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7

Characterization of the Myelin-Associated Glycoprotein Binding Component

By

Maria Elena de Bellard

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
The City University of New York

1999

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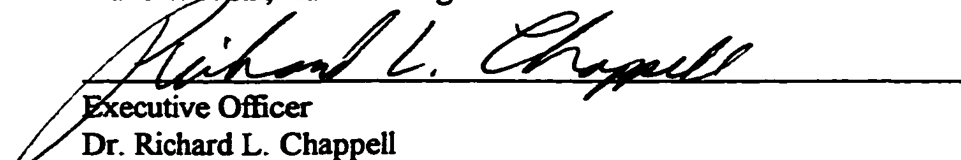

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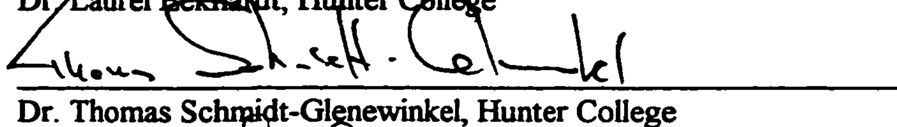

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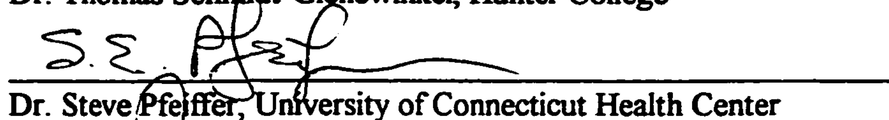

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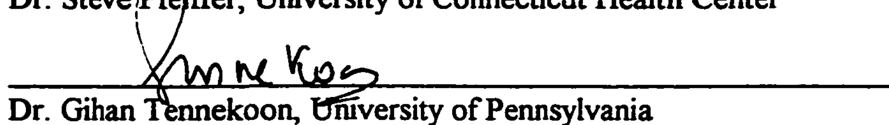

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Abstract

Characterization of the Myelin-Associated Glycoprotein Binding Component

By

Maria Elena de Bellard

Adviser: Distinguished Professor Marie T. Filbin. Ph.D.

The adult mammalian CNS does not regenerate after injury due to several factors, the most outstanding, the presence of myelin-specific inhibitors of axonal growth. Myelin-associated glycoprotein (MAG) is a potent inhibitor of axonal regeneration from both cerebellar and DRG neurons. In contrast, MAG promotes axonal growth from newborn DRG neurons. Here, we show that to date of all the neurons tested, a postnatal switch in response is only observed for DRG neurons; MAG inhibits axonal growth from retinal, SCG, spinal and hippocampal neurons of all postnatal ages. Furthermore, MAG binds to neurons, from which it promotes and inhibits outgrowth, in a sialic acid-dependent, trypsin-sensitive manner. Hence, the interaction is via a sialoglycoprotein. Importantly, both inhibition and promotion of neurite outgrowth by MAG was reduced, or abolished, either by desialylation or by including small sialic acid-bearing sugars in the cultures. MAG contributes to the inhibitory properties of myelin both *in vitro* and *in vivo*. When myelin from MAG-/- mice is used as a substrate, neurites from postnatal cerebellar and

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DRG neurons grow twice as long as on MAG+/+ myelin. Consistent with MAG's dual role, neurites from neonatal DRG neurons grow twice as long on MAG+/+ myelin relative to MAG-/- myelin. In addition, when mice are genetically engineered to express MAG in Schwann cells during PNS regeneration, the growth of axons after injury was dramatically reduced in the transgenic compared with the control mice. Attempts to isolate the neuronal sialoglycoprotein with which MAG interacts, by purifying MAG binding partners from different types of neurons using soluble MAG-Fc, indicated that MAG binds to at least three proteins of approximately 190 and 250kD. The p190 band corresponds to two proteins, one of which is a GPI-linked sialoglycoprotein. Together these results indicate that MAG can significantly contribute to the lack of axonal regeneration given by myelin inhibitors present after injury, and that partial reversal of this inhibition can be achieved by desialylation or adding small sialic acid sugars. It has been the goal of these studies to provide some clues to how lack of regeneration takes place in the nervous system and how to circumvent this problem in the CNS.

