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Inhibition of Axon Growth by Oligodendrocyte Precursor Cells

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The glial scar that forms at the site of injury is thought to be a biochemical and physical barrier to successful regeneration, although the molecules responsible for this barrier function are not well understood. Glia scars contain large numbers of oligodendrocyte precursor cells (OPCs) and these cells can produce several different growth-inhibitory chondroitin sulfate proteoglycans (CSPGs), including NG2, neurocan, and phosphacan. Here, we used membrane-based assays to show that the surface of OPCs is both nonpermissive and inhibitory for neurite outgrowth. Inhibition of growth by OPC is reversed by treatment with antibodies against the NG2 CSPG and the expression of NG2 is sufficient to change a growth-permissive cell surface to a nonpermissive surface. These result suggest that the OPCs that accumulate rapidly at sites of CNS injury can contribute to the creation of an environment that inhibits nerve regeneration and that NG2 is a necessary feature of that environment.

INTRODUCTION

After injury to the adult mammalian CNS, damaged neurons fail to regenerate their axons and the functions carried out by these axons are lost. This failure to regenerate is due, in part, to the formation of a glial scar which is thought to be a physical and biochemical barrier for axonal regeneration (Reier *et al.,* 1983; Fawcett and Asher, 1999). Glial scars contain numerous cellular elements, including damaged oligodendrocytes, hypertrophic astrocytes, activated microglia, oligodendrocyte precursor cells (OPCs), meningeal cells, and proliferating vascular endothelial cells. Each of these cell types may contribute to the growth nonpermissive nature of the damaged CNS. For example, damage to oligodendrocytes in the white matter may release myelin associated growth-inhibitory molecules such as MAG and nogo (Mukhopadhyay *et al.,* 1994; McKerracher *et al.,* 1994; Chen *et al.,* 2000; Grandpre *et al.,* 2000) such that they could interact with regenerating axons. Meningeal fibroblasts may secrete semaphorinIII, an axon repellant (Pasterkamp *et al.,* 1999). Oligodendrocyte precursor cells, which proliferate rapidly after injury and form a dense plaque at the damage site, make several species of growth-inhibitory CSPGs (Levine, 1994; Fawcett and Asher, 1999; Levine *et al.,* 2001). Last, hypertrophic or reactive astrocytes are major features of the mature glial scar and by sealing off the damaged area and recreating the glial limitans, they can form a barrier to axon regrowth (Reier *et al.,* 1983).

Numerous species of CSPGs, including neurocan, phosphacan, versican, and NG2 are enriched at sites of CNS injury (Fawcett and Asher, 1999). These complex, multifunctional molecules are expressed by reactive astrocytes, OPCs, meningeal cells, and they are also found in myelin (Niederost *et al.,* 1999). Several observations implicate CSPGs as scar-associated inhibitors of neuronal regeneration. Davies *et al.* (1997, 1999) found that although transplanted adult sensory neurons can grow axons several centimeters through undamaged CNS white matter tracts, when the regenerating axons confront an area previously damaged and now containing high levels of CSPGs, they stop growing. When removed from the damaged CNS, glial scar tissue is a poor substrate for axon growth and intact CSPGs contribute to this inability to support extensive axon growth (McKeon *et al.,* 1995). Purified preparations of neurocan, phosphacan, and NG2 all inhibit neurite outgrowth from embryonic and neonatal neurons *in vitro* (Dou and Levine, 1994; Friedlander *et al.,* 1994; Milev *et al.,* 1994). Thus, the high levels of CSPGs produced by reactive cells at sites of CNS damage may contribute to a growth nonpermissive environment.

Studies of the properties of isolated and purified molecules can provide important biochemical and structural information, but may not necessarily reflect

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all the properties of the complex cells found at sites of injury that make those molecules. To determine whether OPCs can inhibit axon regeneration, we used membrane stripe and membrane carpet assays (Bastmeyer and Stuermer, 1993; Tuttle *et al.*, 1995). These assays directly test the ability of OPC cell surface to promote or inhibit neurite growth from neonatal neurons. The results presented here show that the surfaces of OPCs are nonpermissive for axon growth and repel growing axons and that the NG2 CSPG is both necessary and sufficient for this activity. This suggests that the OPCs found within the glial scar may be an important factor in the inhibition of regeneration. Therapies designed to prevent the rapid accumulation of OPCs or to prevent the expression of NG2 may be beneficial in promoting CNS axon regeneration.

RESULTS

"Oligosphere" Cells Are Mostly Oligodendrocyte Precursor Cells

Large numbers of OPCs for use in membrane-based assays were generated using the "oligosphere" procedure (Avellana-Adalid *et al.*, 1996). To characterize these cells, we examined the expression of marker antigens associated with different stages of the oligodendrocyte lineage by indirect immunofluorescence after dissociating the "oligospheres" into single cell suspensions. As shown in Fig. 1 and Table 1, 90% of the oligosphere cells express the NG2 CSPG on their surfaces. Most of the NG2-positive cells had a bipolar morphology, characteristic of immature OPCs. These NG2-positive cells were also stained with monoclonal antibody A2B5, a second widely used marker for OPCs, but fewer than 8% of the NG2-positive cells expressed the 04 antigen, a marker for preoligodendrocytes (Table 1). The 04-positive preoligodendrocytes accounted for 8.8% of all the cells present and, among these, 81% also expressed NG2. The major nonoligodendrocyte lineage cell type present in these cultures were GFAP-containing, process-bearing astrocytes (Table 1). These cells, which also expressed NG2 on their surfaces, accounted for 7.6% of all the cells present. Less than 5% of the cells were stained with antibodies against myelin-basic protein and only 1.4% of the cells expressed the OX42 antigen, markers for differentiated oligodendrocytes and microglia respectively. Thus, the overwhelming majority of the cells used in the experiments reported below correspond to OPCs with only a small number of myelinating oligodendrocytes or microglia. These are the types of cells that accumulate at sites of CNS injury (Levine, 1994; Nomura *et al.*, 2000; McTigue *et al.*, 2001).

Oligosphere cells express the NG2 CSPG, which is produced as a fully processed proteoglycan containing chondroitin sulfate glycosaminoglycan (GAG) chains (Fig. 1). After removal of the GAG chains with chondroitinase ABC (ChABC), the core protein had an apparent molecular weight of 300,000 daltons and comigrates on one-dimensional SDS gels with NG2 expressed by B49 cells, the cell line from which NG2 was originally identified (Wilson *et al.*, 1981). Oligodendrocyte precursor cells express at least two other inhibitory CSPGs, phosphacan and neurocan, and these proteins could be detected on immunoblots only after digestion with ChABC (Fig. 1). The major forms of phosphacan expressed by the oligosphere cells were the membrane associated form, referred to as the long receptor form of RTPTβ, and the proteoglycan form, referred to as phosphacan (Maurel *et al.*, 1994). Neurocan was present as two polypeptides with molecular weights of 150,000 and 270,000 daltons. Phosphacan was detected on the cell surface by immunofluorescence staining of living cells (Fig. 1), but, as reported previously (Asher *et al.*, 2000), neurocan immunoreactivity accumulated on the surface of the tissue culture dish and little immunoreactivity was associated with the OPC cell surface (data not shown). The OPC membranes also contained multiple isoforms of NCAM, including polypeptides of 180,000, 140,000, and 120,000 daltons (Fig. 1). Other cell adhesion molecules such as L1, TAG1, and laminin were not detected (data not shown). These results show that oligosphere cells express the inhibitory CSPGs found at the CNS injury sites (Levine, 1994; McKeon *et al.*, 1999; Asher *et al.*, 2000).

