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Molecular and Cellular Neuroscience 24 (2003) 913–925
MCN Molecular and Cellular Neuroscience
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The astrocyte/meningeal cell interface is a barrier to neurite outgrowth which can be overcome by manipulation of inhibitory molecules or axonal signalling pathways

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Received 7 May 2003; revised 26 August 2003; accepted 3 September 2003

Abstract

Invading meningeal cells form a barrier to axon regeneration after damage to the spinal cord and other parts of the CNS, axons stopping at the interface between meningeal cells and astrocytes. Axon behavior was examined using an in vitro model of astrocyte/meningeal cell interfaces, created by plating aggregates of astrocytes and meningeal cells onto coverslips. At these interfaces growth of dorsal root ganglion axons attempting to grow from astrocytes to meningeal cells was blocked, but axons grew rapidly from meningeal cells onto astrocytes. Meningeal cells were examined for expression of axon growth inhibitory molecules, and found to express NG2, versican, and semaphorins 3A and 3C. Astrocytes express growth promoting molecules, including N-Cadherin, laminin, fibronectin, and tenascin-C. We treated cultures in various ways to attempt to promote axon growth across the inhibitory boundaries. Blockade of NG2 with antibody and blockade of neuropilin 2 but not neuropilin 1 both promoted axon growth from astrocytes to meningeal cells. Blockade of permissive molecules on astrocytes with N-Cadherin blocking peptide or anti beta-1 integrin had no effect. Manipulation of axonal signalling pathways also increased axon growth from astrocytes to meningeal cells. Increasing cAMP levels and inactivation of rho were both effective when the cultures were fixed in paraformaldehyde, demonstrating that their effect is on axons and not via effects on the glial cells.

Introduction

Wherever the central nervous system (CNS) is damaged a glial scar will form, and this tissue constitutes a barrier to regenerating axons. The formation of the scar is a complex process, involving astrocytes, oligodendrocyte precursors, meningeal cells, macrophages, and microglia (Fawcett and Asher, 1999). Within this tissue, chondroitin sulphate proteoglycans have been identified as important inhibitory molecules, and many axons stop growing where these molecules are present. In addition, the core of the lesion site may become infiltrated with collagenous tissue and cells of meningeal origin, which reform an accessory glia limitans where they abut neighbouring astrocytes (Shearer and Fawcett, 2001; Hermanns et al., 2001; Mathewson and Berry, 1985). Studies by Davies et al. and Stichel and Muller (Davies et al., 1999; Stichel and Müller, 1998) have suggested that the interface between astrocyte-containing CNS tissue and the invading meningeal cells is an absolute barrier to nerve regeneration. Moreover, when the formation of these collagenous barriers is prevented axons are able to grow across the site of a CNS injury (Stichel et al., 1999). Axon growth on meningeal cells has been studied in vitro (Ness and David, 1997; Hirsch and Bahr, 1999), with both studies showing that they support growth poorly, and they have been shown to express the inhibitory molecule semaphorin

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doi:10.1016/j.mcn.2003.09.004

3A and the inhibitory CSPGs versican and NG2 (Morgenstern et al., 2002; Asher et al., 2001, 2002; Pasterkamp et al., 1998a, 1999; Hirsch and Bahr, 1999). Some of these potential inhibitory molecules have been been demonstrated in the lesion core region in vivo. Most of the class 3 semaphorins are present (De Winter et al., 2002), as are the inhibitory CSPGs NG2 and versican (Jones et al., 2002; Asher et al., 2002).

In the damaged CNS it is clear that some axons stop regenerating precisely at the astrocyte–meningeal cell interface. As with Schwann cell–astrocyte interfaces such as occur at the dorsal root entry zone, these boundaries therefore represent a major impediment to CNS repair. In previous studies we have shown that astrocytes and meningeal cells, when cocultured, separate out into distinct territories with sharp boundaries that are reminiscent of the separation of meningeal cells and astrocytes in vivo (Abnet et al., 1991; Struckhoff, 1995; Franklin et al., 1992). In the present paper we have examined the inhibitory mechanisms for axon growth across these boundaries. We report that axons are inhibited from crossing from astrocytes to meningeal cells, but cross boundaries readily in the other direction. Inhibition by meningeal cells can be ascribed in part to the CSPG NG2 and to class 3 semaphorins, and this inhibition can be overcome by manipulation of axonal growth cone signalling, in particular by raising cyclic nucleotide levels and blocking Rho activity.

Results

Astrocytes and meningeal cells form distinct patches of cells, and a glia limitans-like structure, when cultured together

Meningeal cells and astrocytes were plated as small aggregates. These initially flattened out as small patches of one or the other cell type. By 7 days these patches had begun to grow into contact with one another, and by 10 days the coverslips were completely covered with cells. Staining with anti-glial fibrilliary acidic protein (GFAP, visualising astrocytes), anti-fibronectin (visualising meningeal cells), and anti-RALDH2 (visualising meningeal cells) verified the phase observation that the cell types remained segregated into separate patches. At the boundaries between these patches, as described previously (Abnet et al., 1991), the astrocyte processes were hypertrophic and tended to run parallel to the interface. In addition, some hypertrophic astrocyte processes ran from the interface, and as described previously, these processes ran underneath the meningeal cells (Fig. 1).

Dorsal root ganglion neurites avoid crossing the astrocyte/meningeal cell interface

When DRG explants from P0–P1 rats were grown on astrocyte–meningeal cell monolayers they could fall on astrocytes, meningeal cells, or between the two. Neurites grew well on astrocyte patches with long numerous neurites and less well on meningeal patches where the neurites were visibly shorter, thinner, and fewer in number (Fig. 2). When they reached boundaries they showed a strong preference for growing on astrocytes. When neurites that had been growing on astrocytes reached a boundary with meningeal cells only 24.3% continued to grow straight onto the meningeal cells. The remainder showed avoidance behaviour and either stopped at the interface or, in most cases, turned and grew instead along the edge of boundary on the astrocyte side (Fig. 3).

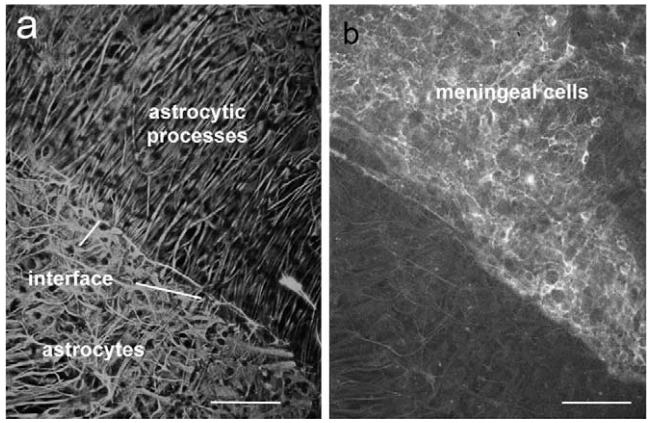

Fig. 1. Astrocytes and meningeal cells separate into patches, with clear boundaries between them. (a) GFAP stain; (b) RALDH2 stain. The astrocyte patch is at the bottom left, the meningeal cells are on the top left. There is a clear boundary on the surface of the culture, but some GFAP+ve astrocyte processes pass under the meningeal cells. Bar = 200 μm.

Some DRGs landed on patches of meningeal cells. Their axons were faced with a boundary between meningeal cells and astrocytes. Here there was almost no axon turning or stopping, and almost all the neurites (86.2%) crossed the interface from meningeal cells onto astrocytes, with a few axons turning to grow parallel to the interface.