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INTRODUCTION

Vertebrate nervous system regeneration is the process by which a neuron is able to regrow an axon after an injury. How this phenomenon takes place and the components involved have been under intense research since the early 1900s when Ramon y Cajal first described it (Ramon y Cajal, 1968). He noticed that regeneration is limited, if it occurs at all, in the central nervous system (CNS) of mammals even though it occurs in the peripheral nervous system (PNS) (Ramon y Cajal, 1968). His early observations pointed out that injured CNS axons start to regenerate as PNS neurons do, but soon afterwards they halt when contacting white matter, as if facing a barrier, and later degenerate. These observations led to the suggestion that the environment through which the CNS neurons must regrow is not favorable, in contrast to the environment encountered by the PNS neurons or embryonic neurons during development (Keirstead, 1992; Savio and Schwab, 1989).

There are several factors now known that can play a critical role in the capacity of the mammalian nervous system to regenerate: neuronal age, availability of trophic/tropic factors, the growth substrate, presence of myelin debris, the extent of the injury, and the degenerative process that takes place distal to the lesion. Of these, the one that stands out most is the developmental age of the neurons. It has been well documented that embryonic cells are more plastic than neonatal or adult neurons (Davies, 1994; Gage, 1995; Shewan et al., 1995; Unsicker, 1985), and neonatal neurons are more plastic than adult neurons (Iwashita, 1994; Nicholls et al., 1994). Dusart showed that age can affect the capacity of neurons to put out neurites as well as affecting their response to the different neurotrophic factors present at the site of the lesion (Dusart et al., 1997). *In vitro* studies with chick retinal ganglion cells showed that there was a considerable loss in their ability to reinnervate tectal membranes with increasing developmental age (Cheng, 1995). This loss of reinnervation is probably due to a change in trophic requirements, membrane receptors for growth factors, and type of adhesion molecules present throughout development (Becker et al., 1998; Tuttle et al., 1995; Yan and Johnson, 1987). For example, 1) mature chick retinal ganglion cells do not put out neurites in response to laminin, even though they express the laminin receptor (Cohen et al., 1986; Neugebauer, 1991). 2) Dorsal root ganglion (DRG) neurons require nerve growth factor (NGF) up to late post-natal age for their survival and to establish their synapses, but

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in adulthood they no longer depend on NGF for survival, though its presence enhances regeneration (Kawaja, 1992; Lindsay, 1988; Yip, 1984).

Neurons clearly change their capacity for axonal regeneration *in vivo* after development ends. In support of this general observation are the transplant studies of embryonic or progenitor neurons, which show that the mature CNS still retains the potential to direct integration and differentiation of some immature neurons after grafting in adult brain (Bjorklund, 1984; Davies, 1994; Perlow, 1879; Shihabuddin, 1995). Therefore, the reason for the lack of regeneration in the adult CNS most likely lies in the change that the neurons undergo after development, as well as in the change of their surrounding environment. Several observations suggest that neurons are expressing extracellular membrane components that make them sensitive to substances present in the CNS, but which are either absent or at much lower concentration in the PNS (Tuttle et al., 1995). Embryonic rat retinal or DRG neurons have been shown to put out more neurites when grown on top of sections from either myelinated or unmyelinated optic nerve than older neurons (Shewan et al., 1995). More recently, Bandtlow showed that rat DRG neurite outgrowth up to E13 and chick retinal neurite outgrowth up to E6 are indifferent to a myelin glycoprotein which induces growth cone collapse, but after that specific age they will start to be inhibited by it (Bandtlow and Loschinger, 1997).

All these observations point to another factor, the substrate, over which the neurons grow after an injury as a pivotal player in the fact that adult mammalian neurons cannot regenerate. In a key experiment, Carbonetto and co-workers showed that embryonic chick DRG neurons grew neurites on PNS sections but avoided CNS tissue (Carbonetto et al., 1987). The suggestion was that there are components (now known as inhibitors of neurite outgrowth) in the CNS myelin, absent or at lower concentration in the PNS myelin, which are partly responsible for the lack of regeneration in the CNS.

Work carried out in Aguayo's laboratory has confirmed these results *in vivo* by showing that adult neurons can regenerate over considerable distances along sciatic nerve grafts. In their experiments axotomized retinal ganglion cells were capable of extending long axons into the superior colliculus and even into the cerebellum, making persistent synapses after innervating and growing along the peripheral nerve grafts (Carter et al., 1989; David and Aguayo, 1985; Vidal Sanz, 1991). More recently, it has been shown that adult DRG neurons microtransplanted into the

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CNS can grow long axons and far from the transplant site along myelin tracts in the CNS (Davies et al., 1997). Axonal outgrowth took place provided the transplant did not cause any gliosis or major damage at the site of injection. Both of these findings reinforce the hypothesis that adult neurons have the ability to re-grow, but the injury and the substrate they encounter while growing play an inhibitory role for axonal regeneration.