Growing Neurites Avoid OPC Membranes

To analyze the behavior of growing axons when encountering OPCs, we used a membrane stripe assay (Bastmeyer and Stuermer, 1993). In this assay, axons growing out of small explants of postnatal rat cerebellum are presented with a choice between growing on stripes of OPC membranes or on stripes of poly-l-lysine (PLL). As shown in Fig. 2A, neurites preferred to grow on lanes coated with PLL and avoided the stripes made with OPC membranes. This preference of neurite outgrowth for the PLL coated lanes is not due simply to the presence of cellular membranes and any mechanical interference with growth since neurites grew freely across and on top of membrane stripes prepared from control CHO cells (Fig. 2B). When the membrane stripes

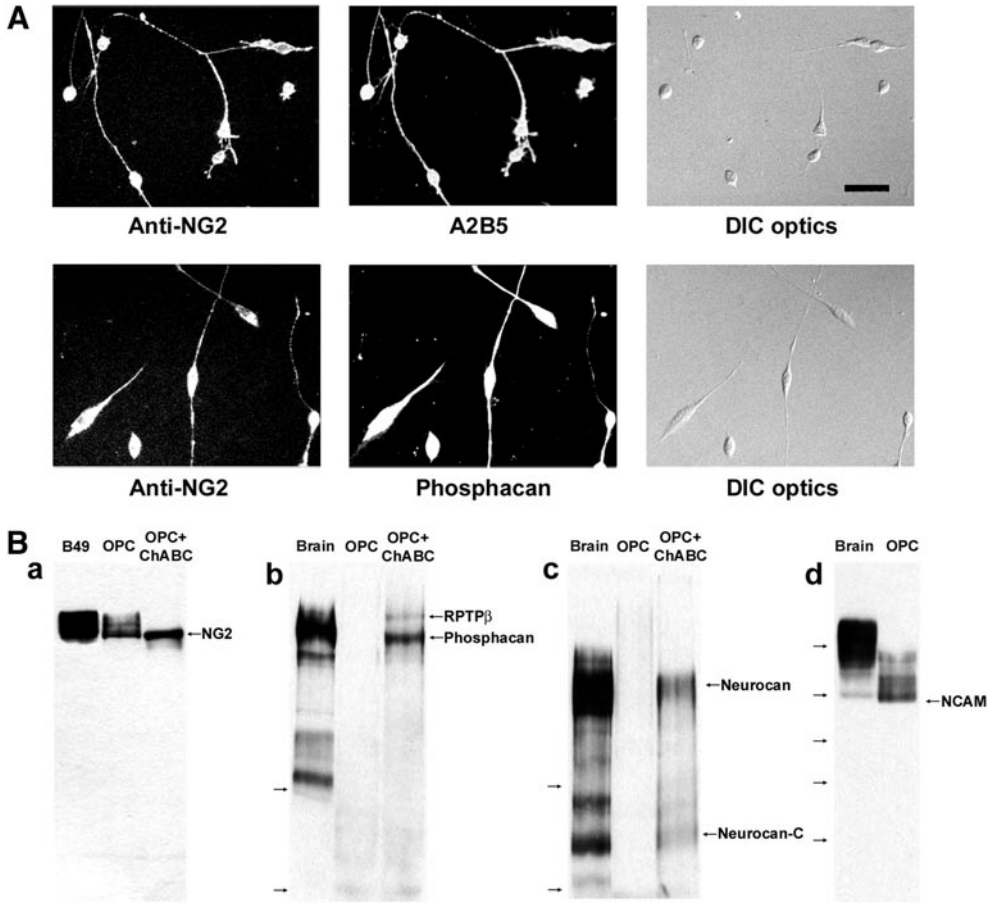

FIG. 1. Characterization of oligosphere cells. (A) Oligospheres were dissociated and immunofluorescently stained with the indicated antibodies as described under Experimental Methods. The NG2-positive cells also express the A2B5 antigen and phosphacan on their surfaces. The third panel shows the cells in DIC optics. Bar, 50 μm. (B) Characterization of oligosphere cells by immuno-blot. Oligospheres were lysed in 1% NP-40 as described under Experimental Methods and selected proteins in the lysates were identified by immunoblotting. The following antibodies were used: (a) anti-NG2, (b) anti-phosphacan, (c) anti-neurocan, and (d) anti-NCAM. NG2 was detected as a 300-kDa core protein proteoglycan, decorated with chondroitin sulfate GAG chains (a). Phosphacan was detected as the membrane associated long receptor form of RPTPβ and the dissociated form referred to as phosphacan (b). Intact neurocan and the C-terminal half of the neurocan (Neurocan-C) were also detected in the lysate (c). Oligosphere cells express three forms of NCAM with molecular weights of 180, 140, and 120 kDa (d). The amount of protein loaded in each lane: OPC, 40 μg; OPC + ChABC, 40 μg, brain extract, 15 μg; B49 cells, 10 μg. The arrows on the left side of each gel indicate the electrophoretic mobility of molecular weight markers. From top to bottom, they are 185, 115, 84, 62, and 51 kDa, respectively.

were prepared from CHO cells that express MAG (Mukhopadhyay *et al.*, 1994), the neurites again avoided the membranes and grew preferentially on the PLL-coated lanes (Fig. 2C). Transfection of the CHO cells with a plasmid encoding the L1 cell adhesion molecule caused the neurites to show a greater preference for the membranes as compared to the PLL substrates (data not shown). Thus, the patterns of neurite outgrowth in this membrane stripe assay reflect accurately the responses of the growth cones to growth-inhibitory and growth-stimulatory signals contained within the membranes.

To compare the neurite outgrowth patterns between different explants grown on different substrates, we used a semiquantitative scoring system based on the patterns of outgrowth (Walter *et al.*, 1987). In this system, a score of 0 indicates uniform outgrowth with no preference for either the PLL substrate or the membranes and 2 indicates maximum avoidance of the membrane stripes. Intermediate effects are scored using 0.5 unit blocks. As shown in Fig. 2D, cerebellar axons avoid the OPC membrane stripes to an extent similar to their avoidance of the MAG expressing CHO membrane stripes. These results indicate that the surface of

TABLE 1
Immunological Characterization of Oligosphere Cells

Antigen	Cell type	Percentage of total cells	Positive cells / total cells counted
NG2	Oligodendrocyte precursor cells	90.0 ± 3.7	2528 / 2801
GFAP	Astrocytes	7.6 ± 2.4	52 / 666
04	Preoligodendrocytes	8.8 ± 5.6	40 / 456
OX42	Microglia	1.4 ± 0.5	7 / 462
Myelin basic protein	Oligodendrocyte	4.7 ± 0.1	28 / 520

Note. Oligosphere cells were dissociated, immunofluorescently stained, and counted as described under Experimental Methods. Data shown is the mean and standard deviation of the mean from two to four different experiments. Each experiment comprised at least two separate cultures.

OPCs is repulsive for the neurites growing from postnatal cerebellar explants.

Anti-NG2 Antibody Blocks the Inhibitory Properties of the OPC Membranes

To determine the role of NG2 in the growth-inhibitory activity of the OPC membranes, membranes were incubated with a total IgG fraction prepared from rabbit anti-NG2 antisera prior to creating the stripes. As controls, OPC membranes were treated with normal rabbit IgG. As illustrated in Fig. 3B, the anti-NG2 antibody treatment made the OPC membrane stripes permissive for neurite outgrowth, resulting in a more uniform pattern of outgrowth. The antibody effectively blocked the inhibitory activity of the OPC membranes when used at a concentration of 400 μg / ml (Fig. 3E and inset). Treatment of the OPC membranes with normal rabbit IgG, also at 400 μg / ml, had no effect on the growth inhibitory activity of the membranes (Figs. 3C and 3E).