There was little Schwann cell migration from the DRG explants compared to cultures on laminin, collagen, or other surfaces, since these cells migrate poorly on astrocytes (Wilby et al., 1999). The axons grew far beyond the halo of Schwann cells migrating from the explants and were not guided by Schwann cells in this choice assay.

Expression of inhibitory molecules by meningeal cells

Primary meningeal cultures produce the chondroitin sulphate proteoglycans versican and NG2

Purified cultures of meningeal cells were examined by immunohistochemistry and Western blotting. Western blots stained with an antibody to the CSPG NG2 showed a high-molecular-weight smear extending from approximately 340 to 380 kDa in both cell extract and conditioned medium lanes (Fig. 4a). This smear resolved to a single band at approximately 320 kDa when the sample was treated with

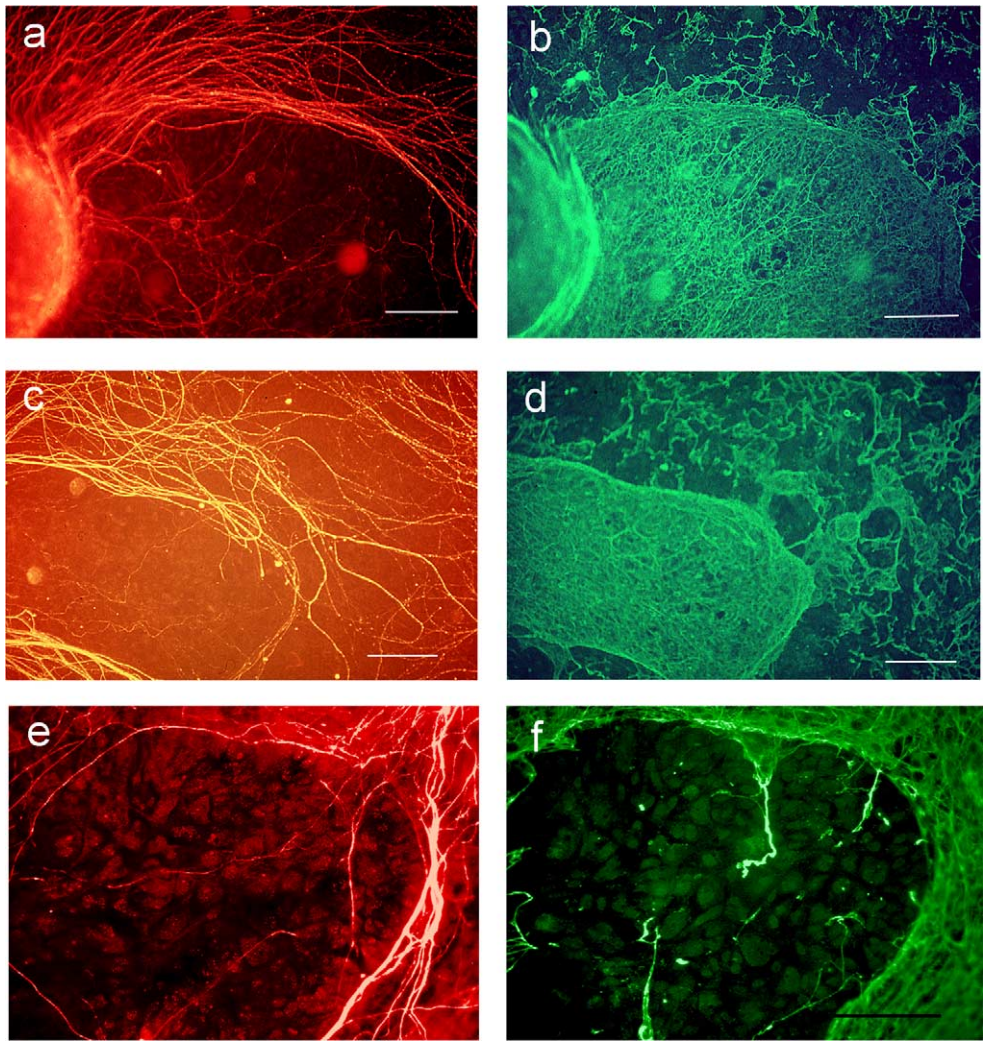

Fig. 2. Axons growing on astrocytes mostly avoid crossing boundaries onto meningeal cells. The meningeal cell patches are labelled with anti-fibronectin in (b) and (d), while the astrocytes are labelled with anti-GFAP in (f) In (a), (c), and (e) axons are seen growing around the boundary between astrocytes and meningeal cells, with a few passing across it. In (a) part of the DRG was resting on a meningeal cell patch, and axons can be seen growing out directly on to these cells. Their growth is sparser and more tortuous than axons on astrocytes. Bar = 200 μm.

chondroitinase ABC, corresponding to the size of the NG2 protein core. Immunocytochemistry of cultures using anti-NG2 showed staining on meningeal cells, with stronger staining of the few contaminating oligodendrocyte lineage cells, which were only seen on top of astrocytes.

Western blots stained with an antibody against versican showed no immunoreactivity in the lanes in which the sample was not digested with chondroitinase, presumably because versican with GAG chains attached is too large to migrate through the gel. On chondroitinase digestion two bands were seen, which migrated at approximately 400 kDa. These bands were of higher molecular weight than the single band obtained from oligodendrocyte lineage cells or from whole brain and were therefore considered to represent the V_0 and V_1 forms of versican (Fig. 4b). Immunohistochemistry for versican showed a streaky distribution over the meningeal cells, with much stronger staining at boundaries which stopped precisely at the edge of the meningeal patches (Fig. 5d). The versican did not appear to be attached to the meningeal cells through hyaluronan, since digestion with hyaluronidase did not remove the versican staining.

Western blots stained with the CS56 antibody, which recognises predominantly chondroitin sulphate-D, showed a smeared band at the same molecular weight as that of the undigested NG2 (Fig. 4c). This immunoreactive band disappeared upon treatment with chondroitinase ABC.

Neurocan was present at low levels in blots (Fig. 4d). As neurocan is known to be produced and secreted at high levels by astrocytes (Asher et al., 2000), it was possible that this neurocan was produced by contaminating astrocytes which were present in small numbers in the meningeal cultures. Meningeal cells were passaged to reduce astrocyte numbers (as determined by GFAP immunoreactivity) and subsequently tested for neurocan expression. The level of neurocan was indeed seen to decrease in relation to the number of GFAP-positive cells between the primary (PY)

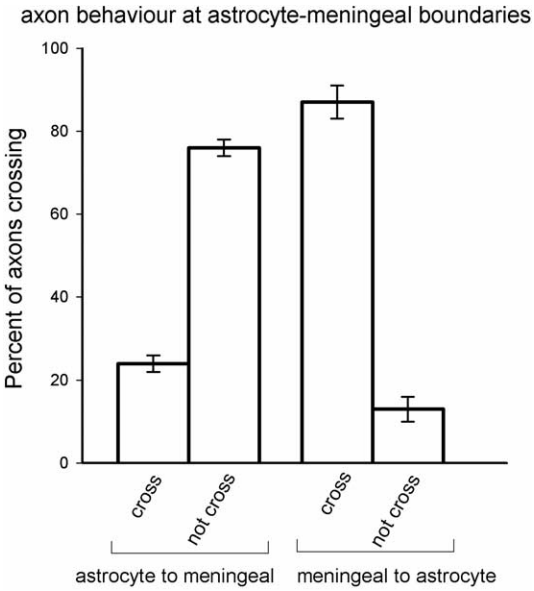

Fig. 3. Quantification of axon behaviour at astrocyte/meningeal cell boundaries.

culture and the passaged (PS) culture (Fig. 4e). Comparison between a primary culture containing 4.4% GFAP+ve cells versus a passaged culture containing 0.1% GFAP-positive cells showed that neurocan levels correlated with astrocyte numbers. We conclude that meningeal cells do not produce neurocan.