The best evidence about the role of the substrate in regeneration was given by the surgical experiments performed by Olson and coworkers (Cheng et al., 1996). After complete spinal cord section, small pieces of peripheral nerves were surgically connected to bridge white matter to gray matter in the spinal cord. After the surgery the animals were able to regain considerable motor function due to successful regeneration of axons. When the surgery bridged white matter to white matter, there was no significant recovery in the motor functions. The condition of the substrate after the injury, especially the presence of myelin debris (plus reactive gliosis and local inflammatory responses on a smaller scale), make a big part of the reason behind the failure of CNS neurons to regenerate. Taken together, these findings imply that there are some axonal inhibitors in CNS myelin that are present and/or released after an injury that can inhibit axonal regeneration, and that these components are not present or released in the PNS grafts used in these experiments nor in the DRG microtransplant experiments. The presence of these inhibitory conditions in the CNS explain why the same adult neurons that will regenerate into PNS nerves will not grow axons into the spinal cord after axotomy of their dorsal roots (Carlstedt et al., 1989).

Myelin debris is not the only substrate neurons encounter after an injury. The most abundant glial cells, astrocytes, also have been shown to play a role after injury to the CNS. Normal astrocytes can be a permissive substrate for the growth of axons (Noble et al., 1984), but after an injury some astrocytes change and become "reactive" (Rudge and Silver, 1990). The astrocytes undergo hypertrophy and hyperplasia in proximity to a lesion, and even though they participate in the removal of cellular debris by phagocytosis, the result of this changing process in the astrocytes is the formation of a scar tissue and their transformation into "reactive" astrocytes. This observation led to the belief that they were forming a barrier to the growing axons thus impairing regeneration (Stensaas, 1987). Opposing this hypothesis were two findings. One showing that when the dorsal root of a DRG is axotomized, the regenerating DRG neurons will not re-innervate the spinal cord again, stopping short of the boundary between PNS and CNS, even

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though there are no reactive astrocytes at that site (Perkins, 1980). The other finding came from the Silver laboratory, which showed that the cause of inhibition of regeneration by "reactive" astrocytes is the presence of non-permissive molecules on their surface or secreted into the extracellular matrix (McKeon et al., 1991; Roux, 1994; Rudge and Silver, 1990), like tenascin, janusin and proteoglycans (Davies et al., 1997; Faissner, 1990; Schachner, 1994; Smith-Thomas, 1994). More recently they have shown that adult DRG neurons can grow long axons out of their microtransplant space in an adult brain, provided there was no production of chondroitin sulfate proteoglycan (CSPG) by the "reactive" astrocytes as a boundary encapsulating the transplant (Davies et al., 1997). In other words, neurons were able to regenerate along these tracts because there was no glial scar with non-permissive molecules, and because along those same tracts a promoting protein, myelin oligodendrocyte glycoprotein (MOG) is expressed. We and others have shown that MOG promotes neurite outgrowth from postnatal cerebellar neurons and embryonic hippocampal neurons (Turnley and Bartlett, 1998).

A neuron will not be able to grow an axon after an injury if it cannot survive the aftermath of the injury itself. It is well known that neurons need certain growth factors for their survival (trophic effect) as well as topographic markers to reach their original targets (tropic effect). Consistent with the differences between regeneration in the CNS and PNS, these factors are either distal or at low concentrations in the mature CNS (Kawaja, 1992), but are present or upregulated in the PNS after an injury (Daniloff, 1986; Moskowitz and Oblinger, 1995; Zhou, 1996).