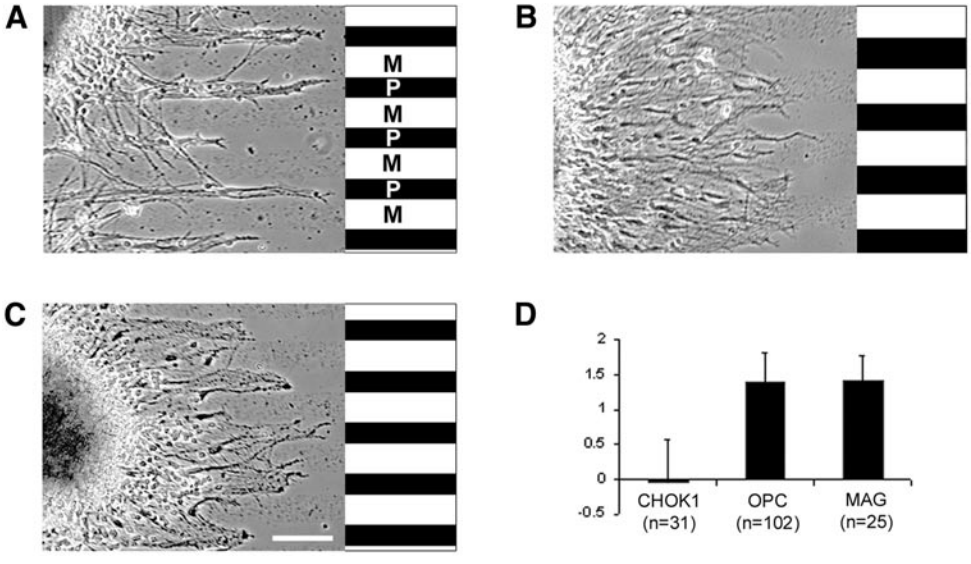

FIG. 2. Neurites avoid OPC membrane stripes. Phase contrast photomicrographs showing neurite outgrowth patterns from cerebellar explants grown on membrane stripes prepared from (A) OPCs, (B) CHO cells, and (C) MAG expressing CHO cells. The black and white bars to the right indicate the position of the membrane stripes (M) and the poly-l-lysine-coated substrate (P). Bar, 100 μm. Neurites avoid growing on membrane stripes prepared from OPCs or MAG expressing CHO cells, but grow over membrane stripes made from untransfected CHO cells. (D) Semiquantitative scoring of the neurite outgrowth patterns on the different membrane stripes. The neurite outgrowth patterns were scored as described under Experimental Methods. The score shown is the mean and standard deviation from the mean for each condition. *n* indicates the total number of explants scored in at least three independent experiments.

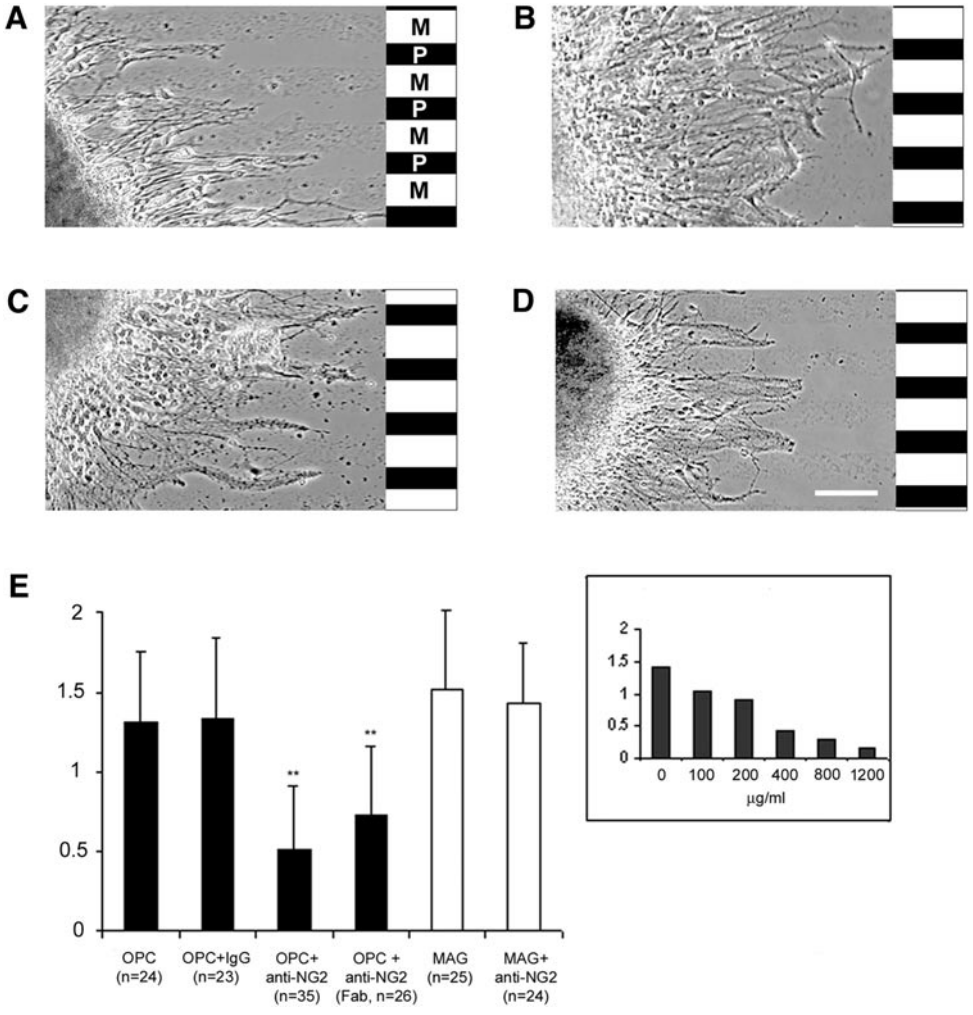

FIG. 3. Anti-NG2 antibodies reverse the inhibition caused by the OPC membrane stripes. Phase contrast photomicrographs showing the neurite outgrowth patterns on (A) OPC membrane stripes, (B) OPC membrane stripes treated with anti-NG2 antibodies, (C) OPC membrane stripes treated with normal rabbit IgG, (D) MAG-CHO membrane stripes treated with the anti-NG2 antibodies. M, Membrane stripes; P, PLL coated stripes. Bar, 100 μm. (E) Semiquantitative summary of the antibody reversal experiments. Antibodies are used at 400 μg/ml except the Fab fragments of the anti-NG2 antibody which were used at 1 mg/ml. Normal IgG treatment did not alter the neurite outgrowth patterns whereas anti-NG2 antibodies significantly lowered the score, indicating that the anti-NG2 antibody treatment made the OPC membrane stripes more permissive for neurite outgrowth. The inset shows the scores of neurite outgrowth patterns when OPC membrane stripes were treated with anti-NG2 antibodies at concentrations ranging from 100 to 1200 μg/ml. Data shown is the mean and the standard deviations of the mean and n represents the total number of explant scored. $^{**}P < 0.001$, Student's t test.

Incubation of the MAG expressing CHO cell membranes with the anti-NG2 antibody did not affect the inhibitory activity of the membranes and neurite outgrowth still occurred preferentially on the PLL substrate. (Fig. 3D). Fab fragments of the rabbit anti-NG2 antibody reversed the neurite outgrowth patterns as effectively as the intact antibody (Fig. 3E), suggesting that the blocking effect of the anti-NG2 antibody is not due to aggregation of the antigen or the shielding of other potential inhibitors physically associated with NG2. These results demonstrate that the NG2 proteoglycan is necessary for the full neurite outgrowth inhibitory activity of the OPC cell surface.