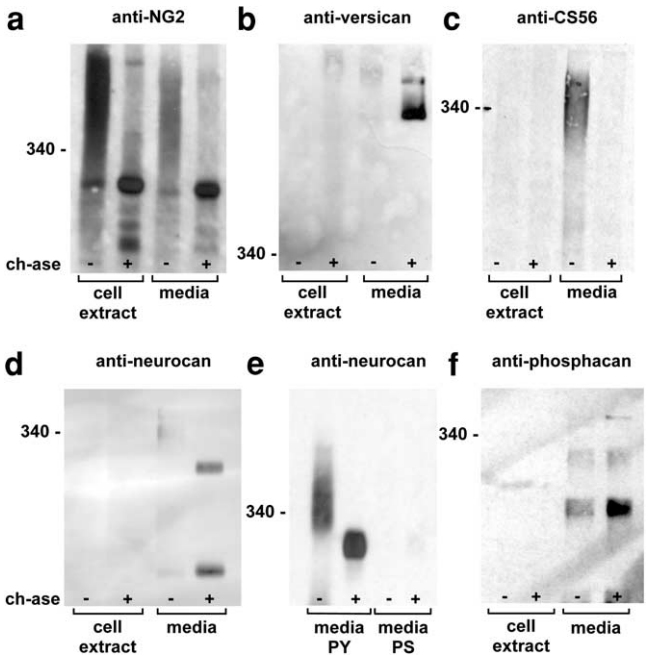

Fig. 4. Western blots of meningeal cell supernatant and cell extract, demonstrating that the cells produce NG2 and versican. A small amount of neurocan was detected in cultures, bottom left, and medium PY lanes, which disappeared when the cells were passaged to removed astrocytes (media PS). The bands in the phosphacan medium lanes do not correspond to the size of phosphacan and are therefore background staining.

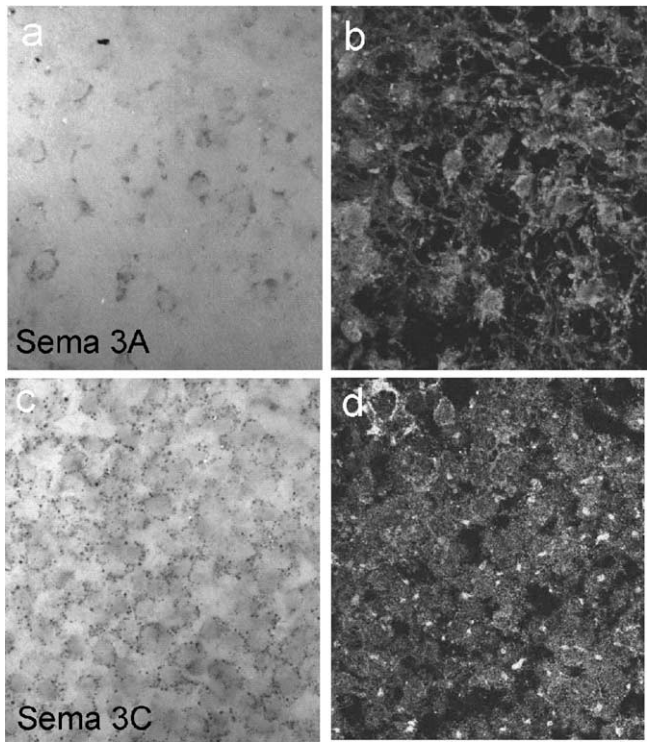

Fig. 5. Meningeal cells express both Sema 3A and Sema 3C. (a) and (c) are in situ hybridizations showing Sema mRNA, while (b) and (d) show that the same cells are immunoreactive to RALDH2, demonstrating that they are meningeal cells.

Neither phosphacan nor brevican were detected in cell extracts or conditioned media from meningeal cells

In situ hybridization on cultures of meningeal cells with probes to semaphorin 3A and 3C revealed that meningeal cells produce both molecules (Niclou et al., accompanying paper and Fig. 5).

Expression of axon-growth-promoting molecules in astrocyte/meningeal cell cultures

In order to look for molecules that might attract axons towards astrocytes rather than meningeal cells, we examined the distribution of known axon-growth-promoting molecules. Immunohistochemistry using the FWCAD anti N-cadherin antibody showed staining on the cell surface of astrocytes only, with no trace on meningeal cells (Fig. 6c). The distribution of laminin and fibronectin on live (nonpermeabilised) cultures showed a thin band of both molecules at the interface between astrocytes and meningeal cells (Fig. 6a), with scattered weak staining over the meningeal area of the culture. When cells were permeabilised by fixation in −20°C methanol prior to immunocytochemistry there was strong staining for both fibronectin and laminin associated with the patches of meningeal cells; this presumably represents matrix laid down between these cells and the coverslip. Tenascin-C, which has both axon growth promoting and inhibitory properties, had a similar distribution pattern

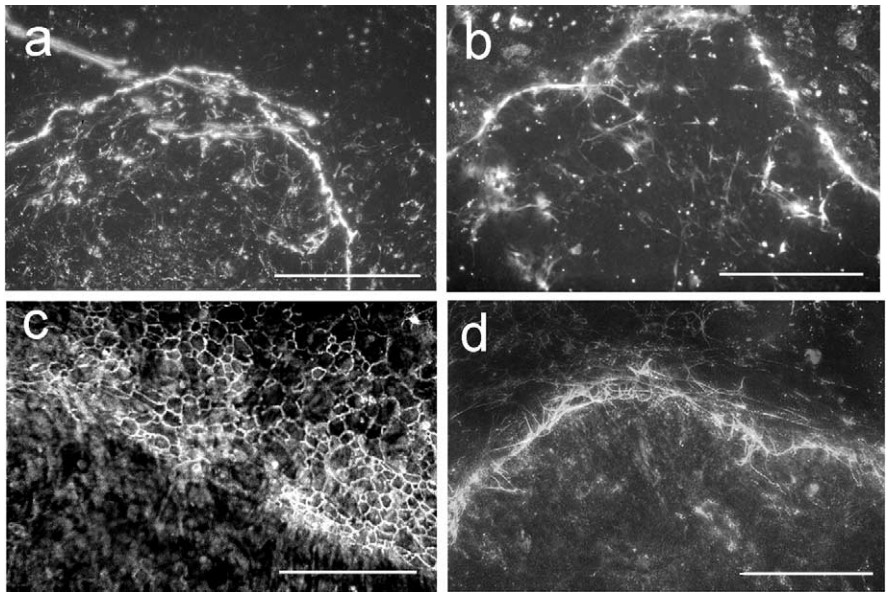

Fig. 6. Distribution of molecules at the astrocyte/meningeal cell boundary. Laminin (a), tenascin-C (b), and versican (d) are concentrated at the interface region. N-Cadherin is seen between and on the surface of astrocytes, but not on meningeal cells (c). Bars = 200 μm.

in nonpermeablised cultures to fibronectin and laminin with a clear deposition at the interface region (Fig. 6b), some staining on the surface of the meningeal cells, and a fine, dim, granular staining on astrocytes. However, in contrast to laminin and fibronectin, upon permeablisation tenascin-C was laid down underneath both meningeal cells and astrocytes.

