lesion site in mice (Steward et al., 1999). Despite these anatomical differences, our data indicate that the cellular and molecular components of neurite growth inhibition addressed in this article do not differ considerably between mice and rat. The bioassays performed with meningeal cells from either rat or mice revealed a similar growth cone collapsing activity (Fig. 2A and 2C) and a similar inhibition of neurite outgrowth (Figs. 3 and 4) from these cells independent of the species used.

Sema3A inhibits neurite outgrowth

It is often suggested that chemorepellents are functional only when they are sensed in a gradient but not when presented at a constant concentration or over a longer period (Bagnard et al., 2000; Loschinger et al., 2000). Since axon extension is a long-term phenomenon, it could be speculated that the continued presence of a chemorepellent leads to adaptation of the growth cone and therefore does not affect growth per se. Our findings that neurite outgrowth is enhanced on a Sema3A-deficient MC substrate and is inhibited on addition of exogenous Sema3A provide evidence that a chemorepellent, rather than merely deviating growing axons, can indeed act as an inhibitor of axon growth. This observation may also better describe the function of chemorepellents in the adult CNS where semaphorins and their receptors are still widely expressed (see references above) and where they are thought to be involved in limiting

sprouting and plasticity and in inhibiting axon regeneration, in the intact and injured brain, respectively (Holtmaat et al., 2002; De Winter et al., 2002a; Holtmaat et al., 2003).

Sema3A is associated with the MC cell surface

In this study Sema3A collapsing activity was present in a crude preparation of membrane proteins while we did not detect significant collapsing activity in MC-conditioned medium. This might be explained by low expression levels compared with a cell line manipulated to express recombinant Sema3A protein. Nevertheless our neurite outgrowth assay shows that the endogenous expression level of Sema3A in MCs is high enough to exert a potent inhibitory effect on primary neurons. In the latter condition, the axons are in direct contact with the cell membrane and ECM, where, if retained locally, even a low expression level may lead to a high protein concentration. Therefore our data suggest that, although secreted, the vast majority of Sema3A stays associated with the cell surface and/or ECM components. Further support of this contention comes from the work of Shearer et al. (submitted) who show an effect of semaphorins on axon crossing that is limited to a sharp boundary between MCs and astrocytes. This implies that after injury Sema3A will probably not disperse throughout the scar or the surrounding intact tissue, but rather form a barrier to regenerating axons at its site of production. The observed abrupt stopping behavior of sprouting axons abutting the lesion core is consistent with this notion (Davies et al., 1999). Moreover, considerable axonal regeneration is obtained after chemical disruption of the collagenous basal lamina in the neural scar, suggesting that together with the collagen network associated inhibitory proteins are eliminated by this treatment (Stichel et al., 1999a, 1999b; Hermanns et al., 2001).

Modulation of Sema3A sensitivity by NGF

When embryonic DRGs were grown under our standard culture conditions (20 ng/ml NGF), their neurites grew poorly on a monolayer of MCs (see Figs. 3B and 4C). This growth capacity on MCs is virtually zero when the NGF concentration is lowered to 0.2 ng/ml (Fig. 4C), while on a laminin substrate this low NGF concentration is sufficient to support neuronal survival and trigger significant neurite outgrowth (60% of control). On removal of Sema3A the neurite outgrowth potential is more strongly increased in low NGF (3.8-fold compared with 1.6-fold in high NGF) (Fig. 4C), indicating that NGF can partly overcome the inhibitory effect of Sema3A. In other words under conditions of diminished neurotrophin support the effect of growth inhibition is amplified. Our data are in line with previous reports showing that DRGs exposed to high NGF are more resistant to Sema3A-induced growth cone collapse (Tuttle and O'Leary, 1998; Dontchev and Letourneau, 2002) and extend the observation to include a long-term

regulatory effect of neurotrophins on Sema3A in neurite outgrowth. This has important implications for regeneration studies in the adult CNS where the presence of growth factors is limited and thus the sensibility to inhibitory chemorepellents may be particularly high.

