Neuron, Vol. 15, 1375–1381, December, 1995, Copyright © 1995 by Cell Press

Lack of Evidence That Myelin-Associated Glycoprotein Is a Major Inhibitor of Axonal Regeneration in the CNS

Udo Bartsch,*† Christine E. Bandtlow,‡† Lisa Schnell,‡ Susanne Bartsch,* Adrian A. Spillmann,‡ Beatrix P. Rubin,‡ Rainer Hillenbrand,* Dirk Montag,* Martin E. Schwab,‡ and Melitta Schachner*

*Department of Neurobiology
Swiss Federal Institut of Technology
Hönggerberg
CH-8093 Zürich
Switzerland

‡Brain Research Institute
University of Zürich
August Forelstrasse 1
CH-8092 Zürich
Switzerland

Summary

The MAG-deficient mouse was used to test whether MAG acts as a significant inhibitor of axonal regeneration in the adult mammalian CNS, as suggested by cell culture experiments. Cell spreading, neurite elongation, or growth cone collapse of different cell types in vitro was not significantly different when myelin preparations or optic nerve cryosections from either MAG-deficient or wild-type mice were used as a substrate. More importantly, the extent of axonal regrowth in lesioned optic nerve and corticospinal tract in vivo was similarly poor in MAG-deficient and wild-type mice. However, axonal regrowth increased significantly and to a similar extent in both genotypes after application of the IN-1 antibody directed against the neurite growth inhibitors NI-35 and NI-250. These observations do not support the view that MAG is a significant inhibitor of axonal regeneration in the adult CNS.

Introduction

Injured axons in the adult mammalian CNS normally fail to regenerate over long distances. Regeneration of CNS neurons can be achieved, however, by providing a microenvironment that is permissive for regeneration, such as a PNS explant (reviewed by Aguayo, 1985). Poor axonal regeneration in the differentiated CNS has been related to the low abundancy of neurotrophic factors or neurite growth–permissive molecules and to the presence of molecules that are inhibitory for axonal growth (for reviews, see Schwab et al., 1993; Schachner et al., 1994).

In the developing nervous system, inhibitory molecules are likely to be involved in the guidance of axons to their appropriate targets (Luo et al., 1993; Taylor et al., 1993; Colamarino and Tessier-Lavigne, 1995; Messersmith et al., 1995; Püschel et al., 1995). In the adult mammalian CNS, neurite growth–inhibitory activity is believed to be mainly, but not exclusively, associated with oligodendrocytes and myelin (for reviews, see Schwab et al., 1993; Keynes and Cook, 1995). In support of this view, axonal regeneration has been observed in fiber tracts in which myelination has been delayed experimentally (Savio and Schwab, 1990; Keirstead et al., 1992; Weibel et al., 1994). Among the molecules hypothesized to contribute to the inhibitory activity of CNS glial cells and CNS myelin are constituents of the extracellular matrix, such as tenascin-C, tenascin-R, and keratan, and chondroitin sulfate proteoglycans (for reviews, see Schwab et al., 1993; Schachner et al., 1994). Two antigenically related neurite growth inhibitors with 35 and 250 kDa molecular weight (NI-35 and NI-250) have been identified in mammalian CNS myelin (Caroni and Schwab, 1988a). The monoclonal antibody (MAb) IN-1 raised against these neurite growth inhibitors neutralizes part of the inhibitory effect of differentiated oligodendrocytes and CNS white matter in vitro and in vivo (Caroni and Schwab, 1988b; Schwab et al., 1993).

Recently, in vitro experiments suggested that myelin-associated glycoprotein (MAG), a well-characterized member of the immunoglobulin gene superfamily represents another and possibly major inhibitor of axonal regeneration (McKerracher et al., 1994; Mukhopadhyay et al., 1994). MAG is exclusively expressed by oligodendrocytes and Schwann cells and is involved in the formation and maintenance of myelin (see Bartsch et al., 1995, for references). The additional role of MAG as an inhibitor of axonal regeneration is indicated by the observation that in vitro MAG exerts a robust inhibitory effect on neurite outgrowth from young cerebellar neurons, adult dorsal root ganglion (DRG) neurons (Mukhopadhyay et al., 1994), and NG108-15 neuroblastoma cells (McKerracher et al., 1994). In the present study, we took advantage of the availability of a MAG-deficient ($MAG^{-/-}$) mouse (Montag et al., 1994) to test in vitro and in vivo the hypothesis that MAG restricts axonal regeneration in the adult CNS.

Results

Behavior of NG108-15, PC12, and 3T3 Cells on Purified MAG and Myelin Extracts

Over 80% of NG108-15 cells plated on poly-L-lysine, bovine serum albumin, liver plasma membrane proteins (10 μg/well), or tissue culture plastic extended long neurites after 16 hr. In contrast, 38% (± 8%) of the cells plated on proteins extracted from CNS myelin of $MAG^{+/+}$ mice (10 μg/well) extended processes longer than the diameter of the cell body, and 50% (± 13%) of the cells were inhibited on immunopurified MAG from mouse brain (8 μg/well), in agreement with McKerracher et al. (1994). Interestingly, however, 41% (± 7%) of the NG108-15 cells plated on myelin preparations from $MAG^{-/-}$ mice (10 μg/well) were still inhibited, indicating the presence of other inhibitory proteins.