Effect on axon guidance of treatments designed to affect growth promoting and growth inhibitory molecules

The results of these interventions are shown in Fig. 8.

Treatments affecting CSPG glycosaminoglycans

Since meningeal cells express inhibitory CSPGs, we treated cultures with the enzyme chondroitinase ABC to remove chondroitin sulphate GAG chains just before or during growth of axons from DRGs. We also inhibited GAG chain sulphation by treating cultures with sodium chlorate for 10 days before the axon growth assay. Both treatments have been shown to reduce inhibition by CSPGs in vitro (Smith-Thomas et al., 1995). In neither case did the treatment result in a statistically significant increase relative to control in the ability of axons to cross from astrocytes to meningeal cells. In the case of sodium chlorate mean neurite crossing at the interface for control was 24.6% ($n = 16$), compared to 30% ($n = 9$) for the treatment group. For chondroitinase, mean neurite crossing at the interface for control was 18.9% ($n = 23$), for chondroitinase buffer 24.7% ($n = 37$), and for chondroitinase 26.1% ($n = 28$). CSPG GAG chains are not, therefore, involved in repelling axons from meningeal cells in this assay.

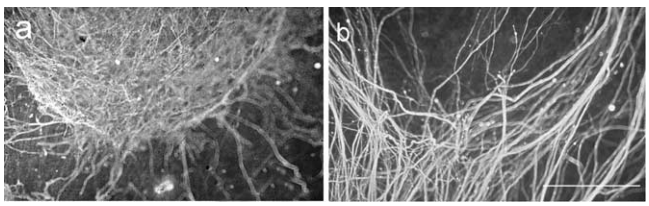

Fig. 7. A variety of treatments can increase the proportion of axons crossing astrocyte/meningeal boundaries, all of them producing this appearance. In this picture the culture was treated with forskolin and NT-3. Many axons in (b) have crossed the boundary onto meningeal cells, but the interface region still remains attractive. Bar = 200 μm.-

Blockade of NG2 increases DRG neurite crossing of the astrocyte/meningeal cell interface

Application of a function-blocking antibody against NG2, an inhibitory CSPG expressed by meningeal cells, resulted in an increase in axon crossing from astrocytes to meningeal cells (Fig. 8) with a mean axon crossing of 23.5% ($n = 7$) in the control compared to 44.2% ($n = 21$) in the treatment group. One-way ANOVA gave $P = 0.002$; Dunnett's method post hoc test showed a significant difference between treatment and control ($P < 0.05$). Previous experiments have shown the specific activity of this antibody in blocking the inhibitory effects of purified NG2 and cell surface NG2 (Fidler et al., 1999; Ughrin et al., 2003; Chen et al., 2002).

Blockade of neuropilin-2 increases neurite crossing at the astrocyte/meningeal cell interface

Meningeal cells express the inhibitory axon guidance molecules semaphorin 3A and 3C. These act via the neu-

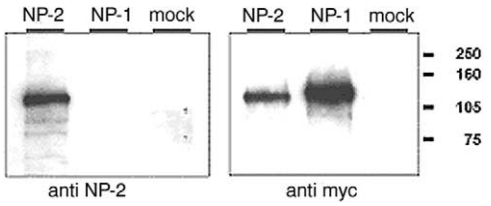

Fig. 8. The NP2 antibody specifically recognizes NP2 in transfected 293T cells. 293T cells were transfected with myc-tagged NP2 or myc-tagged NP1 expression plasmid or mock transfected. Whole cell lysate was harvested and analysed by Western blot with either the anti-NP2 antibody (left) or an anti-myc antibody (right). The NP2 antibody only recognizes the NP2 protein at the appropriate molecular weight, while the myc antibody shows expression of both NP2 and NP1 constructs. Molecular weight markers (kDa) are indicated on the right.

ropilin receptors. Semaphorin 3A signals through a homodimer of neuropilin 1 (NP-1) whereas semaphorin 3F uses a homodimer of neuropilin 2, and semaphorin 3C uses a heterodimer of NP-1 and NP-2. In order to test for a role of semaphorins in guiding axons away from meningeal cells, we used two neuropilin blocking antibodies. The anti-NP-1 has been widely used in blocking experiments (Kolodkin et al., 1997). The anti NP-2 was raised by us and specifically recognises the MAM domain of NP-2 (Fig. 9). This antibody binds to the axons of postnatal DRGs grown in vitro, demonstrating that postnatal sensory neurones, in contrast to embryonic sensory neurones, produce neuropilin-2 (not shown). Application of the NP-1 antibody alone did not result in an increase in neurite crossing from astrocytes on to meningeal cells; the mean control crossing was 24.1% ($n = 42$) compared to 22.6% ($n = 67$) in the presence of NP-1 antibody. However in the presence of NP-2 antibody alone, or with NP-1 and NP-2 antibody in combination (Fig. 8), the mean crossing rate increased from 30.5% in serum controls ($n = 83$) to 43% ($n = 76$) and from 36.8% ($n = 101$) to 49% ($n = 73$), respectively. Addition of the NP-1 antibody to the NP-2 antibody thus produced no additional effect. In the case of NP-2 alone Kruskal–Wallis one-way ANOVA gave $H_1 = 10.15$, with $P = 0.001$; Dunn's post hoc tests showed a significant difference between each treatment relative to control ($P < 0.05$). For NP-1/NP-2 in combination, Kruskal–Wallis one-way ANOVA gave $H_1 = 11.13$, with $P < 0.001$; Dunn's post hoc tests showed a significant difference between each treatment relative to control ($P < 0.05$). This result is consistent with the repulsion of axons by semaphorin 3C produced by meningeal cells.

Neither blockade of N-cadherin nor β-1 integrin promotes neurite crossing of the astrocyte/meningeal cell interface

We next performed experiments to reduce the attractiveness of astrocytes by blocking growth-promoting molecules associated with axon growth on astrocytes. Application of a cyclic N-cadherin blocking peptide which has been proven in previous experiments to reduce adhesion among Schwann cells, oligodendrocytes, and axons (Schnaedelbach et al., 2001; Williams et al., 2000) was made to reduce the attractive effect of this molecule on astrocytes. This treatment did not alter neurite behavior at the interface. Mean neurite crossing from astrocytes to meningeal cells in the control group was 29.3% ($n = 65$), compared to 31.4% ($n = 85$).

Application of β-1 integrin blocking antibody to inhibit the possible attractive effects of laminin, fibronectin, and tenascin at the interfaces did not significantly alter neurite crossing at the interface. In control groups the mean crossing from astrocytes to meningeal cells was 25.4% ($n = 107$) compared to the β-1 integrin antibody treated groups, where mean crossing was 27.8% ($n = 122$).

Treatments affecting axonal signalling pathways

The response of axons to inhibitory signals can be altered by treatments modulating growth cone signalling pathways. We applied treatments affecting the signalling pathways most closely involved in inhibitory mechanisms in axons, those going via cAMP and Rho. These results are summarised in Fig. 8.