In this context it is noteworthy that the repulsive effect of myelin-associated inhibitors such as MAG and NogoA is also modulated by neurotrophin treatment through a mechanism that involves cAMP and protein kinase A (Cai et al., 1999). Neurotrophins are known to increase cAMP which in turn can inhibit the small GTPase rhoA, an important effector of cytoskeletal changes in response to inhibitory cues (Luo, 2000). Since intracellular levels of cyclic nucleotides can affect the response to guidance molecules (Song et al., 1998), it is likely that neurotrophin-induced elevation of cAMP interferes with signaling by chemorepellents. Interestingly it was shown that increased levels of cGMP convert the response to Sema3A from repulsion to attraction in *Xenopus* neurons (Song et al., 1998), while a recent study shows that the chemokine SDF-1 reduces growth cone responsiveness to multiple chemorepellents, including Sema3A, through elevated cAMP levels (Chalasanani et al., 2003). Therefore the elevation of cAMP levels may be a general mechanism in mammalian neurons to reduce their sensitivity to inhibitory guidance cues. Indeed, recent work has shown that increasing cAMP levels or inhibiting rhoA by the enzyme C3 transferase enhances axon extension in cultured neurons (Niederost et al., 2002) and stimulates regenerative fiber growth in vivo (Qiu et al., 2002; Neumann et al., 2002; Lehmann et al., 1999; Dergham et al., 2002).

In conclusion, our results show that Sema3A, a chemorepellent during nervous system development, is a major neurite growth-inhibitory molecule in meningeal fibroblasts and is therefore likely to contribute to the inhibitory properties of the neural scar. Similar to axon growth in the developing nervous system, it appears that the balance between growth inhibition and growth promotion regulates the regenerative capability of injured neurons. We propose that a therapeutic approach to stimulate axon regeneration should combine the stimulation of neurite extension while at the same time specifically interfere with growth inhibition.

Experimental methods

Cell culture

Meningeal cells and astrocytes were prepared from newborn rat pups (P0–P2). Briefly, meninges were removed from the cortices and incubated for 30 min at 37°C in Ca²⁺- and Mg²⁺-free Hanks' buffered salt solution (HBSS) containing 0.125% trypsin, 0.5 mM EDTA, and 0.25% collagenase. Meninges were triturated gently in the presence of 80 µg/ml DNase and 100 µg/ml soybean trypsin inhibitor, centrifuged, and resuspended in DMEM containing 10%

FCS and penicillin/streptomycin (PS). The suspension was passed several times through 19- and 25-gauge needles and seeded at a density of two brains per 25-cm² flask. Medium was refreshed the next day; the first cell passage was carried out after 7 to 10 days. Cells were used for experiments between passages 2 and 5. Astrocytes were prepared from the cortices following the same procedure as described for meningeal cells, except that collagenase was omitted. Meningeal cells from mouse pups were isolated as above, except that they were prepared individually per pup and seeded at a density of 1 brain per 3.5-cm dish. Sema3A-knockout mice were from either a C57BL/6 or a CD1 background. The pups were from heterozygous litters and were genotyped by PCR on tail DNA as described (Taniguchi et al., 1997). Only cultures from wild-type and homozygous knockout mice were carried further.

Immunocytochemistry

For immunocytochemistry cells were fixed 30 min in 4% PFA, rinsed, blocked for 30 min in Tris-buffered saline containing 0.1% Triton X-100 (T-TBS) and 2% FCS, incubated with the primary antibody in blocking solution overnight at 4°C, and washed in T-TBS followed by incubation with a Cy3-labeled secondary antibody (Jackson). The following antibodies were used: anti-vimentin (mAb clone V9, Roche 814318); anti-fibronectin (goat, Calbiochem 341653), anti-chondroitin sulfate (clone CS-56, Sigma C8035), anti-GFAP (cow, Dako Z0334), anti-neurofilament (mAb, 2H3 clone, Developmental Studies Hybridoma Bank, University of Iowa), anti-Raldh2 (rabbit polyclonal; a gift from Dr. P. McCafferey, Harvard University) (Berggren et al., 1999).

In situ hybridization

For in situ hybridization, meningeal cells were plated at low density on poly-L-lysine-coated glass coverslips. Cells were fixed for 12 min in 4% PFA, 0.2% Triton X-100, washed, permeabilized, and prehybridized for 3 h at room temperature in hybridization buffer (50% formamide, 5× SSC, 5× Denhardt's, 200 µg/ml hsDNA, and 250 µg/ml tRNA). The buffer was replaced with hybridization buffer containing digoxigenin-labeled Sema3A or Sema3C riboprobes and incubated overnight at 60°C. Probes were prepared as described previously (De Winter et al., 2002b). Coverslips were washed for 30 min at 60°C in 0.2× SSC/50% formamide, brought to room temperature by rinsing in 150 mM NaCl, 100 mM Tris, pH 7.5, and incubated for 30 min in the same buffer containing 1% blocking reagent (Roche, No. 1096176) and 0.3% Triton X-100. A horseradish peroxidase (HRP)-conjugated anti-digoxigenin antibody (1:100; Roche, No. 1207733) was applied for 1 h in blocking buffer, followed by 3 × 5-min rinses. An amplification step was performed with biotinyl-tyramide (1:50; TSA-Biotin System NEL700A, NEN Life Science Products) fol-