We therefore tested different cell types for their responsiveness to MAG as an inhibitory substrate molecule. Nei-

†These authors contributed equally to this work.

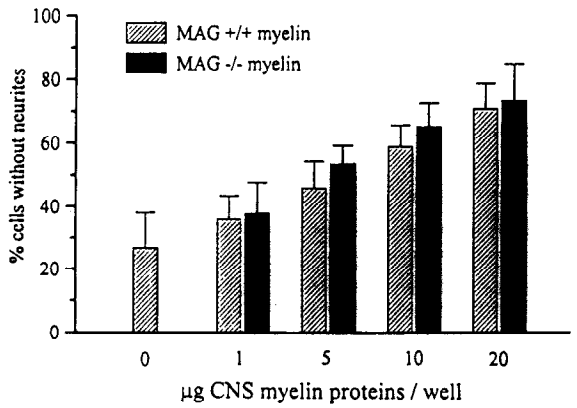

Figure 1. Concentration-Dependent Inhibition of Neurite Outgrowth from PC12 Cells by CNS Myelin Proteins of $MAG^{+/+}$ and $MAG^{-/-}$ Mice

Nerve growth factor–primed PC12 cells were plated at equal numbers on plastic culture dishes coated with increasing concentrations of CNS myelin proteins of $MAG^{+/+}$ or $MAG^{-/-}$ animals. The percentage of process-bearing PC12 cells was determined after 24 hr; 200–300 cells were counted per well. Values represent the mean ± SEM of 3–6 wells.

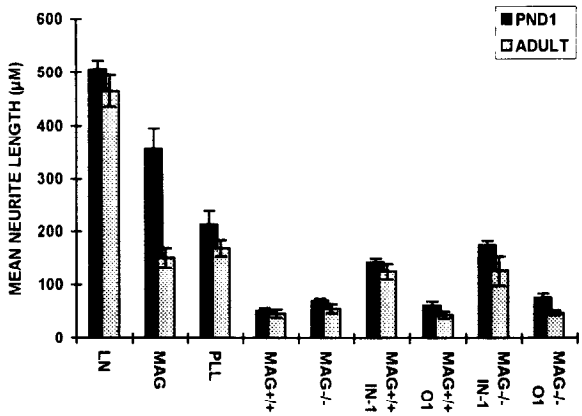

Figure 2. Quantitative Analysis of Neurite Outgrowth from PND1 and Adult DRG Neurons on Various Substrates

Neurite extension of PND1 and adult DRG neurons was determined on the following substrates: laminin (LN), 8 μg/cm² purified MAG, poly-L-lysine (PLL), and 5 μg/cm² myelin proteins from $MAG^{+/+}$ or $MAG^{-/-}$ mice in the presence or absence of MAb IN-1 or the control antibody O1, respectively. Three sets of experiments were performed in duplicates. Error bars represent SEM.

ther 3T3 fibroblasts nor PC12 cells showed any impairment in their spreading or neurite formation when plated on highly purified MAG, even at concentrations of up to 10 μg of protein per well (Rubin et al., 1995). However, concentration-dependent inhibition of cell spreading or neurite growth was observed on myelin proteins from $MAG^{+/+}$ mice with a half-maximal inhibition at ~5 μg of protein per well for fibroblast spreading (data not shown) and ~6 μg per well for PC12 cell process formation (Figure 1). Importantly, a similar concentration-dependent inhibition was observed when myelin proteins from $MAG^{-/-}$ mice were used as a substrate with half-maximal inhibition at 4.8 and 6.5 μg of protein per well for fibroblast spreading (data not shown) and PC12 cell process formation (Figure 1), respectively. Treatment of myelin proteins with MAb IN-1, but not with the control MAb O1, reduced the inhibitory properties of wild-type and MAG-deficient myelin significantly and to a similar extent (data not shown).

Neurite Outgrowth from DRG Neurons of Different Ages on Myelin Proteins

After 16 hr in culture, neurites of DRG neurons prepared from 1-day-old (PND1) animals were about twice as long on MAG as those cultured on poly-L-lysine (Figure 2). In contrast, neurite elongation from adult DRG neurons on MAG was similarly poor as on poly-L-lysine and reduced by ~56% when compared with laminin (Figure 2). Having confirmed the age-dependent responsiveness of these neurons for MAG (Johnson et al., 1989; Schneider-Schaulies et al., 1991; Mukhopadhyay et al., 1994), we investigated neurite outgrowth from DRG neurons on myelin proteins extracted from the CNS of $MAG^{+/+}$ and $MAG^{-/-}$ mice. Neurite outgrowth from adult DRG neurons was strongly reduced to a similar extent on both types of myelin extracts (Figure 2). Interestingly, reduction of neurite outgrowth was now also observed for PND1 DRG neurons (Figure 2). Again, neurite growth was inhibited to a similar extent on both types of myelin extracts (Figure 2). Inhibition of neurite growth by CNS myelin proteins (from $MAG^{+/+}$ and $MAG^{-/-}$ mice) was concentration dependent with half-maximal inhibition observed at ~4 μg of protein per well for PND1 DRG neurons and ~3 μg per well for adult DRG neurons. Outgrowth inhibition for PND1 and adult DRG neurons, however, could partially be reversed in the presence of MAb IN-1 (Figure 2). Mab O1 did not neutralize the inhibition of neurite outgrowth by CNS myelin proteins (Figure 2).