Elevation of cAMP increases the ability of neurites to cross from astrocytes to meningeal cells

Elevation of cAMP and cGMP has been shown to affect axonal responses to environmental guidance cues and to allow axons to overcome inhibitory influences (Cai et al., 2001; Song and Poo, 1999). We therefore increased levels of these signalling molecules by using IBMX (nonselective phosphodiesterase (PDE) inhibitor), forskolin (activator of

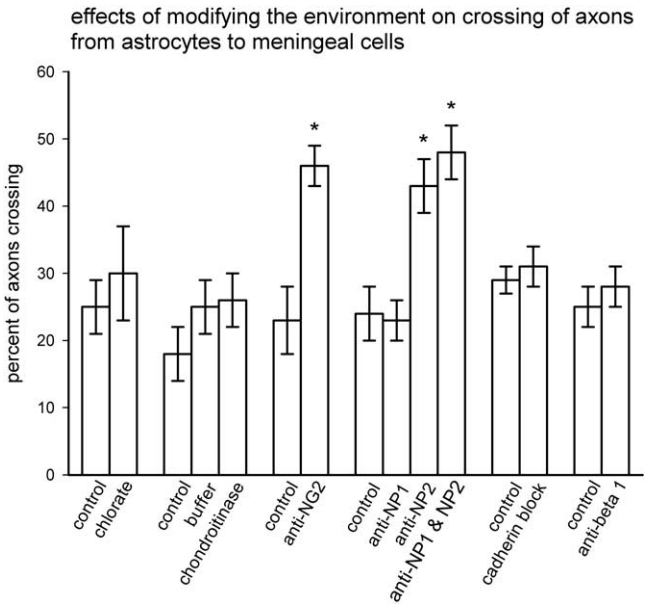

Fig. 9. Treatments that neutralise the inhibitory effects of NG2 or semaphorins increase the proportion of axons that can cross astrocyte/meningeal cell boundaries.

adenylyl cyclase), and rolipram (inhibits PDE IV, increasing cAMP). Treatment with these molecules produced a significant increase in neurite boundary crossing (Fig. 8). The mean crossing rate in controls was 25.9% ($n = 89$); with the addition of IBMX this was raised to 51.5% ($n = 18$), with forskolin to 35.9% ($n = 80$), with IBMX and forskolin together to 49.7% ($n = 20$). Kruskal–Wallis one-way ANOVA gave $H_5 = 46.3$, with $P < 0.001$; Dunn's post hoc tests showed a significant difference between each treatment relative to control ($P < 0.05$).

The raising of cyclic nucleotides could have affected the behaviour of the axons, of the glial cells, or of both. In order to determine whether the promotion of boundary crossing was a direct effect on axons, we examined axon behaviour on cultures in which the glia were killed by fixation in periodate–lysine–paraformaldehyde fixative. In order to look at the effects of cAMP alone we used rolipram to inhibit PDE IV. In control fixed cultures, axons still showed the same behaviour, preferring to stay on astrocytes rather than crossing onto meningeal cells, although the degree of preference was somewhat less. Treatment of the cultures with rolipram increased axon crossing from astrocytes to meningeal cells in a dose-dependent fashion. Rolipram (1 μM) increased mean neurite crossing from a control value of 28.4% ($n = 14$) to 49.5% ($n = 9$), and 10 μM rolipram increased crossing to 71.4%. Kruskal–Wallis one-way ANOVA gave $H_2 = 19.5$, with $P < 0.001$; Dunn's post hoc tests showed a significant difference between 10 μM rolipram relative to control ($P < 0.05$).

Neurotrophins have been reported to increase cyclic nucleotide levels in sensory neurons (Cai et al., 1999) and might therefore have been expected to increase boundary crossing. We saw no effect of NGF alone (not shown). However, treatment of the cultures with NT3 increased boundary crossing from 25.9 to 36.9% ($n = 52$). Further addition of forskolin increased this to 42.1% ($n = 38$) (Fig. 5). We conclude that a variety of treatments that raised axonal cAMP increased the ability of axons to cross the inhibitory boundary from astrocytes to meningeal cells.

Inactivation of Rho increases the ability of axons to cross from astrocytes to meningeal cells

Signalling via Rho has been implicated in the response of axons to inhibitory molecules, and inactivation of Rho by ribosylation with C3-ADP-ribosyltransferase has enabled axons to overcome inhibitory substrates in vivo and in vitro (Dergham et al., 2002; Lehmann et al., 1999) Cell permeant forms of this enzyme have recently been produced by linking the enzyme to a cell permeant peptide sequence (Winton et al., 2002). We used one of these, C3-7, to treat cocultures of astrocyte, meningeal cells, and DRGs. We treated the cultures in two different ways. When C3-7 was added directly into the growth medium and left there while axons grew, there was a significant increase in neurite crossing at 10 μg/ml (Fig. 10), from 32.9% ($n = 82$) to 49.7% ($n = 55$) (significant by one-way ANOVA, $P = < 0.001$, and post hoc Tukey test, $P < 0.05$). There was a smaller effect with 1 μg/ml, where mean crossing was 38.1% ($n = 69$). In experiments where the C3-7 construct was incubated with the DRGs prior to plating there was no difference in crossing from astrocytes to meningeal cells between the groups (control 32.5% ($n = 40$), 1 μg/ml C3-7 31.7% ($n = 55$), and 10 μg/ml C3-7 32.3% ($n = 61$)).

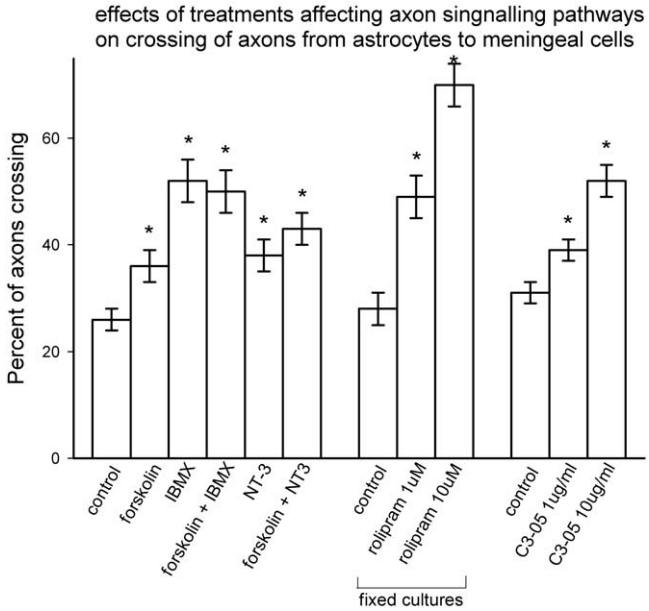

Fig. 10. Several treatments that affect cAMP levels or inactivate Rho can increase the proportion of axons crossing from astrocytes to meningeal cells.

Effects of treatments on the boundaries

The segregation of the astrocytes and meningeal cells into separate territories, the formation of the hypertrophic astrocyte processes, and the laying down of inhibitory molecules took some time, and cocultures were 2 weeks old by the time of our assays. We did not assay the effects of our various treatments on formation of boundaries and territories. None of the treatments had a clear effect on the boundaries over the 2 days of our assays, except for elevation of cAMP which caused the astrocytes to become more process-bearing and hypertrophic, but without breaking down the astrocyte/meningeal cell boundaries.

Discussion

In this study we set out to establish an in vitro model of the astrocyte/meningeal cell interface which is formed in vivo following an injury and which is a major impediment to axon regeneration. As described previously, the two cell types partition in culture, and at the interface between the two cell types a glia-limitans-like structure formed (Abnet et al., 1991; Struckhoff, 1995). That there must be an active

interaction between these two cell types in the formation of a glia limitans is supported by in vitro experiments in which Ness and David (Ness and David, 1997) showed that meningeal cells induce a change in the morphology of adjacent astrocytes and by in vivo experiments in which the selective removal of meningeal cells inhibits the change in astrocyte morphology required for glia limitans formation at the surface of the cerebellum (Sievers et al., 1994).