lowing the manufacturer's instructions. Finally, a streptavidin-alkaline phosphatase complex was applied and the color reaction was developed with NBT and BCIP substrates. The amplification step leads to a characteristic staining pattern of large dots surrounding the cell nucleus, which results from the high concentration of alkaline phosphatase in one particular spot.

RT-PCR

Total RNA was isolated from cultured newborn rat meningeal cells and from rat brain (P9) using TRIzol reagent (Invitrogen) following the manufacturer's guidelines. First-strand DNA was synthesized with 1 μ g total RNA using Superscript II (Invitrogen) and oligo(dT) primers. Control PCRs were performed with RNA samples where the reverse transcriptase step was omitted. A 25-cycle PCR was carried out using Super Taq (Sphaero Q) and supplemented with 2.5 mM MgCl₂. The primers were as follows: Sema3A, 5' agaagggtgtccttggtcca; Sema3A, 3' cactgggtgtacatggctgg; Sema3C, 5' ctgcatcgctgccatatact; Sema3C, 3' gggagcacactcaaggaag. The expected amplicon sizes were 194 bp for Sema3A and 252 bp for Sema3C. The PCR fragments were sequenced to ensure specificity of the PCR.

Calcium phosphate transfection

Sema3A and Sema3C proteins were obtained by transient Ca²⁺ phosphate transfection of 293T cells using myc-tagged chicken Sema3A or Sema3C constructs (a gift of J.A. Raper) (Koppel et al., 1997). In brief, for a 6-cm dish 6 μ g DNA was vigorously mixed with 0.1 \times TE, 2.5 M CaCl₂, and 2 \times HBS (281 mM NaCl, 1.5 mM Na₂HPO₄, pH 7.12) and immediately added to the culture dish. The transfection proceeded for 12–16 h, medium was replaced with DMEM containing PS and 2% FCS, and conditioned medium (CM) was harvested after another 24 h. Semaphorin expression in conditioned medium was verified by Western blot analysis using an anti-myc antibody (Santa Cruz, clone 9E10) (not shown). For the neurite outgrowth assay, CM from transfected cells was applied to the DRG culture at a 1:1 dilution with the neuronal culture medium (see below).

DRG culture and collapse assay

Dorsal root ganglia (DRGs) were isolated from E15 rat embryos, stripped from surrounding roots, and plated as whole explants on laminin-coated glass coverslips. For the collapse assay they were cultured for 24 h in DMEM/F12 containing N2 supplements (Sigma, No. 17502), 2 mM glutamax, 20 ng/ml NGF (Roche, No. 1014331), and PS. Membranes from meningeal cells were prepared by lysing a confluent 10-cm dish in 1 ml cold lysis buffer (20 mM HEPES, pH 7.4, 2 mM MgCl₂, 1 mM EDTA, pH 8, and protease inhibitor cocktail) (Roche, No. 1697498). The cells were allowed to swell for 5 min, then passed three times

through a 20-gauge needle. Lysed cells were spun briefly (1000 g for 5 min at 4°C) to pellet unbroken cells and nuclei. The membranes were pelleted at 100,000g for 1 h at 4°C, and the pellet was washed twice with lysis buffer, carefully adding and removing the buffer without disturbing the pellet. Proteins were extracted by resuspending the membrane pellet in 200–400 μ l extraction buffer (0.5 \times PBS, 10 mM Tris 7.4, 3% sodium cholate) and passing it several times through a 23-gauge needle. Undissolved material was spun down and the supernatant was dialyzed overnight in 1 \times PBS, 5 mM Tris, pH 7.4, 0.01% sodium cholate at 4°C. Protein concentration was determined using a Lowry assay and the extract was applied to E15 DRG cultures at the appropriate concentrations. After a 30-min incubation DRGs were fixed in 4% PFA, 10% sucrose in PBS. The percentage of collapse was determined by counting the number of collapsed versus noncollapsed growth cones. Routinely about 80 growth cones per DRG and 3 DRGs per condition were counted. Each experiment was replicated at least three times.