Neurites from Dissociated Cerebellar Neurons Avoid OPC Membranes

In the cerebellar explant cultures used above, there was an extremely variable extent of glial cell migration (data not shown). As the patterns of glial migration

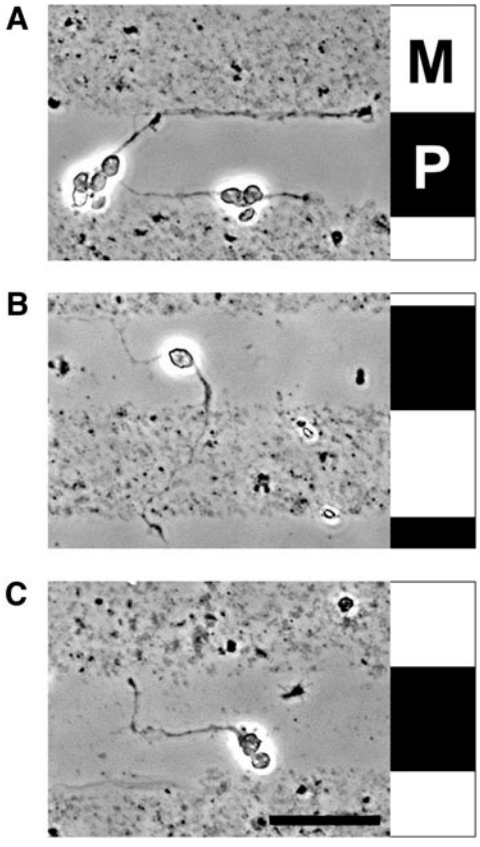

FIG. 4. The behavior of growing neurites from cerebellar granule neurons at the border between PLL and membrane stripes. The axons either turn at the border and/or track along the border (A), cross the border (B), or stop at the border (C). M, membrane stripes; P, PLL stripes. Bar, 50 μm.

could effect the patterns of neurite growth, we prepared dissociated cerebellar granule neurons and seeded these cells onto coverslips containing stripes of OPC membranes. In this way, we could measure the behavior of neurites growing from single neurons. We use cerebellar granule neurons as they are the predominant cell type whose axons grow out of cerebellar explants cultures (Fischer *et al.*, 1986). The dissociated cells did not stick to the membrane stripes, but rather adhered to the PLL coated interstripes. Therefore, we restricted our analysis to those neurites that originated on the PLL interstripes and approached or made contact with the OPC membrane stripes. As shown in Fig. 4, the neurites displayed several different behaviors. The majority of the neurites turned and tracked along the border (Fig. 4A); a smaller portion of the neurites appeared to stop at the border (Fig. 4C), and still fewer neurites crossed the border and grew onto the OPC membrane stripes (Fig. 4B). As shown in Table 2, 83% of the neurites turned and tracked along the borders, 9% stopped at the border, and 8% crossed the border. When confronting membranes from control CHO cells, 60% of the neurites crossed onto the membranes and only 19% turned and tracked along the borders (Table 2). When the OPC membranes were treated with anti-NG2 antibodies (400 μg/ml) prior to making the stripes, the number of granule cells that adhered to the OPC membrane stripes increased from 19 to 43% of total cells. In addition, 39% of the neurites now crossed the border and extended on top of the OPC membranes, a fivefold increase compared to untreated membrane stripes. The percentage of neurites that turned and tracked along the border decreased from 83 to 55%, whereas the number of neurites that appeared to stop did not change. These results show that neurites growing from single granule neurons avoid OPC membranes in a manner similar to that observed for large groups of neurites and that much of this avoidance is due to the presence of NG2 in the membranes.

GAG Chains Are Not Necessary for the Inhibition of Neurite Outgrowth by OPC Membranes

Proteoglycans are complex macromolecules containing both a protein core and covalently attached GAG chains. Previous studies had shown that the chondroitin sulfate GAG chains associated with the NG2 proteoglycan are not necessary for its neurite outgrowth inhibiting activity (Dou and Levine, 1994). To determine whether the chondroitin sulfate GAG chains associated with proteoglycans expressed on the OPC surface are necessary for neurite growth-inhibitory activity, we treated the OPC membranes with ChABC prior to making the stripes. As shown in Fig. 5, removal of the chondroitin sulfate chains did not alter the neurite outgrowth patterns on OPC membrane stripes. This

TABLE 2

Behavior of Single Cerebellar Cells When Encountering Membrane Stripes

	Turn/track	Cross	Stop	*n*
OPC membranes	83.0	8.0	9.8	499
OPC membranes + anti-NG2	54.9	39.4	5.7	574
CHO membranes	18.8	60.1	20.8	154

Note. The percentage of single cerebellar granule cells exhibiting the three different growth behaviors are shown. *n* indicates the total number of cells scored from two independent experiments.

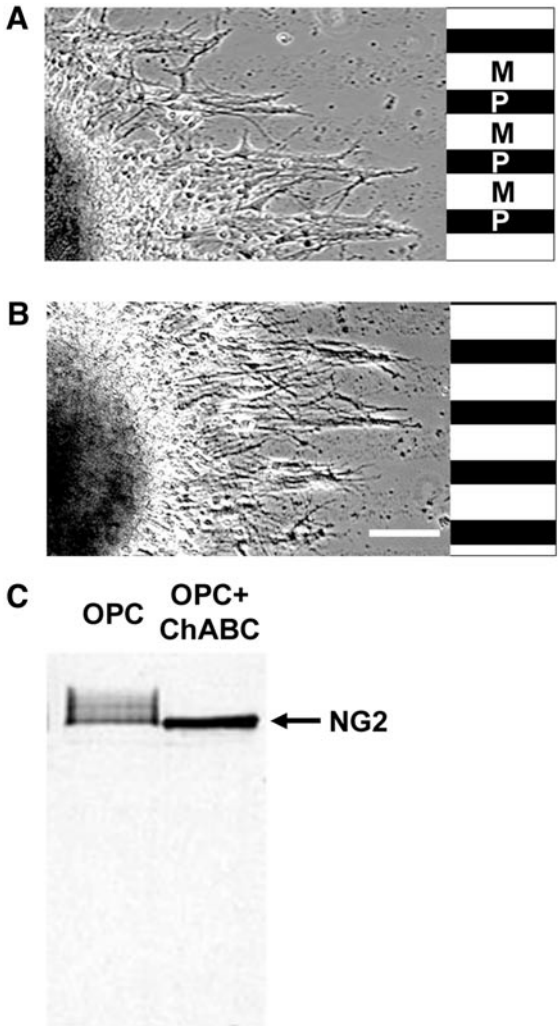

FIG. 5. Chondroitinase ABC treatment does not alter the repulsion from OPC membranes. (A) Neurite growth pattern on OPC membrane stripes without ChABC digestion. The mean score for 18 explants is 1.42. (B) Neurite growth pattern on OPC membrane stripes treated with ChABC. The mean score for the neurite outgrowth pattern (20 explants) is 1.4. (C) An anti-NG2 immunoblot of the untreated and ChABC treated OPC membranes (40 μg/lane). After the ChABC treatment, the high molecular weight smear is reduced and the amount of NG2 core protein (arrow) is increased. The ChABC treatment removes the chondroitin sulfate GAG chains from the NG2 core protein but does not change the repulsive properties of the membranes. M, membrane stripes; P, PLL-coated stripes. Bar, 100 μm.

result suggests that CS GAGs are not necessary for the growth inhibitory activities of the OPC membrane.