As in vivo, meningeal cells were unattractive to axons in our assay. The astrocyte/meningeal boundary presented a barrier to axon growth, with most axons being unable to cross from astrocytes to meningeal cells, while growth in the other direction from meningeal cells to astrocytes was not inhibited. These boundaries are not therefore simply a mechanical barrier, but rather a selective barrier in which axons express a strong preference for astrocytes over meningeal cells. A similar avoidance of meningeal cells is seen when retinal ganglion cells are grown on mixed glial cultures (Hirsch and Bahr, 1999). In the damaged CNS meningeal cells invade many CNS injuries, and the astrocyte meningeal boundary is a three-dimensional structure which often spreads across axon pathways in a position to impede axon regeneration. Its effects on axon regeneration may therefore be due to both the unattractiveness of meningeal cells for axons and the mechanical effects on axon growth.

We next sought to define the molecular features that underlie the axonal preference for astrocytes over meningeal cells. We identified several candidate molecules. We showed that meningeal cells produce the inhibitory CSPGs NG2 and versican, and an accompanying paper (Niclou et al., 2003) demonstrates that meningeal cells produce class 3 semaphorins in vitro, as they do in vivo (DeWinter et al., 2002). Interestingly the splice variants of versican produced by meningeal cells are V_0 and V_1, whereas the isoform produced by oligdendrocyte lineage cells and found throughout the CNS is V_2 (Asher et al., 2002; Morgenstern et al., 2002). Removal of the CSPG GAG chains with chondroitinase or prevention of GAG sulfation did not make the meningeal cells less unattractive to axons, but a blocking antibody to the CSPG NG2 was effective. The inhibitory effects of NG2 are mediated both by the protein core and by GAG chains (Ughrin et al., 2003); our result would suggest that NG2 on meningeal cells exerts much of its inhibitory effect independently of the GAG chains. We attempted to block the inhibitory effects of class 3 semaphorins by blocking their receptors, the neuropilins. Semaphorin 3A signals through a homodimer of neuropilin 1 whereas semaphorin 3F uses a homodimer of neuropilin 2, and semaphorin 3C uses a heterodimer of NP-1 and NP-2 (Giger et al., 2000; Chen et al., 1998; Kolodkin et al., 1997). Rat postnatal DRG neurites have been shown to express NP-1 (Reza et al., 1999) and our immunohistochemistry shows them also to express NP-2, so the axons would be sensitive to semaphorins 3A, 3C, and 3F. We found that an antibody to NP-2 increased the ability of axons to grow onto meningeal cells, but blocking NP-1 had no effect. This suggests that semaphorin 3C and/or 3F is an active chemorepulsive molecule in our tissue culture model. The main inhibitory molecules produced by meningeal cells are therefore NG2 and semaphorin 3C/3F, and these together are the main factors inhibiting axon growth onto meningeal cells.

Axon guidance involves choices between alternative environmental cues, and axons therefore respond more to differences than to absolute levels of attractiveness. Inhibitory molecules expressed in particular places therefore have the properties of guidance molecules, as has been suggested for NG2. The unwillingness of axons to leave an astrocyte environment to grow into a meningeal cell environment could therefore also be explained by the presence of attractive molecules on astrocytes. We examined the expression of some potentially relevant molecules. Fibronectin, laminin, and tenascin were present in larger amounts on meningeal cells than astrocytes, and were also concentrated in a band at the interfaces between the cells. Axon growth on these molecules is β-1 integrin dependent, and while a β-1 blocking antibody reduced axon growth overall on our cultures, it did not affect the proportion of axons able to cross astrocyte/meningeal cell boundaries. N-cadherin was exclusively on astrocytes in our culture model and has been shown to promote axon growth on astrocytes (Neugebauer et al., 1988), yet cadherin-blocking peptides did not make astrocytes sufficiently unattractive that axons grew more readily onto meningeal cells.

Instead of altering the environments that block axon regeneration, an alternative is to manipulate the axonal response to them. Axonal guidance and inhibitory molecules act through a restricted number of signalling pathways, and it has been shown that modulating these pathways can allow axons to grow on inhibitory substrates and to regenerate in the damaged CNS (Qiu et al., 2002; Cai et al., 2001; Lehmann et al., 1999; Neumann et al., 2002). We found that either increasing levels of cAMP in the axons or blocking the Rho pathway using C3 ribosyltransferase both allowed an increased proportion of axons to grow from astrocytes to meningeal cells.

An objective of the present study was to identify and prioritise treatments that might allow axons to regenerate through the lesion core in vivo. Most of the interventions that we have used in our experiments have yet to be tested in animal models. In almost all the experiments that have promoted axon regeneration in spinal cord injuries, the axons have grown around rather than through the meningeal-cell-containing lesion core, as is seen following treatment with chondroitinase ABC (Bradbury et al., 2002). The exceptions to this are treatments which act primarily on the axons to increase their regenerative potential or on signalling from the environment. Thus, papers in which axons appear to be regenerating into the lesion core report regeneration in the spinal cord following blocking of Rho signalling (Dergham et al., 2002), increasing cAMP in sensory neurons (Qiu et al., 2002; Neumann et al., 2002), infusion of

NT-3 (Bradbury et al., 1999), and following a conditioning lesion of the peripheral nerve (Neumann and Woolf, 1999).

The invasion of CNS injuries by meningeal cells is an important reason for the failure of regeneration. Meningeal cell invasion is particularly marked in spinal cord injuries in which the meningeal surface is cut (De Winter et al., 2002). Our tissue culture model reproduces many but not all of the inhibitory influences at these sites. It does not reproduce the three-dimensional aspects of a CNS injury, and in particular it does not reproduce the laying down of a collagenous membrane across the path of regenerating axons, dissolution of which has been shown to promote axon regeneration (Klapka et al., 2003; Hermanns et al., 2001). Also it is also probable that CSPGs are present trapped between the cells of a tissue in much larger amounts than in a monolayer culture, in which most of these molecules are diluted away in the medium (Asher et al., 2000). A full characterization of the effects of CSPGs at astrocyte/meningeal cell boundaries could only, therefore, be made in a three-dimensional culture model or in vivo. However, our model has allowed us to characterise some of the inhibitory mechanisms within the lesion core of CNS injuries and to identify treatments that may allow axons to overcome them. The best way to overcome the inhibitory effects of the meningeal cell lesion core is to prevent its formation. This has been achieved to some extent by treatment with high doses of steroid (Li and David, 1996) and by inhibitors of collagen formation (Klapka et al., 2003; Hermanns et al., 2001). Failing this our results suggest that treatments to block the effects of NG2 and type III semaphorins should make the tissue less inhibitory, and treatments to affect axon growth cone signalling pathways should allow axons to disregard these inhibitory molecules.

Experimental methods

All animal care was carried out in accordance with the Animals (Scientific) Procedures Act, 1986.

Meningeal cell cultures

The meninges were stripped from the cortices of neonatal Wistar rat pups and incubated with 0.1% trypsin (Sigma, Poole, Dorset, UK) and 0.01% collagenase (Sigma) at 37°C for 30 min. Then 0.001% DNase was added, and the tissue was pelletted by centrifugation. The supernatant was discarded and the cells were triturated in a solution containing 50 mg soyabean trypsin inhibitor (Roche, Lewes, East Sussex, UK), 300 mg BSA (Sigma), and 20 μg DNase per 100 ml of Hanks' balanced salt solution (HBSS; Life Technologies, Paisley, UK). The cells were then resuspended in CDMEM (Dulbecco's modified Eagle's medium with Glutamax I (Life Technologies) containing 10% fetal calf serum (FCS; Harlan SeraLab, Loughborough, UK) supplemented with antibiotics) and plated onto uncoated 25-cm^2 flasks or 13-mm poly-D-lysine (Sigma) coated glass coverslips, as required.