Neuropilin-1 blocking experiment

The neuropilin-1 blocking antibody was a gift from Dr. A. Kolodkin (Baltimore, MD, USA). The antiserum was purified by protein A-Sepharose chromatography as described (Kolodkin et al., 1997). For the collapse assay, the IgG fraction was added to the DRGs simultaneously with the protein extract at the indicated concentration. As a control the IgG fraction of a nonblocking NPI antibody generated in our laboratory was used (Pasterkamp et al., 1998).

Neurite outgrowth assay

Meningeal cells and astrocytes were plated on poly-L-lysine-coated glass coverslips (12 mm) at a density of 2 \times 10⁵ cells/coverslip for MCs and 4 \times 10⁵ cells/coverslip for astrocytes, and grown for 48 h in DMEM containing 10% FCS to reach confluency. Two days after preparation of the coverslips, E15 rat DRGs were isolated and seeded on a confluent monolayer of meningeal cells or astrocytes or on control laminin-coated glass coverslips. DRGs were grown in DMEM/F12 containing N2 supplements (Sigma, No. 17502), 2 mM glutamax, 20 ng/ml NGF (Roche, No. 1014331), and PS. After fixation with 4% PFA in PBS containing 10% sucrose, neurites were visualized with the 2H3 anti-neurofilament antibody (2H3 ascites 1:1000; Dev. Stud. Hybridoma Bank, University of Iowa) as described. Fluorescence photographs were taken with the same intensity settings for all DRGs and quantification of neurite growth was performed with ImagePro software. The outgrowth area of each ganglion and the ganglion itself were outlined manually and the area of fluorescence above background level was determined within the marked area. To normalize the data the fluorescence area obtained on a

laminin substrate was set at 100% for each experiment and the fluorescent area obtained for each experimental condition was calculated as a percentage thereof. Each experiment was carried out at least three times, and in each experiment 8 to 12 ganglia per condition were measured. Results were analyzed statistically using combined Student *t* tests. Significance was set at $P < 0.05$.