Expression of the NG2 CSPG by HEK293 Cells

The data presented above demonstrates that NG2 is required for the maximal inhibitory activity of the OPC surface. To determine whether the expression of NG2 is sufficient to inhibit neurite outgrowth, we transfected HEK293 cells with a full length NG2 cDNA under control of the CMV promoter and selected a permanent, high expressing clone which we termed 293-NG2 cells. Figure 6B shows that the 293-NG2 cells expressed a form of the NG2 proteoglycan that in immunoblots is similar to the form of NG2 expressed by the B49 cell line. NG2 is produced by 293 cells as an intact CSPG and ChABC digestion removed the GAG chains, resulting in a core protein with an apparent molecular weight of 290,000 daltons. The surface expression of NG2 was confirmed by immunofluorescence staining of live cells using a polyclonal anti-NG2 antibody (Fig. 6A). There was no staining of the untransfected 293 cells (Fig. 6A) and control transfected cells (not shown). Thus, the NG2 proteoglycan is properly expressed and processed by the transfected 293 cells.

293-NG2 Cell Surface Is Inhibitory for Neurite Outgrowth

To test the effects of cell surface expression of NG2 on neurite outgrowth, we grew cerebellar explants on uniform carpets made from membrane prepared from either the 293-NG2 cells or untransfected 293 cells. After 40–44 h growth, neurite length was measured. As shown in Fig. 7, cerebellar explants extended long neurites on membrane carpets made from untransfected 293 cells (Fig. 7A), while the neurite outgrowth on membrane carpets made from 293-NG2 cells was considerably less robust (Fig. 7B). Measurements of neurite length showed that neurite outgrowth on membrane carpets prepared from 293-NG2 cells was 35% shorter than on control membranes carpets. (Fig. 7C). This extent of inhibition is quantitatively similar to that seen when explants were grown on CHO cells transfected to express high levels of MAG. Treatment of the 293-NG2 cell membranes with the rabbit anti-NG2 antibody prior to making the carpets reversed this growth inhibition and neurite length was indistinguishable from that on control, 293 membrane carpets (data not shown). Thus, the NG2 proteoglycan is sufficient to inhibit neurite outgrowth when it is expressed on the cell surface.

DISCUSSION

We used membrane stripe and membrane carpet assays to show that OPCs can inhibit axon growth and repel growing axons *in vitro*. These inhibitory and repulsive properties are dependent upon the presence of

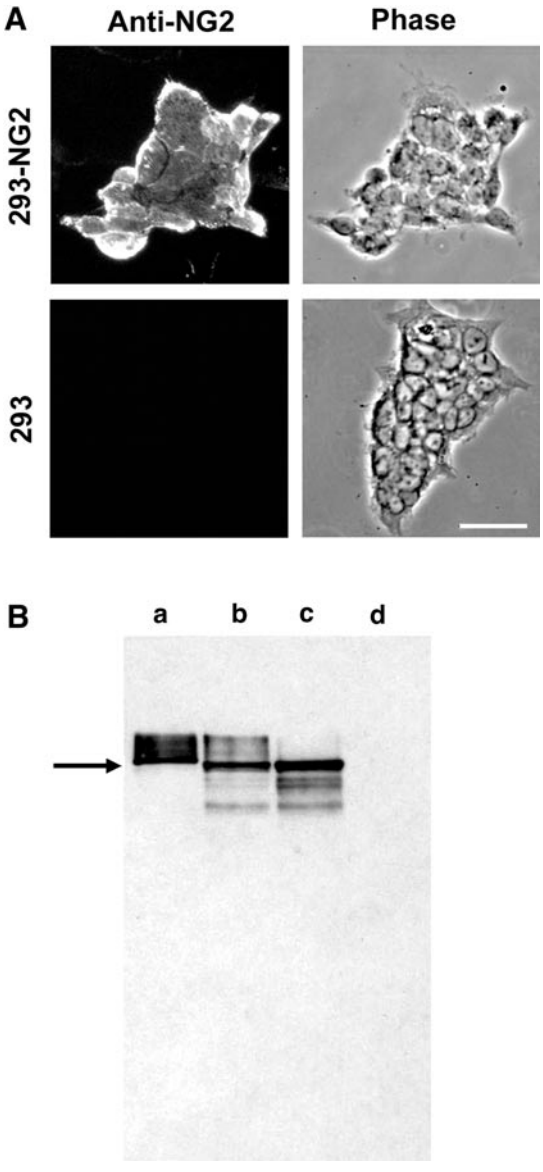

FIG. 6. Characterization of the NG2-expressing 293 cells (293-NG2 cells). (A) Living 293 cells were immunofluorescently stained with a rabbit anti-NG2 antibody. 293-NG2 cells express NG2 on their surface and untransfected 293 cells do not stain. Bar, 50 μm. (B) Anti-NG2 immuno-blot. Cell lysates were separated on a 6% SDS–PAGE and probed with rabbit anti-NG2. Lane a, B49 cell lysates; lane b, 293-NG2 cell membrane lysates; lane c, 293-NG2 cell membrane lysates digested with ChABC for 1 h at 37°C; lane d, untransfected 293 cell membrane lysates. The arrow points to the core protein of NG2 (290 kDa). The amount of protein loaded in each lane: a, 15 μg; b, 5 μg; c, 5 μg; d, 10 μg.

NG2 on the cell surface. Furthermore, the expression of NG2 is sufficient to change a growth permissive surface into one which is non-permissive for axon elongation. These data suggest that OPCs, which are abundant at sites of injury within the CNS, may be a major impediment to successful axon regeneration.

Oligosphere Cells Are a Model for Reactive Glia

Oligodendrocyte precursor cells are a newly recognized cellular component of the adult CNS whose functions in the normal brain are, for the most part, unknown (Nishiyama *et al.*, 1999; Dawson *et al.*, 2000; Levine *et al.*, 2001). After several types of experimentally induced injuries, there is an activation of OPCs (Levine, 1994; Nishiyama *et al.*, 1997; Kierstead *et al.*, 1998; Levine *et al.*, 1998; Ong and Levine, 1999; Levine and Reynolds, 1999; McTique *et al.*, 2001). This activation is rapid in its onset, confined spatially to the damaged area, and is usually accompanied by robust cell division and a highly reproducible sequence of morphological changes. The multipolar OPCs initially loose their processes, the cell bodies enlarge, and, as cell division ensues, many unipolar and bipolar cells appear. There is also increased cell surface immunostaining for NG2 although this increase has not been quantitated. Thus, when it first forms, the glial scar contains a population of rapidly dividing, immature appearing OPCs.

The diverse species of chondroitin sulfate proteoglycans associated with glial scars are thought to inhibit axon regeneration and reactive OPCs may be an important source of these growth-inhibitory molecules (Levine, 1994; Fawcett and Asher, 1999; Fidler *et al.*, 1999; Levine *et al.*, 2001). The goals of this study were to determine whether the OPC cell surface inhibits and repels growing axons and to assess the specific role of NG2 in these processes. We used the "oligosphere" method to generate the large number of cells needed (Avellana-Adalid *et al.*, 1996). In this method, cells are grown in suspension which allows for extensive cell proliferation without differentiation. Cells prepared in this way express marker antigens characteristic of immature OPCs (NG2 and A2B5), but very few of the cells expressed either 04, GFAP, or myelin basic protein antigens. Based on our characterization of rat oligosphere cells, we conclude that this method produces cells that correspond closely to the immature OPCs that accumulate at sites of injury.