Growth Cone Collapse

Addition of inhibitory material prepared by CHAPS solubilization of bovine myelin caused growth cone collapse and retraction of PND1 DRG neurites within minutes (Figure 3) (Bandtlow et al., 1993). A similar responsiveness was observed for adult DRGs, with 78% of the analyzed growth cones collapsing within 20 min upon application of 8 μg of bovine material (Figure 3). No such morphological changes were evoked in PND1 and adult DRG neurons by immunopurified MAG, even at concentrations of 5 μg of protein (Figure 3). In contrast, CHAPS-solubilized myelin proteins from $MAG^{+/+}$ and $MAG^{-/-}$ mouse spinal cord contained potent growth cone–collapsing activities. Interestingly, both sources had similar quantities of growth cone–collapsing activity, with 7 μg of protein causing collapse and retraction of DRG neurites of both developmental ages (Figure 3). The collapsing activities present in $MAG^{+/+}$ and $MAG^{-/-}$ myelin proteins could be neutralized by about 50% with the IN-1 antibody (Figure 3).

Neurite Outgrowth from Cerebellar Neurons on Optic Nerve Cryosections

Strong inhibition of neurite elongation by MAG has been reported for young cerebellar neurons (Mukhopadhyay et al., 1994). To confront these cells with $MAG^{+/+}$ or $MAG^{-/-}$

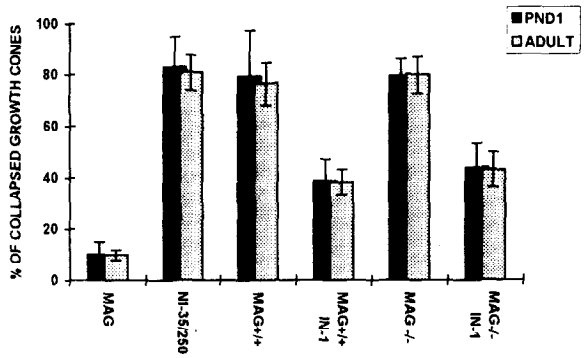

Figure 3. Quantitative Analysis of Growth Cone Collapse Response of PND1 and Adult DRG Neurons

Growth cone collapse of PND1 and adult DRG neurons was analyzed in response to addition of 5 μg of purified MAG, 8 μg of semipurified bovine NI-35/NI-250, or 7 μg of CHAPS-solubilized myelin proteins from $MAG^{+/+}$ or $MAG^{-/-}$ mice in the presence or absence of the neutralizing IN-1 antibody. At least ten individual growth cones were monitored for 60 min for each substance tested.

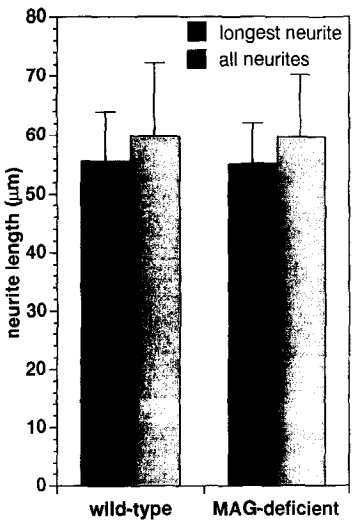

Figure 4. Quantitative Analysis of Neurite Outgrowth from Cerebellar Neurons on Cryosections from Optic Nerves of Adult $MAG^{+/+}$ and $MAG^{-/-}$ mice

Cerebellar neurons were cultured for 48 hr on cryosections and stained with anti-L1 antibodies. In total, 579 and 668 neurons were analyzed on cryosections from $MAG^{+/+}$ and $MAG^{-/-}$ mice, respectively. Values show the mean length (± SD) of the longest neurite (black bars) and of all neurites per cell (gray bars) measured in four independent experiments.

myelin, cerebellar neurons were cultured on cryosections from heavily myelinated optic nerves of these mice. No significant differences in the density of adherent cells were apparent between substrates. More importantly, quantitative analysis of neurite length revealed that the mean length of the longest neurite per cell was not significantly different between neurons cultured on cryosections from $MAG^{+/+}$ (55.5 ± 8.4 μm, mean ± SD; n = 579 neurons) and $MAG^{-/-}$ (55.1 ± 7.1 μm; n = 668 neurons; Figure 4) mice. Similarly, the average length of all neurites per cell (59.7 ± 12.5 μm for $MAG^{+/+}$ mice and 59.7 ± 10.3 μm for $MAG^{-/-}$ mice; Figure 4) and the number of neurites per cell (data not shown) were not significantly different between substrates.