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References

- Abnet, K., Fawcett, J.W., Dunnett, S.B., 1991. Interactions between meningeal cells and astrocytes in vivo and in vitro. *Brain Res. Dev. Brain Res.* 59, 187–196.
- Asher, R.A., Morgenstern, D.A., Moon, L.D., Fawcett, J.W., 2001. Chondroitin sulphate proteoglycans: inhibitory components of the glial scar. *Prog. Brain Res.* 132, 611–619.
- Asher, R.A., Morgenstern, D.A., Shearer, M.C., Adcock, K.H., Pesheva, P., Fawcett, J.W., 2002. Versican is upregulated in CNS injury and is a product of oligodendrocyte lineage cells. *J. Neurosci.* 22, 2225–2236.
- Avnur, Z., Geiger, B., 1984. Immunocytochemical localization of native chondroitin-sulfate in tissues and cultured cells using specific monoclonal antibody. *Cell* 38, 811–822.
- Bagnard, D., Thomasset, N., Lohrum, M., Puschel, A.W., Bolz, J., 2000. Spatial distributions of guidance molecules regulate chemorepulsion and chemoattraction of growth cones. *J. Neurosci.* 20, 1030–1035.
- Berggren, K., McCaffery, P., Drager, U., Forehand, C.J., 1999. Differential distribution of retinoic acid synthesis in the chicken embryo as determined by immunolocalization of the retinoic acid synthetic enzyme, RALDH-2. *Dev. Biol.* 210, 288–304.
- Berry, M., Maxwell, W.L., Logan, A., Mathewson, A., McConnell, P., Ashhurst, D.E., Thomas, G.H., 1983. Deposition of scar tissue in the central nervous system. *Acta Neurochir. Suppl. (Wien)* 32, 31–53.
- Bradbury, E.J., Moon, L.D., Popat, R.J., King, V.R., Bennett, G.S., Patel, P.N., Fawcett, J.W., McMahon, S.B., 2002. Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature* 416, 636–640.
- Cai, D., Shen, Y., De Bellard, M., Tang, S., Filbin, M.T., 1999. Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism. *Neuron* 22, 89–101.
- Carbonell, A.L., Boya, J., 1988. Ultrastructural study on meningeal regeneration and meningo–glial relationships after cerebral stab wound in the adult rat. *Brain Res.* 439, 337–344.
- Chalasanani, S.H., Sabelko, K.A., Sunshine, M.J., Littman, D.R., Raper, J.A., 2003. A chemokine, SDF-1, reduces the effectiveness of multiple axonal repellents and is required for normal axon pathfinding. *J. Neurosci.* 23, 1360–1371.
- Chen, H., Chedotal, A., He, Z., Goodman, C.S., Tessier-Lavigne, M., 1997. Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. *Neuron* 19, 547–559.
- Chen, H., He, Z., Bagri, A., Tessier-Lavigne, M., 1998. Semaphorin–neuropilin interactions underlying sympathetic axon responses to class III semaphorins. *Neuron* 21, 1283–1290.
- Davies, S.J., Goucher, D.R., Doller, C., Silver, J., 1999. Robust regeneration of adult sensory axons in degenerating white matter of the adult rat spinal cord. *J. Neurosci.* 19, 5810–5822.
- Dergham, P., Ellezam, B., Essagian, C., Avedissian, H., Lubell, W.D., McKerracher, L., 2002. Rho signaling pathway targeted to promote spinal cord repair. *J. Neurosci.* 22, 6570–6577.
- De Winter, F., Holtmaat, A.J., Verhaagen, J., 2002a. Neuropilin and class 3 semaphorins in nervous system regeneration. *Adv. Exp. Med. Biol.* 515, 115–139.
- De Winter, F., Oudega, M., Lankhorst, A.J., Hamers, F.P., Blits, B., Ruitenbergh, M.J., Pasterkamp, R.J., Gispens, W.H., Verhaagen, J., 2002b. Injury-induced class 3 semaphorin expression in the rat spinal cord. *Exp. Neurol.* 175, 61–75.
- Dontchev, V.D., Letourneau, P.C., 2002. Nerve growth factor and semaphorin 3A signaling pathways interact in regulating sensory neuronal growth cone motility. *J. Neurosci.* 22, 6659–6669.
- Fawcett, J.W., Asher, R.A., 1999. The glial scar and central nervous system repair. *Brain Res. Bull.* 49, 377–391.
- Gavazzi, I., Stonehouse, J., Sandvig, A., Reza, J.N., Appiah-Kubi, L.S., Keynes, R., Cohen, J., 2000. Peripheral, but not central, axotomy induces neuropilin-1 mRNA expression in adult large diameter primary sensory neurons. *J. Comp. Neurol.* 423, 492–499.
- Giger, R.J., Pasterkamp, R.J., Heijnen, S., Holtmaat, A.J., Verhaagen, J., 1998. Anatomical distribution of the chemorepellent semaphorin III/collapsin-1 in the adult rat and human brain: predominant expression in structures of the olfactory–hippocampal pathway and the motor system. *J. Neurosci. Res.* 52, 27–42.
- Hermanns, S., Klapka, N., Muller, H.W., 2001. The collagenous lesion scar: an obstacle for axonal regeneration in brain and spinal cord injury. *Restor. Neurol. Neurosci.* 19, 139–148.
- Hirsch, S., Bahr, M., 1999. Immunocytochemical characterization of reactive optic nerve astrocytes and meningeal cells. *Glia* 26, 36–46.
- Holtmaat, A.J., De Winter, F., De Wit, J., Gorter, J.A., da Silva, F.H., Verhaagen, J., 2002. Semaphorins: contributors to structural stability of hippocampal networks. *Prog. Brain Res.* 138, 17–38.
- Holtmaat, A.J., Gorter, J.A., De Wit, J., Tolner, E.A., Spijker, S., Giger, R.J., Lopes da Silva, F.H., Verhaagen, J., 2003. Transient downregulation of Sema3A mRNA in a rat model for temporal lobe epilepsy. A novel molecular event potentially contributing to mossy fiber sprouting. *Exp. Neurol.* 182(1), 142–50.
- Kapfhammer, J.P., Grunewald, B.E., Raper, J.A., 1986. The selective inhibition of growth cone extension by specific neurites in culture. *J. Neurosci.* 6, 2527–2534.
- Kolodkin, A.L., 1998. Semaphorin-mediated neuronal growth cone guidance. *Prog. Brain Res.* 117, 115–132.
- Kolodkin, A.L., Levengood, D.V., Rowe, E.G., Tai, Y.T., Giger, R.J., Ginty, D.D., 1997. Neuropilin is a semaphorin III receptor. *Cell* 90, 753–762.
- Koppel, A.M., Feiner, L., Kobayashi, H., Raper, J.A., 1997. A 70 amino acid region within the semaphorin domain activates specific cellular response of semaphorin family members. *Neuron* 19, 531–537.
- Lehmann, M., Fournier, A., Selles-Navarro, I., Dergham, P., Sebok, A., Leclerc, N., Tigyi, G., McKerracher, L., 1999. Inactivation of Rho signaling pathway promotes CNS axon regeneration. *J. Neurosci.* 19, 7537–7547.
- Loschinger, J., Weth, F., Bonhoeffer, F., 2000. Reading of concentration gradients by axonal growth cones. *Philos. Trans. R. Soc. London Ser. B* 355, 971–982.
- Luo, L., 2000. Rho GTPases in neuronal morphogenesis. *Nat. Rev. Neurosci.* 1, 173–180.
- Luo, Y., Raible, D., Raper, J.A., 1993. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* 75, 217–227.