OPCs Are Both Repulsive and Nonpermissive for Axon Growth

The membrane stripe assay shows that OPC membranes are repulsive to growth cones and that axons

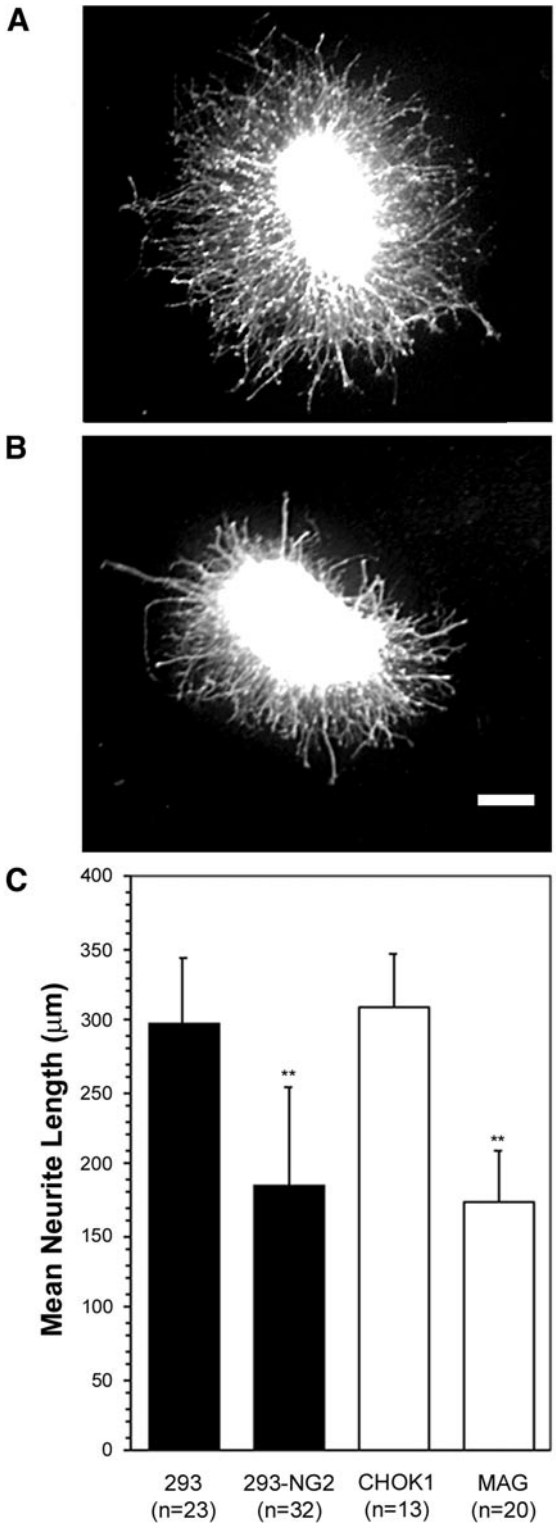

FIG. 7. 293-NG2 cell membranes are inhibitory for neurite outgrowth. Cerebellar explants from P5–7 rats were cultured for 40–44 h on membrane carpets prepared from (A) untransfected 293 cells or (B) NG2 expressing 293 cells. Bar, 200 μm. Neurite outgrowth is more robust on membrane carpet made from untransfected 293 cells. (C) Quantitation of neurite outgrowth on membrane carpet substrates. Mean neurite length was determined as described under Methods. Neurite growth on membrane carpets prepared from 293-NG2 cells is reduced 35% as compared to untransfected 293 cells. A similar degree of inhibition was observed when membrane carpets were prepared from MAG-expressing CHO cells. Data shown is the mean and standard deviations of the mean and n equals the total number of the explant measured. $^{**}P < 0.001$, Student's *t* test.

prefer to grow on areas of the coverslips that do not contain the OPC membranes. Growth cones avoid MAG-expressing membranes to the same extent but grow readily over membranes prepared from untransfected CHO cells. This is the case for neurites emerging from small explants of neonatal cerebellum or from purified granule neurons. In the explant cultures, migrating glial cells also tend to avoid the OPC membrane stripes (data not shown). The use of glial free cultures, however, rules out the possibility that the avoidance of the OPC membrane by growing neurons is due to a preference for growing on migrating glia. The predominant response of single cells was to grow along the border between the membranes and the PLL-coated substrate. This response is similar to the turning behavior of chick DRG neurons when encountering a border between fibronectin and substrate-bound CSPGs (Challacombe *et al.,* 1996) and to the behavior of "primed" sensory neurons whose axons often grow around the glial scar in vivo (Neumann and Woolf, 1999).

The requirement for NG2 in the OPC cell membrane was established by using neutralizing antibodies against NG2. Polyclonal antibodies and Fab fragments reverse the repulsive activity of NG2 and both axon bundles and single cells grow across the OPC membranes after treatment with the antibodies. Antibody treatment also promoted the adhesion of granule cells to the membrane stripes. Thus, when access to NG2 is blocked, OPC membranes are no longer repulsive and non-adhesive. The extent of this reversal was approximately 63% but using greater amounts of antibody could increase this to as much as 85%. Any residual growth inhibitory activity that was not blocked by the anti-NG2 antibodies may be due to either phosphacan or versican, both of which are expressed by OPCs and both of which can inhibit neurite outgrowth (Milev *et al.,* 1994; Canoll *et al.,* 1996; Asher *et al.,* 1999; Schmalfeldt *et al.,* 2000). Although OPCs express neurocan, another growth-inhibitory CSPG, it is secreted by the cells and only small amounts are associated with the membranes used here (Asher *et al.,* 2000). These same

anti-NG2 antibodies had no effect on the repulsive activity of MAG, ruling out the possibility that the antibodies were promoting growth independently of antigen binding. The Fab fragments were slightly less effective, reflecting perhaps some denaturation of the antibodies during the proteolytic cleavage and purification. The use of Fab fragments also rules out any potential masking of additional membrane components by the polyclonal antibodies.

Together, these observations suggest that NG2 is responsible for most of the growth-inhibitory properties of immature OPCs. This conclusion is in agreement with the work of Fidler *et al.* (1999), who suggested that NG2 is responsible for most of the growth inhibitory properties of Neu7 cell line, an astrocytic line whose surface does not support axon growth from neonatal DRG neurons. Previously, we showed that immunoaffinity purified NG2 and recombinant fragments of NG2 are inhibitory to axon growth (Dou and Levine, 1994; Ughrin *et al.*, 1999). Using the carpet assay, we have now demonstrated that NG2 in a membrane environment is non-permissive for neurite extension and that the expression of NG2 can convert a growth-permissive substrate into a nonpermissive one. Membranes prepared from oligosphere cells were also inhibitory to growth and, as was the case with the stripe assay, treatment of the membrane carpets with anti-NG2 antibodies reversed this inhibition (data not shown). Assays using cell membranes as substrates reflect accurately the growth modulatory properties of the cells or tissue under study (Walter *et al.*, 1987; Drescher *et al.*, 1995; Nakamoto *et al.*, 1996; Wang and Anderson, 1997; Shen *et al.*, 1998). Therefore, from these results using the membrane stripe and carpet assays, we conclude that the surface of OPCs is non-permissive for neurite growth and NG2 is both necessary and sufficient for this inhibition.