Axonal Regrowth in Injured Optic Nerve and Corticospinal Tract

To analyze whether MAG restricts axonal regeneration in vivo, regrowth of lesioned retinal ganglion cell (RGC) axons and corticospinal tract (CST) axons was compared between $MAG^{+/+}$ and $MAG^{-/-}$ mice.

Anterograde axonal tracing was used to monitor regrowth of RGC axons 2 weeks after an intraorbital optic nerve crush. When straight-running axons were observed at the chiasmal end of the nerve, the crush was considered as incomplete, and animals were excluded from the analysis. In contrast, regrowing RGC axons followed an irregular course, and occasionally a growth cone–like structure was observed at their tips (data not shown). In $MAG^{+/+}$ mice, the number of axons regrowing across the lesion site was low, and axons extended only for short distances into the distal stump. Similar results were obtained for $MAG^{-/-}$ mice. Quantitative analysis confirmed that regrowth of RGC axons was not improved in $MAG^{-/-}$ animals when compared with age-matched $MAG^{+/+}$ mice. The mean length of regrown axons in $MAG^{+/+}$ animals was 250 μm (n = 23) compared with 210 μm (n = 15) in $MAG^{-/-}$ mice (Figure 5a). However, the number and length of regrowing RGC axons increased considerably and to a similar extent in $MAG^{+/+}$ and $MAG^{-/-}$ mice after application of MAb IN-1. In $MAG^{+/+}$ animals, the mean axon length in distal stumps was 780 μm (n = 12), with a maximal length of 1200 μm, while in $MAG^{-/-}$ mice the mean axon length was 950 μm (n = 5), with a maximal length of 1650 μm (Figure 5a).

Regrowth of CST axons was assessed in mice lesioned at 5–7 weeks of age. Following laminectomy of a single vertebra at lower thoracic levels, the dorsal half of the spinal cord was transected bilaterally, thus interrupting both corticospinal tracts, which run in the dorsal funiculus in the mouse, as well as the very minor dorsolateral corticospinal axon population. In about 50% of the mice, the enlargement of the lesion by secondary tissue damage and scarring had created a situation that did not allow CST axons to reach the caudal aspects of the lesion area. In the remaining animals, regenerative sprouts of CST axons could be seen to grow through ventral and ventrolateral tissue bridges in $MAG^{+/+}$ (n = 10) and $MAG^{-/-}$ (n = 12) mice. In the absence of MAb IN-1, elongation of these fibers was usually restricted to <1 mm in both $MAG^{+/+}$ and $MAG^{-/-}$ mice (Figure 5b). In contrast, a majority of animals showed elongation of a small proportion of the CST axons over long distances in the presence of MAb IN-1 (Figure 5b). The maximal distances covered by regenerating fibers varied from animal to animal, and no effect of the absence of MAG could be observed.

Discussion

In the present study, we used the MAG-deficient mouse to analyze in vitro and in vivo whether MAG is a significant

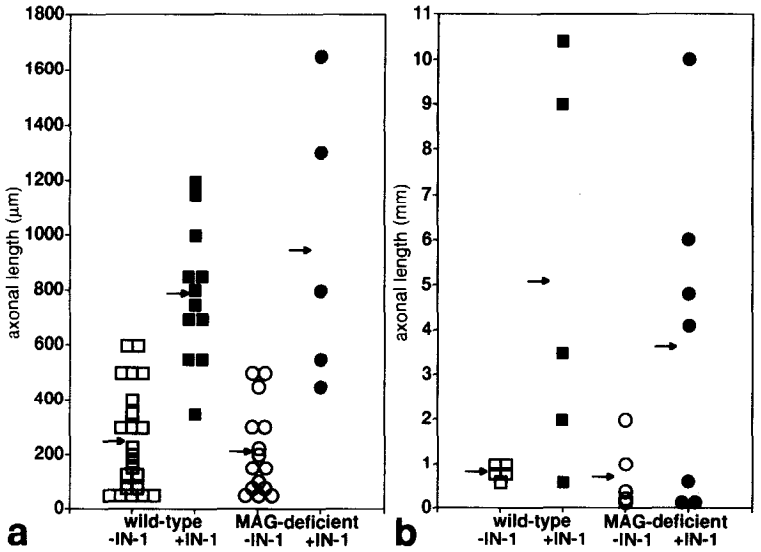

Figure 5. Analysis of Axonal Regrowth in MAG$^{+/+}$ and MAG$^{-/-}$ Mice In Vivo

The extent of regrowth of lesioned RGC (a) and CST (b) axons in MAG$^{+/+}$ (squares) and MAG$^{-/-}$ (circles) mice was determined in the absence (open symbols) and presence (closed symbols) of MAb IN-1. Each symbol represents the length of the longest regrown axon per animal, measured from the lesion site. Mean values are indicated by arrows.

inhibitor of axonal regeneration in the adult mammalian CNS.