Astrocyte cell cultures

The cortices of neonatal Wistar rat pups were removed, the meninges were peeled off, and the remaining tissue was diced. The tissue was incubated at 37°C in 0.1% trypsin for 20 min, followed by the addition of 0.001% DNAse (Sigma). The tissue was pelletted and triturated in an HBSS solution containing soyabean trypsin inhibitor, BSA, and DNAse as above, and the cells were then resuspended in CDMEM. The cells were plated onto either poly-D-lysine-coated 75-cm^2 flasks (protocol modified from McCarthy and de Vellis, 1980) or 13-mm poly-D-lysine-coated glass coverslips.

Meningeal cell–astrocyte cocultures

Astrocytes which had been growing in culture for 7 days and had reached confluence were shaken overnight to remove any loosely attached oligodendrocyte progenitor cells or microglia and then trypsinised off. The cells were spun down and resuspended in 1 ml of CDMEM and then left to aggregate at 37°C for 3 h with intermittent agitation. Concurrently meningeal cells which had been growing in culture for 7 days and had also reached confluence were similarly trypsinised, resuspended, counted, and left to aggregate for 3 h at 37°C.

Following the 3-h aggregation period, the two cell types were plated at a ratio of 0.85×10^5 astrocytes:0.85×10^4 meningeal cells/13-mm poly-D-lysine-coated coverslip. These cocultures were left to grow for 10–14 days, with the medium being changed every 2–3 days as required. After this time neonatal (P0–P1) DRGs were placed on the cocultures. The DRG/cocultures were grown in CDMEM supplemented with 10 ng/ml nerve growth factor (NGF; Serotec, Oxford) (CDMEM/NGF) for 48 h, after which they were fixed in −20°C methanol and appropriately stained (detailed below).

Immunocytochemistry

Cultures were stained either live or after cold methanol fixation. For live staining antibodies were diluted in 2% FCS/HBSS. Primary antibodies were applied for 20 min, and then, after washing, the secondary antibody was applied for a further 20 min. For staining after fixation, primary antibodies were diluted in 3% BSA (Fluka)/PBS and left on for 45 min. Secondary antibodies along with Hoescht nuclear stain (1:5000; Sigma) were left on for 45 min.

Conditioned medium

The CDMEM medium from confluent meningeal cells (approximately 7 days old) was removed, the cultures were

washed in HBSS, and then DMEM supplemented with 1% FCS and 1% ITS+ (containing insulin, transferrin, selenium, bovine serum albumin, linoleic acid; Collaborative Biomedical Products, Bedford, MA) was added. The cells were then left for a further 72 h whereupon this medium was collected, a cocktail of protease inhibitors (Complete; Roche) was added, and the medium was immediately centrifuged at 1000 rpm for 10 min. The supernatant was removed and frozen at −64°C. The monolayers of cells which remained in the flasks were used for the cell extraction described below.

Cell extraction

Following removal of the medium the confluent 25-cm^2 flasks of meningeal cells were washed twice in room temperature PBS, and then 2.5 ml of PBS was placed into each flask and the cells were scraped off using a rubber policeman. The scraped cells were collected and centrifuged at 1000 rpm for 10 min, whereupon the supernatant was discarded and the remaining pellet of cells was resuspended in 1% Triton X-100 lysis buffer (50 mM Tris, 150 mM sodium chloride, 1% Triton X-100, pH 7.4), supplemented with a cocktail of protease inhibitors (Complete). The cells were allowed to lyse for 1 h at 4°C with constant inversion before being microcentrifuged at 13,000 rpm for 10 min and the pellet (containing cell debris) was discarded. The supernatant, composed of dissolved intracellular cell proteins, was then frozen at −64°C.

Western blot

Half of each of the samples were subject to chondroitinase ABC (Roche) treatment for 3 h at 37°C and the remaining half were left undigested. Samples were boiled in nonreducing buffer (10% sodium dodecyl sulphate [SDS; BDH] in Tris HCl [Sigma], pH 6.8, with glycerol and bromphenol blue [BDH]) and SDS–polyacrylamide electrophoresis (SDS–PAGE) then carried out on a 4% 37.5:1 acrylamide–bisacrylamide gel at 5 mamp for 16–18 h at room temperature. Proteins were transferred to nitrocellulose membrane (Hybond-C; Amersham, Buckinghamshire, UK) using a wet-transfer method for 14–16 h at 4°C with a constant current of 150 mamp. For Western blot all washes and incubations were carried out at room temperature in Tris-buffered saline containing Tween 20 (BDH) termed TBS/Tween 20 (0.9% sodium chloride, 10 mM Tris HCl, 0.05% Tween 20). The membrane was washed briefly for 2 × 5 min and then blocked with 5% dried milk/TBS/Tween 20 (except the membrane used for the versican antibody which was washed in TBS/Tween 20 alone) for 1 h. After another brief wash the membranes were incubated for 2 h with primary antibody. Membranes were then washed for 6 × 5 min and incubated with the corresponding conjugated secondary antibody, either goat anti-rabbit or horse anti-mouse Ig horseradish peroxidase conjugates (1:2000; Vector, Peterborough, UK), for 1 h. Finally the membranes were washed for 6 × 5 min and developed using enhanced chemiluminescence (ECL) reagents (National Diagnostics, Hull, UK, and also Amersham).

Antibody details

Neuropilin-2 antibody production

Neuropilin-2 (NP-2) antibodies were produced similarly to the neuropilin-1 antibodies as described previously (Pasterkamp et al., 1998b). An 807-bp. PCR fragment corresponding to the MAM domain of rat NP-2 (amino acids C592-L860 (Kolodkin et al., 1997)) was generated using the primers GAA TTC GGA TCC TGT GAC TGG ACA GAC TCA AAG and GAG CTC AAG CTT CAG GAT GGG GTC CAG TGT GTA and was cloned in the *Bam*HI and *Hin*dIII sites of the pQE30 vector (Qiagen, Hilden, Germany). This was used to produce 6-histidine-tagged NP-2 fragments in *Escherichia coli* which were purified on a Ni–NTA–agarose column according to specifications of the manufacturer (Qiagen). Rabbits were immunized with ~0.5 mg of purified protein in complete Freund's adjuvant and boosted two times in incomplete Freund's adjuvant. NP-2 antibodies were affinity purified on a NP-2 protein immunosorbent column according to a method described previously (Oestreicher et al., 1983). In short, NP-2 protein fragments were coupled to CNBr-activated sepharose 4B in 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 9.0, and subsequently washed with 1 M glycine, pH 8.0, 1 M NaCl in 0.1 M sodium acetate, pH 4.0, and 0.1 M sodium borate, pH 8.5. The Sepharose was packed in a column, and 3 ml of serum was added. The column was washed with PBS, and specifically bound antibodies were eluted with 100 mM ammonium formate, pH 2.7. Eluents were neutralized with 1 M ammonia and concentrated by lyophilization.

Neuropilin-2 Western blot. Myc-tagged constructs of full-length rat NP-1 and NP-2 were transfected in 293T cells using a Ca–phosphate transfection method. Cells were harvested 48 h after transfection and the cell lysate was prepared. About 5 μg protein per sample was separated by SDS–PAGE and blotted onto nitrocellulose filters. The blot was either incubated with the anti-myc antibody (1:1000; clone 9E10, Santa Cruz) or with the rabbit anti-NP-2 antibody (1:1000). A secondary HRP-coupled antibody was applied and detected with a chemiluminescence detection kit (enhanced luminol reagent, Perkin–Elmer).