Contribution of GAG Chains to OPC-Mediated Inhibition

In the stripe assay, removal of the chondroitin sulfate GAG chains had no effect on the ability of membranes to repulse axons. This result was not unexpected since we had shown previously that the chondroitin sulfate GAG chains are not necessary for inhibition by immunoaffinity purified NG2 (Dou and Levine, 1994). Two regions of the extracellular portion of the NG2 core protein, the N-terminal domain 1 and juxtamembrane region (domain 3), are each sufficient to inhibit neurite growth in vitro and to induce growth cone collapse and these regions do not contain consensus sequences for GAG chain attachment (Nishiyama *et al.*, 1991; Ughrin *et al.*, 1999). OPCs express predominantly chondroitin 4-sulfate chains which have little or no effect on neurite extension stimulated by cell adhesion molecules of the Ig super-family such as L1 unless used at high concentrations (Gallo and Bertolotto, 1990; Dou and Levine, 1995). Thus, the GAGs carried on NG2 may make only a minor contribution to the repulsive properties of OPCs. There is, however, ample evidence in the literature that chondroitin sulfate GAG chains can either inhibit or stimulate neurite growth (Verna *et al.*, 1989; Snow *et al.*, 1990; Dou and Levine, 1995; Clement *et al.*, 1999). These effects vary according to the structure of the GAG chain, the type of test neuron used, and the presence or absence of other growth-promoting molecules (Dou and Levine, 1995; Garwood *et al.*, 1999; Hynds and Snow, 2001). More important, the treatment of reactive glial cells in vitro or the lesioned nigrostriatal pathway in vivo with chondroitinase ABC can allow limited axon regrowth (McKeon *et al.*, 1995; Moon *et al.*, 2001), suggesting that GAGs may inhibit regeneration in vivo. We think these apparent contradictory results do not indicate that NG2 is an insignificant factor in the inhibition of axon regeneration at glial scars for the following 2 reasons. First, NG2 is one of several inhibitory CSPGs identified in scar tissue (Bovolenta *et al.*, 1993; McKeon *et al.*, 1995; Asher *et al.*, 2000). NG2 rapidly accumulates at injury sites but begins to decline within 2–3 weeks (Levine, 1994). Therefore, in experiments where filters were implanted into the CNS for 30 days and then removed and digested with chondroitinases and shown to be growth permissive, the GAG lyases would probably not have as significant an effect on NG2 as on other inhibitory proteoglycans that appear at later stages during the wound healing process (Bovalenta *et al.*, 1993; McKeon *et al.*, 1995). Second, the mechanisms by which chondroitinase ABC promotes regrowth are not understood. One of the major functions of proteoglycans and of their GAG chains is to organize the extracellular matrix that coats the surfaces of cells. After injection into the brain parenchyma, chondroitinase enzymes degrade both GAG chains and the extracellular matrix (Bruckner *et al.*, 1998; Moon *et al.*, 2001). This enzymatic degradation of the extracellular matrix almost certainly changes the environment through which axons must grow. Whether such treatments affect the high levels of NG2 associated with the glial scar is unknown. It is also difficult to predict how alterations in the extracellular environment affect the physiology of neurons and their ability to regenerate. Thus, while chondroitin sulfate GAG chains may contribute to the inhibitory properties of the ma-

ture glial scar, that they contribute to the inhibitory properties of OPCs and of NG2 seems unlikely.

Glial Scars, Proteoglycans, and Axon Regeneration

A major conclusion from this study is that the OPCs that accumulate at newly forming glial scars can be inhibitory to axon regeneration. Much of this inhibition is due to the expression of NG2. Oligodendrocyte precursor cells produce neurocan and phosphacan, two other inhibitory CSPGs, and, as these CSPGs also accumulate at sites of CNS injury, we cannot rule out a role for these and perhaps other molecules as growth inhibitors *in vivo* (McKeon *et al.*, 1999; Asher *et al.*, 2000; Plant *et al.*, 2001).

Much attention has been focused on the myelin-associated inhibitors of regeneration MAG and nogo, although their roles *in vivo* are not well understood (Bandtlow and Schwab, 2000; Qiu *et al.*, 2000; Fournier and Strittmatter, 2001). Blocking nogo with antibodies promotes the regeneration of descending corticospinal axons but has no effects on the regeneration of ascending sensory axons (Schnell and Schwab, 1990; Oudega *et al.*, 2000). Results from the analysis of different strains of mice lacking MAG show only a minor enhancement of CNS regeneration (Bartsch *et al.*, 1995). After some types of injury, myelin components are cleared rapidly from the injury site, so that at 7 days after the initial damage, OPCs, along with microglia, may be the predominant cell types found within the scar (Levine, 1994; Stichel *et al.*, 1995). Thus, as axons begin to regenerate into the damaged area, they would encounter a barrier of OPCs. Meningeal fibroblasts within glial scars may also act as barriers to regeneration, especially for sensory axons (Pasterkamp *et al.*, 2001). This barrier function could be mediated by both semaphorinIII and NG2 since meningeal cells also produce NG2 (Levine and Nishiyama, 1996; Pasterkamp *et al.*, 1999). As glial scars mature, hypertrophic astrocytes become the predominant cell type and their growth-inhibitory properties are well documented (Reier *et al.*, 1983; McKeon *et al.*, 1991; Bahr *et al.*, 1995). Together, these results indicate that the local conditions at the site of injury are likely critical for the success or failure of any attempts at axon regeneration and repair and that these conditions change as glial scars develop.

Given that at least four different cell types and numerous molecules can function as growth inhibitors at glial scars, what is necessary to overcome these barriers and allow nerves to regenerate? Experimental procedures such as depletion of glia, prior "conditioning" lesions of peripheral nerve, and administration of GAG lyases can promote nerve regeneration in animal models but may have limited applicability in clinical settings (Neumann and Woolf, 1999; Moon *et al.*, 2000, 2001). Early intervention appears critical for neurological recovery after spinal cord injury and large doses of methylprednisolone given soon after injury may reduce inflammatory responses (Bracken *et al.*, 1990, Constantini and Young, 1994). Inflammation triggers the release of cytokines, some of which increase the expression of inhibitory CSPGs (Fok-Seang *et al.*, 1998; Asher *et al.*, 2000). One area for additional investigation is the relationship between the inflammatory response and the expression of inhibitory molecules. Strategies that enhance growth promoting mechanisms within neurons may also be suitable for clinical application. Among these, manipulations of intracellular levels of cyclic nucleotides are perhaps most promising since raising intracellular cAMP levels allows neurons to overcome MAG-induced growth inhibition and partially restores growth on NG2-coated surfaces (Dou and Levine, 1997; Cai *et al.*, 1999). The success of such potential treatments rests on identifying the inhibitory molecules found at glial scars, understanding which neuronal populations respond to these cues, and determining the cellular mechanisms that underlie these responses.

EXPERIMENTAL METHODS

Tissue culture. Oligodendrocyte precursor cells were prepared by the shaking method as previously described (McCarthy *et al.*, 1980) and expanded as "oligospheres" (Avellana-Adalid *et al.*, 1996). Oligospheres were grown in Dulbecco's modified Eagle's medium (DMEM, Mediatech Inc.) supplemented with N2 mixture (Gibco) and 30% B104 conditioned medium (prepared according to Louis *et al.*, 1992) for 7–9 days before harvesting for membrane preparation. The HEK293 cell line was grown in DMEM with 10% fetal bovine serum (FBS, JRH Biosciences). CHO cells were grown in RPMI-1640 medium (Mediatech, Inc.) with 10% FBS. MAG expressing CHO cells (a gift from M. Filbin) were maintained in DMEM supplemented with 10% FBS and other additives as described (Mukhopadhyay *et al.*, 1994). Cerebellar explants were prepared by removing the cerebella from P5–P7 rats. After removing the meninges and blood vessels, the cerebella were chopped sagittally into 150-μm wide slices using a tissue chopper (The Mickel Laboratory Engineering Co. LTD). Those pieces were teased into smaller pieces using blunt forceps and small cresent-shaped pieces were manually selected for use as explant cultures. The ex-

plants were grown in DMEM with 10% FBS, 20 mM KCl, and 10 ng/ml basic fibroblast growth factor (bFGF, PeproTech Inc.).