The effect of MAG on neurite outgrowth from DRG neurons is of particular interest. Neurite elongation from young postnatal DRG neurons in vitro is significantly enhanced by MAG (Johnson et al., 1989; Schneider-Schaulies et al., 1991), whereas neurite outgrowth from adult DRG neurons cultured either on transfected chinese hamster ovary (CHO) cells expressing the 72 kDa isoform of MAG or on recombinant MAG is significantly reduced when compared with control substrates (Mukhopadhyay et al., 1994). We observed a similar age-dependent behavior of DRG neurons on purified MAG. Age-dependent differences in the extent of neurite elongation from DRG neurons on cryosections from peripheral nerves were believed to support the view that MAG is crucial in determining the extent of neurite growth. Extensive neurite outgrowth from embryonic DRG neurons but only poor outgrowth from adult DRG neurons was observed on cryosections from normal sciatic nerves (a MAG-positive substrate). In contrast, robust neurite outgrowth from adult DRG neurons has been observed on cryosections from predegenerated sciatic nerves (considered to be a MAG-negative substrate; Bedi et al., 1992). More recently, however, neurite outgrowth from DRG neurons of different ages has been analyzed on optic nerve cryosections from neonatal (a MAG-negative substrate; Johnson and Quarles, 1986) and adult (a MAG-positive substrate) rats (Shewan et al., 1995). On both kinds of cryosections, neurite outgrowth was extensive for embryonic DRG neurons, decreased with increasing age of the nerve cells, and was poor for adult DRG neurons (Shewan et al., 1995). These results do not support a close and causal relation between presence or absence of MAG and the ability of DRG neurons to extend neurites on complex substrates. Accordingly, we observed that myelin extracts from the CNS of MAG$^{-/-}$ mice reduced neurite elongation and induced growth cone collapse of adult DRG neurons to a similar extent as do myelin extracts from MAG$^{+/+}$ mice. Inhibitory activity of myelin extracts from both genotypes, however, could be partially neutralized to a similar extent by application of MAb IN-1. These results demonstrate that MAG does not interfere significantly with neurite elongation from adult DRG neurons as soon as physiologically more appropriate substrates are used.

Neurite outgrowth from NG108-15 neuroblastoma cells is reduced on recombinant MAG, whereas it is increased on bovine myelin preparations after immunodepletion of MAG (McKerracher et al., 1994). In agreement with these observations, we found reduced neurite outgrowth from these cells when purified MAG was used as a substrate. In contrast to the results obtained with myelin fractions immunodepleted of MAG, however, we did not observe a significant difference in neurite outgrowth when NG108-15 cells were cultured on myelin extracts from either MAG$^{-/-}$ or MAG$^{+/+}$ mice. Possibly, other molecules inhibiting neurite growth were removed or functionally destroyed during the immunodepletion procedure. Neurite outgrowth assays with the immunodepleted myelin extracts after addition of purified MAG or of the previously removed material might help to clarify this point. Alternatively, a major role of MAG as an inhibitor of axonal regeneration might not become apparent in the MAG$^{-/-}$ mouse, since other neurite growth–inhibitory molecules that compensate for the lack of MAG-related inhibitory activity could be up-regulated in the mutant. However, one has then to assume that the hypothetical up-regulation of one or more other inhibitory molecules compensates exactly for the lack of the inhibitory activity of MAG, since we found no differences in the inhibitory activity between CNS tissue of MAG$^{+/+}$ and MAG$^{-/-}$ mice in any of our experiments. Finally, we consider a significant up-regulation of NI-35 and NI-250 in the MAG mutant as unlikely, since IN-1 antibodies neutralize the inhibitory activity of MAG$^{+/+}$ and MAG$^{-/-}$ CNS to a similar extent in all our experiments.

Neurite outgrowth from cerebellar neurons is reduced by ~70% when MAG-transfected CHO cells are used as a substrate (Mukhopadhyay et al., 1994). Axons of these nerve cells are normally not in contact with myelin. To

confront cerebellar neurons with MAG-negative myelin in a functional context that resembles the environment in the intact organism, cells were cultured on heavily myelinated optic nerve cryosections from $MAG^{-/-}$ mice. On these substrates, an inhibitory activity of MAG was no longer apparent, again suggesting that MAG plays only a minor role in the control of neurite elongation on complex substrates. That the contribution of other molecules to the inhibitory activity of oligodendrocytes and CNS myelin is more relevant than that of MAG is also indicated by the observation that neurite outgrowth from PC12 cells and spreading of 3T3 fibroblasts is similar on myelin extracts from $MAG^{+/+}$ and $MAG^{-/-}$ mice but improved after application of MAb IN-1.

Regeneration experiments in the living animal are the crucial tests of whether a certain molecule plays a physiological role in determining the extent of axonal regeneration. We therefore decided to use the $MAG^{-/-}$ mouse to study axonal regeneration in two myelinated fiber tracts in vivo, the optic nerve and the CST. The length of the longest regrown axons distal to the lesion site was measured to monitor success of axonal regeneration. The extent of axonal regrowth in both fiber tracts was poor in the $MAG^{-/-}$ mutant and similar to that observed in $MAG^{+/+}$ mice. As a positive control, axonal regeneration was analyzed in the same fiber tracts after application of IN-1 antibodies. In antibody-treated animals, axonal regrowth was considerably improved in both fiber tracts, in agreement with previous studies in the rat (Schnell et al., 1994; Weibel et al., 1994). Importantly, axonal regrowth after application of MAb IN-1 increased to a similar extent in $MAG^{-/-}$ and $MAG^{+/+}$ mice. Thus, we found no evidence for a major role of MAG in restricting axonal regeneration in vivo.