Western blot

Versican monoclonal antibody (mAb) 12C5 (supernatant 1:10; (Asher et al., 1991)), chondroitin sulphate mAb CS56 (1:1000; Sigma), NG2 polyclonal antibody (pAb) (2 microgram/ml; (Levine and Card, 1987)), phosphacan mAb 3F8 (1:100;(Rauch et al., 1991)), neurocan mAb 1G2 (1:20; (Matsui et al., 1994)), brevican mAb (1:250; Transduction Laboratories, Affiniti Research Products Ltd., Mamhead,

UK), Rho pAb (1:1000; Santa Cruz Biotechnology, California, USA), and for control studies rabbit immunoglobulin (Ig) (Dako, Ely, UK), mouse IgM (ICN Biomedicals Ltd., Thame, UK), or mouse IgG (Sigma) were used as appropriate.

Immunocytochemistry

Immunocytochemistry was carried out on living (unfixed) cells: versican mAb 12C5 (supernatant 1:1), NG2 mAb D31.10, phosphacan mAb 3F8 (1:100), chondroitin sulphate mAb CS56 (1:100), O-2A mAb A2B5 (supernatant 1:3; American Type Culture Collection, Manassas, VA), and neurocan mAb 1G2 (supernatant 1:1).

Immunocytochemistry was carried out on methanol fixed cells: glial fibrillary acidic protein (GFAP) mAb (1:20; Roche), GFAP pAb (1:200; Dako), RALDH-2 pAb (1:1000, a gift from P. McCaffery, Harvard University, USA), anti-N-cadherin (1:200, FWCAD, a gift from F. Watt, ICRF, London), neurofilament pAb (1:100; Sigma-Genosys, Pampisford, UK), or neurofilament-associated-protein mAb 3A10 (supernatant 1:5; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA).

Immunocytochemistry was carried out on living and fixed cells: fibronectin pAb (1:200; Dako), laminin pAb (1:500; a gift from A. Faissner, Ruhr University, Bochum), and tenascin-C (1:200, a gift from A. Faissner).

Secondary and tertiary antibodies (used on either living or fixed, depending on whichever condition the primary antibody had been used under) used were biotinylated anti-mouse/anti-rabbit immunoglobulin (1:100; Amersham) or anti-mouse/anti-rabbit Ig FITC conjugates (1:200; Jackson) and Cy3–streptavidin (1 μg/ml; Amersham)

Controls. Controls of rabbit Ig (Dako), mouse IgM (ICN), or mouse IgG (Sigma) were used as appropriate.

Treatments applied to cocultures

Chlorate. Cocultures were grown in CDMEM for 1–2 days, and then the medium was changed to 20 mM sodium chlorate (Sigma)/CDMEM. Control cultures were grown in CDMEM alone. At 10–14 days DRG explants were plated onto the monolayers and grown in NGF/CDMEM without chlorate for 48 h.

Chondroitinase ABC. Cocultures were grown in CDMEM for 10–14 days, treated for 3 h at 37°C with chondroitinase ABC (Roche; 0.2U/ml/coverslip), diluted in DMEM/1% ITS+ (Collaborative Biomedical Products), and then washed in HBSS. Control cultures were incubated either in DMEM/1% ITS+ alone ('buffer') or CDMEM ('normal') for 3 h and then washed in HBSS. DRG explants were plated and left to grow in NGF/CDMEM for 24–36 h.

NG2 blocking antibody. Cocultures were grown in CDMEM for 10–14 days and then anti-NG2 (0.2 mg/ml; Levine and Card., 1987) in CDMEM/NGF was applied to the cultures when the DRG explants were plated. Control cultures were grown in CDMEM/NGF alone with rabbit serum and both sets were left for 48 h.

Neuropilin 1 (NP-1)/neuropilin 2 (NP-2) blocking antibodies. DRG explants were plated onto 10- to 14-day-old cocultures and then incubated with either 200 μg/ml of purified rabbit anti-NP-1 (gift from A. Kolodkin (Kolodkin et al., 1997)) in CDMEM/NGF, 200 μg/ml of purified rabbit anti-NP-2 (described above) in CDMEM/NGF, or both 200 μg/ml purified rabbit anti-NP-1 and 200 μg/ml purified rabbit anti-NP-2 in CDMEM/NGF. Control groups were treated with 200 μg/ml of rabbit immunoglobulin in CDMEM/NGF.

N-Cadherin blocking peptide. Cocultures were grown in CDMEM for 10–14 days and then when DRG explants were plated either 0.3 mg/ml N-cadherin blocking peptide (cyclic HAVD peptide from Adherex Co. (Ottawa, Canada) shown to block N-cadherin in previous experiments from our laboratory and elsewhere (Wilby et al., 1999; Schnaedelbach et al., 2001) was added to each well, and the explants were left to grow for 48 h.

β-1 integrin blocking antibody. Cocultures were grown in CDMEM for 10–14 days, and then when DRG explants were plated either 10 μg/ml β-1 integrin blocking antibody (PharMingen) in CDMEM/NGF was added to each well, and the explants were left to grow for 48 h.

NT-3/forskolin/IBMX. Cocultures were grown in CDMEM for 10–14 days, and then when DRG explants were plated a number of treatments were applied: 20 ng/ml NT-3/DMEM, 5 μM forskolin/DMEM, 20 ng/ml NT-3/5 μM forskolin/DMEM, 1 mM IBMX/DMEM, 1 mM IBMX/5 μM forskolin, or DMEM alone (control). DRG explants were grown in one of the six treatments for 48 h.

Rolipram treatment of DRGs plated on fixed monolayers. Cocultures were grown for 10–14 days in CDMEM and then fixed in 0.075 M L-lysine (Sigma)/0.01 M sodium periodate (Sigma)/0.037 M sodium phosphate buffer/0.8% paraformaldehyde for 10 min. After being washed DRGs were plated onto the monolayers in either CDMEM, 1 μM rolipram/CDMEM, 10 μM rolipram/CDMEM, or DMSO (equivolume)/CDMEM and left for 48 h.

Cell-permeant C3 (C3-7). Cocultures were grown in CDMEM for 10–14 days, and then when the DRG explants were plated two different methods were used to get the C3-7 construct (Bioaxone, Montreal, Canada (Winton et al., 2002)) into the neurites. In the first, C3-7 was added to the growth medium (CDMEM/NGF) at either 1 or 10 μg/ml and left on the cultures for 48 h. In the second method, DRGs were collected following dissection and incubated at 37°C for 1 h in a small volume of HBSS, again at either 1 or 10 μg/ml. Following this incubation they were plated onto the cocultures and grown in CDMEM/NGF for 48 h.

Quantification of axon behaviour

Axon behaviour was quantified only in places where they reached an immunohistochemically identified astrocyte/meningeal cell boundary nearly at right angles to the orientation of the boundary. Each boundary, representing a patch of meningeal cells, was counted as a single result. At each boundary the proportion of axons choosing either to cross or not cross onto the meningeal patch was calculated, with the percentage crossing for each boundary used for statistical analysis. Results were analysed using *t* tests or ANOVA as appropriate using Sigmastat.

Acknowledgments

This work was funded by the Medical Research Council, the Wellcome Trust, the International Spinal Research Trust and Action Research, and the Christopher Reeve Paralysis Foundation. The authors thank Dorothy Gibson for her technical assistance.

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