- Miyazaki, N., Furuyama, T., Amasaki, M., Sugimoto, H., Sakai, T., Takeda, N., Kubo, T., Inagaki, S., 1999a. Mouse semaphorin H inhibits neurite outgrowth from sensory neurons. *Neurosci. Res.* 33, 269–274.
- Miyazaki, N., Furuyama, T., Sakai, T., Fujioka, S., Mori, T., Ohoka, Y., Takeda, N., Kubo, T., Inagaki, S., 1999b. Developmental localization of semaphorin H messenger RNA acting as a collapsing factor on sensory axons in the mouse brain. *Neuroscience* 93, 401–408.
- Moon, L.D., Asher, R.A., Rhodes, K.E., Fawcett, J.W., 2001. Regeneration of CNS axons back to their target following treatment of adult rat brain with chondroitinase ABC. *Nat. Neurosci.* 4, 465–466.
- Morgenstern, D.A., Asher, R.A., Fawcett, J.W., 2002. Chondroitin sulphate proteoglycans in the CNS injury response. *Prog. Brain Res.* 137, 313–332.
- Murakami, Y., Suto, F., Shimizu, M., Shinoda, T., Kameyama, T., Fujisawa, H., 2001. Differential expression of plexin-A subfamily members in the mouse nervous system. *Dev. Dyn.* 220, 246–258.
- Ness, R., David, S., 1997. Leptomeningeal cells modulate the neurite growth promoting properties of astrocytes in vitro. *Glia* 19, 47–57.
- Neumann, S., Bradke, F., Tessier-Lavigne, M., Basbaum, A.I., 2002. Regeneration of sensory axons within the injured spinal cord induced by intraganglionic cAMP elevation. *Neuron* 34, 885–893.
- Niederost, B., Oertle, T., Fritsche, J., McKinney, R.A., Bandtlow, C.E., 2002. Nogo-A and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA and Rac1. *J. Neurosci.* 22, 10368–10376.
- Noble, M., Fok-Seang, J., Cohen, J., 1984. Glia are a unique substrate for the in vitro growth of central nervous system neurons. *J. Neurosci.* 4, 1892–1903.
- Owesson, C., Pizzey, J., Tonge, D., 2000. Sensitivity of NGF-responsive dorsal root ganglion neurons to semaphorin D is maintained in both neonatal and adult mice. *Exp. Neurol.* 165, 394–398.
- Pasterkamp, R.J., De Winter, F., Holtmaat, A.J., Verhaagen, J., 1998. Evidence for a role of the chemorepellent semaphorin III and its receptor neuropilin-1 in the regeneration of primary olfactory axons. *J. Neurosci.* 18, 9962–9976.
- Pasterkamp, R.J., Giger, R.J., Ruitenbergh, M.J., Holtmaat, A.J., De Wit, J., De Winter, F., Verhaagen, J., 1999. Expression of the gene encoding the chemorepellent semaphorin III is induced in the fibroblast component of neural scar tissue formed following injuries of adult but not neonatal CNS. *Mol. Cell Neurosci.* 13, 143–166.
- Qiu, J., Cai, D., Dai, H., McAtee, M., Hoffman, P.N., Bregman, B.S., Filbin, M.T., 2002. Spinal axon regeneration induced by elevation of cyclic AMP. *Neuron* 34, 895–903.
- Raper, J.A., 2000. Semaphorins and their receptors in vertebrates and invertebrates. *Curr. Opin. Neurobiol.* 10, 88–94.
- Reier, P.J., Lawrence, F.E., Jakeman, L., 1989. Reactive astrocyte and axonal outgrowth in the injured CNS: is gliosis really an impediment to regeneration, in: Seil, F.J. (Ed.), *Frontiers of Clinical Neuroscience*. Alan R. Liss, New York, pp. 183–209.