In summary, our observations strongly argue against a major role of MAG as an inhibitor of axonal regeneration in the lesioned adult mammalian CNS. A comparison of inhibitory activity of MAG and neurite growth inhibitors NI-35 and NI-250 in vitro, for instance, demonstrates a strong effect of NI-35 and NI-250 on all substrates tested, while inhibitory effects of MAG become apparent only when the purified molecule is offered as a substrate. More importantly, we found no evidence that MAG prevents regeneration in vivo. The identification of additional oligodendrocyte- or astrocyte-derived neurite growth inhibitors thus remains a major challenge for future investigations.

Experimental Procedures

Animals

Generation of mice deficient for MAG and genotyping of animals have been described (Montag et al., 1994). Age-matched $MAG^{+/+}$ (C57BL/6 or $MAG^{-/-}$ littermates) animals were used as a control.

Cell Culture

Rat PC12 cells were a gift of Dr. Moses Chao (Cornell University, NY). Cells were grown in RPMI-1640 medium (GIBCO) with 10% horse serum and 5% fetal calf serum and induced to differentiate with 100 ng/ml nerve growth factor. Cells of the NG108-15 line were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum and 1% hypoxanthine-aminopterin-thymidine (GIBCO) and were induced to form processes by reducing the serum to 5% concurrent with the addition of 1 mM dibutyrylic cyclic adenosine monophosphate (Sigma). NIH 3T3 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. DRG neurons from PND1 and adult rats were prepared and cultured as described (Unsicker et al., 1985). Small cerebellar neurons were prepared from 6- to 7-day-old ICR mice (Keilhauer et al., 1985) and cultured in basal medium Eagle with 10% horse serum. Hybridoma cells secreting MAb IN-1 (Caroni and Schwab, 1988b) were cultured in Iscove's modified Eagle's medium containing 5% fetal calf serum.

Isolation and Purification of MAG

MAG was isolated from brain extracts of adult mice by immunoaffinity purification (Poltorak et al., 1987), except that the buffer for elution of MAG from the immunoaffinity column contained either 0.1% deoxycholate or 0.8% β-octylglycoside.

Myelin Preparations from $MAG^{+/+}$ and $MAG^{-/-}$ Mice

Spinal cords of 4-week-old $MAG^{+/+}$ or $MAG^{-/-}$ mice were used for the preparation of myelin (Caroni and Schwab, 1988a). Proteins were solubilized with a CHAPS-containing buffer (Rubin et al., 1995). Protein determinations were carried out using a protein assay kit (Bio-Rad) and bovine serum albumin (type IV) as a standard. Extracts of bovine spinal cord were prepared in a similar fashion.

Neurite Outgrowth Assay on Purified MAG or Myelin Preparations

Neurite outgrowth assays were performed in 4-well dishes (Greiner) coated overnight with various concentrations of extracts derived from myelin of $MAG^{+/+}$ or $MAG^{-/-}$ mice. Immunoaffinity-purified MAG was coated on precoated poly-L-lysine or on plastic tissue culture dishes. About 5000 cells in 80 μl of medium were plated per well, and assays were stopped by addition of formalin after 24 hr in culture. Cells bearing neurites longer than the diameter of the cell body were scored to determine the percentage of cells that had successfully initiated neurite outgrowth. To assess inhibitory activity that can be neutralized by MAb IN-1 (Caroni and Schwab, 1988b), substrate-coated wells were incubated with IN-1 hybridoma supernatant for 20 min at 37°C and briefly washed with Hank's balanced salt solution before cells were applied in the presence of IN-1 hybridoma supernatant (1:1, v:v). As a control antibody, O1 hybridoma supernatant was used (Sommer and Schachner, 1981).

3T3 Spreading Assay

Spreading of 3T3 cells was determined by counting the numbers of round and flat cells 60 min after plating (Caroni and Schwab, 1988a).

Growth Cone Collapse Assay

CHAPS-solubilized spinal cord myelin proteins of $MAG^{+/+}$ or $MAG^{-/-}$ mice and bovine were supplemented with phosphatidyl choline at a protein:lipid ratio of 1:10 and dialysed against PBS for 24–48 hr, followed by L15 (GIBCO) without additives for a minimum of 3 hr. Unincorporated proteins were separated by a sucrose gradient. Protein incorporation into lipid vesicles was calculated by determining the concentration of protein before dialysis and of unincorporated protein after sucrose gradient centrifugation. The DRG growth cone collapse assay was performed as described (Bandtlow et al., 1993).