Dissociated cerebellar granular neurons were prepared from P4 rat cerebella using discontinuous Percoll gradients as described previously (Hatten *et al.*, 1985). Neurons were seeded onto 15-mm-round coverslips (FGR Steinmetz) with cell membrane stripes at the density of 36,000 cells/coverslip in DMEM + 10% FBS, 20 mM KCl, and 10 ng/ml bFGF. Dissociated oligosphere cells were prepared by incubating oligospheres with trypsin-EDTA (Gibco) for 10 min at 37°C. Following trypsin digestion, the oligospheres were dissociated by passage through flame-narrowed Pasteur pipettes. The dissociated oligosphere cells were seeded on coverslips first coated with poly-l-lysine (PLL, 50 μg/ml), then coated with fibronectin (0.5 μg/cm^2, Roche) at the density of 10,000 cells per coverslip. The cultures were maintained in DMEM + N2 supplement + 30% B104 medium for 48 h prior to immunofluorescence staining. All chemical reagents were purchased from Sigma unless specified otherwise.

Immunofluorescent staining. The immunofluorescent staining methods were similar to those described previously (Levine and Stallcup, 1987). The following antibodies were used: rabbit anti-NG2 antibody (1:300, Levine and Card, 1987), monoclonal anti-MBP (1:25, Chemicon), monoclonal A2B5 antibody (1:500, DHSB), monoclonal OX42 antibody (1:200, Serotec), monoclonal anti-phosphacan antibody (1:100, Chemicon), monoclonal anti-neurocan (1:100, Chemicon), monoclonal anti-GFAP antibody (1:100, Roche), and monoclonal anti-O4 (1:5, supernatant, a gift from S. Pfeiffer). CY3-conjugated goat anti-mouse (Jackson Labs) and FITC-conjugated goat anti-rabbit (SBTA, Fisher) antibodies were used as secondary antibodies. Cultures were examined using a Zeiss Axioplan microscope equipped with fluorescence and Normarski optics.

Western blot analysis. Cell lysates were prepared by incubating cells in solutions of 1% NP-40, 0.15 M NaCl, 10 mM Tris, pH 8.0, and protease inhibitors. After 15 min on ice, the lysate was centrifuged at 14,000*g* for 10 min and the supernatant removed and kept. Protein concentration was determined by the Bradford assay (Bio-Rad). Proteins were separated by 6% acrylamide gel in reducing conditions and immunoblotted as described previously (Levine *et al.*, 1998) using ECL reagents (Amersham). Primary antibodies include a polyclonal anti-NG2 antibody (1:2000), monoclonal anti-NCAM antibody (5B8, 1:1000, DSHB), monoclonal anti-phosphacan antibody (1:500, Chemicon), and monoclonal anti-neurocan antibody (1:500, Chemicon).

Membrane stripe assay. Cells were washed with PBS twice and resuspended into 1.5 ml of homogenizing buffer (10 mM Tris, 1.5 mM $CaCl_2$, pH 7.5). A cocktail of protease inhibitors (Complete, Mini EDTA-free protease inhibitor Cocktail tablets, Roche) was added and cells were homogenized in a Dounce homogenizer. The homogenate was centrifuged in a Sorvall RC5C centrifuge at 1200 rpm for 5 min at 4°C to remove nuclei and unbroken cells. The supernatant was centrifuged again at 12,000 rpm for 30 min at 4°C to pellet the cell membranes. The pellet was resuspended in PBS and the protein concentration determined. Cell membranes were prepared fresh for each experiment and used within 4–6 h after preparation. Membrane stripes were prepared on PLL coated coverslips (50 μg/ml) using a silicon matrix as described by Bastmeyer and Stuermer (1993). Briefly, the matrix consists of parallel channels separated by bars. At one end, the parallel channels merge into an inlet channel. When the matrix was pressed against a coverslip, the bars are in contact with the coverslip and protect the coverslip from the cell membranes. Membranes (7–10 μl of cell membranes, 500 μg/ml in PBS) was injected through the inlet channel and spread through the entire channel system. The coverslip and matrix were centrifuged at 3000*g* for 15 min (Beckman GS-6 centrifuge) to adhere the membranes to the coverslip. Removal of the silicon matrix leaves a patterned substrate with cell membrane stripes alternating with PLL coated stripes. The striped substrates were washed and incubated in PBS prior to plating the explants. P5~P7 cerebellar explants were plated on the striped substrate and allowed to grow for 40~44 h. The growth patterns of neurites extending from the explants were monitored under phase contrast using a Zeiss Axiovert microscope. The neurite outgrowth patterns were analyzed based on their preference for the membrane stripes using the semiquantitative score system such as that used by Walter *et al.* (1987). A five-step scale was used with 0 representing no preference between membrane stripes and PLL coated stripes and 2 for maximal avoidance of the membrane stripes.

When dissociated cerebellar neurons were used in the membrane stripe assay, cerebellar neurons were allowed to grow for 40~44 h. The cultures were fixed with 10% formalin in PBS and observed under phase contrast using a Zeiss Axiovert microscope. Phase contrast images of the culture were taken using a CCD camera (Hamamatsu). The neurite responses to the border between PLL and membrane stripes were classified as turning or tracking, stopping and crossing.

Chondroitinase treatment and antibody treatment. Chondroitinase digestion was performed by incubating cell membranes with the protease-free chondroitinase ABC (ChABC, Roche) in 37°C for 1 h. Generally, 50 μg of membrane protein were incubated with 0.01 unit of ChABC. The reaction was stopped by adding 1 mM $CaCl_2$ to the reaction mixture and the entire mixture was used to make membrane stripes. For antibody treatment, cell membranes were incubated with antibodies in the indicated concentration on ice for 30 min and the mixture was used to make the membrane stripes. An IgG fraction was purified from a polyclonal anti-NG2 antiserum using a protein A column (Pierce). The Fab form of the antibody was prepared by digesting the IgG fraction with immobilized papain (Pierce) and removing the Fc and IgG forms by protein A chromatography.

Preparation of NG2 expressing 293 cells. The full-length NG2 cDNA (Nishiyama *et al.*, 1991) was removed from the pBluescript vector by partial digestion with *Xba*I and *Hin*dIII. The insert was gel purified and ligated into the pCDNA3 vector (Invitrogen) previously digested with *Xba*I and *Hin*dIII. The orientation of the NG2 cDNA in the plasmid was confirmed by restriction enzyme digestion and partial DNA sequencing. HEK293 cells were transfected with 0.5 μg of DNA per 10-cm dish using the calcium phosphate precipitation method followed by a glycerol shock. Transfected cells were selected in medium containing G418 (800 μg/ml, Gibco). Colonies were picked, expanded, and tested for expression of NG2 by immunoblotting and immunofluorescence staining.

Membrane carpet assay. Cell membranes, prepared as described above, were made into "carpets" using a nylon matrix as described by Walter *et al.* (1987). Cerebellar explants were placed on the uniform carpet in DMEM with 10% FBS, 20 mM KCl, and 10 ng/ml bFGF. After 40~44 h, the explants and their growing processes were visualized using 5-carboxyfluorescein diacetate as described previously (Tuttle *et al.*, 1995). The neurite growth was measured from digital images. The distance from the edge of the body of the explant to the tip of the longest process was measured at eight positions around the explant, separated by 45° of arc using Metamorph imaging software (Universal Imaging Corp.). The average of those eight measurements was calculated as mean neurite outgrowth length for each explant.

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