- Reza, J.N., Gavazzi, I., Cohen, J., 1999. Neuropilin-1 is expressed on adult mammalian dorsal root ganglion neurons and mediates semaphorin3a/collapsin-1-induced growth cone collapse by small diameter sensory afferents. *Mol. Cell Neurosci.* 14, 317–326.
- Ross, R., 1968. The fibroblast and wound repair. *Biol. Rev. Camb. Philos. Soc.* 43, 51–96.
- Rudge, J.S., Silver, J., 1990. Inhibition of neurite outgrowth on astroglial scars in vitro. *J. Neurosci.* 10, 3594–3603.
- Shearer, M.C., Fawcett, J.W., 2001. The astrocyte/meningeal cell interface—a barrier to successful nerve regeneration? *Cell Tissue Res.* 305(2), 267–273.
- Shearer, M.C., Niclou, S.P., Brown, D., Asher, R.A., Holtmaat, A.J., Verhaagen, J., Fawcett, J.W. The astrocyte/meningeal cell interface is a barrier to neurite outgrowth which can be overcome by manipulation of inhibitory molecules or axonal signalling pathways (submitted).
- Sievers, J., Pehlemann, F.W., Gude, S., Berry, M., 1994. Meningeal cells organize the superficial glia limitans of the cerebellum and produce components of both the interstitial matrix and the basement membrane. *J. Neurocytol.* 23, 135–149.
- Song, H., Ming, G., He, Z., Lehmann, M., McKerracher, L., Tessier-Lavigne, M., Poo, M., 1998. Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. *Science* 281, 1515–1518.
- Spencer, T., Domeniconi, M., Cao, Z., Filbin, M.T., 2003. New roles for old proteins in adult CNS axonal regeneration. *Curr. Opin. Neurobiol.* 13, 133–139.
- Steward, O., Schauwecker, P.E., Guth, L., Zhang, Z., Fujiki, M., Inman, D., Wrathall, J., Kempermann, G., Gage, F.H., Saatman, K.E., Raghupathi, R., McIntosh, T., 1999. Genetic approaches to neurotrauma research: opportunities and potential pitfalls of murine models. *Exp. Neurol.* 157, 19–42.
- Stichel, C.C., Hermanns, S., Luhmann, H.J., Lausberg, F., Niermann, H., D'Urso, D., Servos, G., Hartwig, H.G., Muller, H.W., 1999a. Inhibition of collagen IV deposition promotes regeneration of injured CNS axons. *Eur. J. Neurosci.* 11, 632–646.
- Stichel, C.C., Niermann, H., D'Urso, D., Lausberg, F., Hermanns, S., Muller, H.W., 1999b. Basal membrane-depleted scar in lesioned CNS: characteristics and relationships with regenerating axons. *Neuroscience* 93, 321–333.
- Tanelian, D.L., Barry, M.A., Johnston, S.A., Le, T., Smith, G.M., 1997. Semaphorin III can repulse and inhibit adult sensory afferents in vivo. *Nat. Med.* 3, 1398–1401.
- Taniguchi, M., Yuasa, S., Fujisawa, H., Naruse, I., Saga, S., Mishina, M., Yagi, T., 1997. Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection. *Neuron* 19, 519–530.
- Tuttle, R., O'Leary, D.D., 1998. Neurotrophins rapidly modulate growth cone response to the axon guidance molecule, collapsin-1. *Mol. Cell Neurosci.* 11, 1–8.
- Wang, X., Chun, S.J., Treloar, H., Vartanian, T., Greer, C.A., Strittmatter, S.M., 2002. Localization of Nogo-A and Nogo-66 receptor proteins at sites of axon-myelin and synaptic contact. *J. Neurosci.* 22, 5505–5515.
- Zhao, D., McCaffery, P., Ivins, K.J., Neve, R.L., Hogan, P., Chin, W.W., Drager, U.C., 1996. Molecular identification of a major retinoic-acid-synthesizing enzyme, a retinaldehyde-specific dehydrogenase. *Eur. J. Biochem.* 240, 15–22.