Neurite Outgrowth Assay on Optic Nerve Cryosections

Optic nerves from $MAG^{+/+}$ and $MAG^{-/-}$ mice (at least 2 months old) were quickly removed and frozen in Hank's balanced salt solution. Longitudinal cryosections 16 μm in thickness were mounted onto poly-L-lysine-coated coverslips, and about 80,000 cerebellar neurons were plated onto each coverslip. After 2 days, cultures were fixed and stained with polyclonal antibodies to L1. The length of neurites longer than the diameter of the cell body and not in contact with other neurites or cell bodies was determined.

Optic Nerve and Spinal Cord Lesions

$MAG^{+/+}$ and $MAG^{-/-}$ mice (all at least 7 weeks old) were deeply anesthetized, and optic nerves were crushed intraorbitally (Bartsch et al., 1992). In some animals, 1–2 μl of vitreous fluid was removed at the time of lesioning, and about the same volume of a dense cell suspension of IN-1 hybridoma cells was injected. An axonal tracer (biotin-N-hydroxysuccinimidester [Sigma]; Halfter, 1987) was injected into the vitreous fluid 2 weeks later. After an additional 30 hr, optic nerves were fixed

in 4% paraformaldehyde, and the tracer was visualized in longitudinal cryosections using fluorescein isothiocyanate–conjugated avidin (Vector). For each animal, the longest regrown axon in the distal stump of the optic nerve (measured from the lesion site) was determined at a magnification of 200×.

MAG$^{-/-}$ or MAG$^{+/+}$ mice at ages of 5–7 weeks were anesthetized, and a partial laminectomy was performed at thoracic level 7. The spinal cord was lesioned with fine iridectomy scissors by transecting both dorsal and dorsolateral funiculi under visual control. IN-1 hybridoma cells were injected as a dense cell suspension (5 μl; 10^7 cells) into the left parietal cortex of some animals at the time of the spinal cord lesion (Schnell and Schwab, 1990). Following postoperative survival times of 2–3 weeks, animals were anesthetized and injected with wheat germ agglutinin–horseradish peroxidase (5% in water; Sigma) into the right motor cortex (total of 0.75 μl in 3–4 injection sites). After 24 hr, animals were perfused with 1.25% glutaraldehyde and 1% formaldehyde; the thoracic and lumbar parts of the spinal cord were dissected; 50 μm thick frozen sections were cut in the sagittal plane, mounted as a continuous section series on slides, and reacted for horseradish peroxidase activity with tetramethyl benzidine as a substrate. The maximal elongation distances of CST axons were measured in millimeters from the site of CST transection on number-coded section series.

Acknowledgments

The authors are grateful to Dr. N. Mantei for critically reading the manuscript and to E. Gui-Xia Yu and B. Niederöst for excellent technical assistance. The study was supported by a grant of the Swiss Federal Institute of Technology (to U. B. and D. M.). The work of C. E. B., L. S., A. A. S., B. P. R., and M. E. S. was supported by grants of the Swiss National Science Foundation (number 31-29981.90 to M. E. S. and number 31-42299.94 to C. E. B.) and an anonymous donation (Union Bank of Switzerland).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *"advertisement"* in accordance with 18 USC Section 1734 solely to indicate this fact.

Received July 14, 1995; revised September 5, 1995.

References

Aguayo, A.J. (1985). Axonal regeneration from injured neurons in the adult mammalian central nervous system. In Synaptic Plasticity, W. C. Cotman, ed. (New York: Guilford Press), pp. 457–484.

Bandtlow, C.E., Schmidt, M.F., Hassinger, T.D., Schwab, M.E., and Kater, S.B. (1993). Role of intracellular calcium in NI-35-evoked collapse of neuronal growth cones. Science *259*, 80–83.

Bartsch, U., Bartsch, S., Dörries, U., and Schachner, M. (1992). Immunohistological localization of tenascin in the developing and lesioned adult mouse optic nerve. Eur. J. Neurosci. *4*, 338–352.

Bartsch, U., Montag, D., Bartsch, S., and Schachner, M. (1995). Multiply myelinated axons in the optic nerve of mice deficient for the myelin-associated glycoprotein. Glia *14*, 115–122.

Bedi, K.S., Winter, J., Berry, M., and Cohen, J. (1992). Adult rat dorsal root ganglion neurons extend neurites on predegenerated but not on normal peripheral nerves *in vitro*. Eur. J. Neurosci. *4*, 193–200.

Caroni, P., and Schwab, M.E. (1988a). Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. J. Cell Biol. *106*, 1281–1288.

Caroni, P., and Schwab, M.E. (1988b). Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. Neuron *1*, 85–96.

Colamarino, S.A., and Tessier-Lavigne, M. (1995). The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons. Cell *81*, 621–629.

Halfter, W. (1987). Anterograde tracing of retinal axons in the avian embryo with low molecular weight derivatives of biotin. Dev. Biol. *119*, 322–335.

Johnson, D., and Quarles, R.H. (1986). Deposition of the myelin-associated glycoprotein in specific regions of the developing rat central nervous system. Dev. Brain Res. *28*, 263–266.

Johnson, P.W., Abramow-Newerly, W., Seilheimer, B., Sadoul, R., Tropak, M.B., Arquint, M., Dunn, R.J., Schachner, M., and Roder, J.C. (1989). Recombinant myelin-associated glycoprotein confers neural adhesion and neurite outgrowth function. Neuron *3*, 377–385.

Keilhauer, G., Faissner, A., and Schachner, M. (1985). Differential inhibition of neurone–neurone, neurone–astrocyte and astrocyte–astrocyte adhesion by L1, L2 and N-CAM antibodies. Nature *316*, 728–730.

Keirstead, H.S., Hasan, S.J., Muir, G.D., and Steeves, J.D. (1992). Suppression of the onset of myelination extends the permissive period for the functional repair of embryonic spinal cord. Proc. Natl. Acad. Sci. USA *89*, 11664–11668.

Keynes, R.J., and Cook, G.M.W. (1995). Repulsive and inhibitory signals. Curr. Opin. Neurobiol. *5*, 75–82.

Luo, Y., Raible, D., and Raper, J.A. (1993). Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell *75*, 217–227.

McKerracher, L., David, S., Jackson, D.L., Kottis, V., Dunn, R.J., and Braun, P.E. (1994). Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron *13*, 805–811.

Messersmith, E.K., Leonardo, E.D., Shatz, C.J., Tessier-Lavigne, M., Goodman, C.S., and Kolodkin, A. (1995). Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. Neuron *14*, 949–959.

Montag, D., Giese, K.P., Bartsch, U., Martini, R., Lang, Y., Blüthmann, H., Karthigasan, J., Kirschner, D.A., Wintergerst, E.S., Nave, K.-A., Zielasek, J., Toyka, K.V., Lipp, H.-P., and Schachner, M. (1994). Mice deficient for the myelin-associated glycoprotein show subtle abnormalities in myelin. Neuron *13*, 229–246.

Mukhopadhyay, G., Doherty, P., Walsh, F.S., Crocker, P.R., and Filbin, M.T. (1994). A novel role for myelin-associated glycoprotein, MAG, as an inhibitor of axonal regeneration. Neuron *13*, 757–767.

Poltorak, M., Sadoul, R., Keilhauer, G., Landa, C., Fahrig, T., and Schachner, M. (1987). Myelin-associated glycoprotein, a member of the L2/HNK-1 family of neural cell adhesion molecules is involved in neuron–oligodendrocyte and oligodendrocyte–oligodendrocyte interactions. J. Cell Biol. *105*, 1893–1899.

Püschel, A.W., Adams, R.H., and Betz, H. (1995). Murine semaphorin D/collapsin is a member of a diverse gene family and creates domains inhibitory for axonal extension. Neuron *14*, 941–948.

Rubin, B.P., Spillmann, A.A., Bandtlow, C.E., Keller, F., and Schwab, M.E. (1995). Inhibition of PC12 cell attachment and neurite outgrowth by detergent solubilized CNS myelin proteins. Eur. J. Neurosci., in press.

Savio, T., and Schwab, M.E. (1990). Lesioned corticospinal tract axons regenerate in myelin-free rat spinal cord. Proc. Natl. Acad. Sci. USA *87*, 4130–4133.

Schachner, M., Taylor, J., Bartsch, U., and Pesheva, P. (1994). The perplexing multifunctionality of janusin, a tenascin-related molecule. Persp. Dev. Neurobiol. *2*, 33–41.

Schneider-Schaulies, J., Kirchhoff, F., Archelos, J., and Schachner, M. (1991). Down-regulation of myelin-associated glycoprotein on Schwann cells by interferon-γ and tumor necrosis factor-α affects neurite outgrowth. Neuron *7*, 995–1005.

Schnell, L.E., and Schwab, M.E. (1990). Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. Nature *343*, 269–272.

Schnell, L.E., Schneider, R., Kolbeck, R., Barde, Y.A., and Schwab, M.E. (1994). Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. Nature *367*, 170–173.

Schwab, M.E., Kapfhammer, J.P., and Bandtlow, C.E. (1993). Inhibitors of neurite growth. Annu. Rev. Neurosci. *16*, 565–595.

Shewan, D., Berry, M., and Cohen, J. (1995). Extensive regeneration *in vitro* by early embryonic neurons on immature and adult CNS tissue. J. Neurosci. *15*, 2057–2062.

Sommer, I., and Schachner, M. (1981). Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. Dev. Biol. *83*, 311–327.

Taylor, J., Pesheva, P., and Schachner, M. (1993). Influence of janusin and tenascin on growth cone behavior *in vitro*. J. Neurosci. Res. *35*, 347–362.

Unsicker, K., Skaper, S.D., Davis, G.E., Manthorpe, M., and Varon, S. (1985). Comparison of the effects of laminin and the polyornithine-binding neurite promoting factor from RN22 Schwannoma cells on neurite regeneration from cultured newborn and adult rat dorsal root ganglion neurons. Brain Res. *349*, 304–308.

Weibel, D., Cadelli, D., and Schwab, M.E. (1994). Regeneration of lesioned rat optic nerve fibers is improved after neutralization of myelin-associated neurite growth inhibitors. Brain Res. *642*, 259–266.