A very important aspect for survival of the injured neurons is the response of their neighboring cells after the lesion. Not all neurons are equally sensitive to absence of growth factors, nor will they respond similarly to an injury (Bunge, 1987; Jacobson, 1991; Lasek and Katz, 1987). Aguayo found that after axotomy many retinal ganglion cells die even when allowed to grow on a PNS graft, and only 20% of the surviving neurons will regenerate axons and form synapses under these conditions (Aguayo et al., 1991). This low survival and regenerative rate can be improved if PNS explants, which secrete a cohort of trophic factors (Funakoshi et al., 1993), are grafted into the vitreous body of the eye, enabling the survival and potentiating the growth of retinal neurons (Berry et al., 1996). Ramon y Cajal described many years ago that in spinal cord and peripheral nerve, the extent of the traumatic degeneration at the neuronal body is proportional to the axonal diameter (Ramon y Cajal, 1968). That is, large neurons with large axons are more

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likely to die than the smaller ones after an injury. The DRG neurons which are bipolar, do not have the same response in synthesis and transport of cytoskeletal components at their peripheral or central projecting axons after axotomy (Lasek and Katz, 1987). Traumatic insults to the spinal cord induce both an immediate mechanical damage and subsequent tissue degeneration, as well as apoptosis, which together might contribute to spinal cord cell loss usually observed in these cases (Liu et al., 1997).

The ultimate issue in CNS regeneration is recovery of lost function. Thus any factor that improves or gives recovery is extremely important. The different experiments which show partial recovery emphasize the significance of allowing at least few axons to succeed by regenerating after an injury (Bregman et al., 1997; Cheng et al., 1996; Schwab et al., 1993). This success in axonal regeneration can happen provided that two aspects are taken into account. One is the innate plasticity of the nervous system; the other is overcoming the inhibitory molecules found in CNS myelin debris. In the CNS many uninjured neurons will react to a lesion by sprouting and reshaping their connections when other neighboring cells have been lost, giving some recovery of the lost function (Singer, 1982). This plasticity of neighboring neurons might be related to trophic factors like BDNF, GDNF and NT-3 that are secreted by the surrounding glial cells in response to the injury and which have been shown to improve regeneration and/or survival of neurons (Bregman et al., 1995; Bregman et al., 1997; Cheng et al., 1996; Kobayashi et al., 1997; Lindholm, 1987). If taking advantage of the trophic factors effect to enhance regeneration seems promising, overcoming myelin inhibitors is another issue. In order to neutralize or eliminate the inhibitors present in myelin they first have to be identified and well characterized.

Several studies have shown the relevance of mammalian CNS myelin debris as inhibitor of axonal regeneration. Shimizu (1990) and Keirstead (1992, 1995) have shown a close correlation between failure of regeneration of spinal cord tracts and the presence of myelin (Keirstead, 1992; Keirstead et al., 1995; Shimizu, 1990). The immunological elimination of myelin within the hatchling chick spinal cord shortly after a thoracic transection, facilitated partial neuroanatomical regeneration with some functional recovery (Keirstead et al., 1995). In addition, when oligodendrocyte development is arrested in neonatal rat's optic nerves and myelination does not occur, the resultant unmyelinated nerves in these animals had higher fiber counts fluctuating throughout the length of the nerves, indicative of an increased number of sprouts in these

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unmyelinated nerves (Colello and Schwab, 1994). Myelin has been known for years to be responsible for the saltatory transmission of the nerve impulse. The results from these experiments suggested that it may have another physiological function: stabilizing the axon and preventing supernumerary sprouts throughout its length (Colello et al., 1994; Colello and Schwab, 1994; Schwegler et al., 1995). This function has been thought to take place through the presence of inhibitors of axonal growth (Schulz et al., 1998; Schwab, 1996). Thus when myelin is not present, there is an increase in the number and caliber of fibers in the CNS.

If axonal regeneration in the CNS does not take place in great part because of myelin, why is it possible to have axonal regeneration in the PNS that also has myelin with almost the same components that CNS does? It has been shown that peripheral nerve grafts are a suitable substratum for regeneration because of two processes that take place in the PNS, which do not happen in the CNS. One crucial difference is given by the myelinating glia of the PNS; the Schwann cells. Schwann cells can undergo a series of changes that favor the growth of axons (Berry et al., 1996; Li and Raisman, 1994), changes that oligodendrocytes in the CNS do not experience (Carbonetto, 1991). Schwann cells after an injury will de-differentiate, begin to secrete NGF, and form the Bungner tubes, along with taking part in the removal of myelin by macrophages. It is in these permissive paths (Bungner tubes) and conditions (with trophic and tropic factors more abundant than in the CNS) where the DRG neurons and motor neurons axons will regenerate (Bunge, 1993; Son and Thompson, 1995). In brief, neurons of PNS nerves always find a prepared and favorable pathway for regeneration, while neurons in the CNS do not find such conditions after an injury (Griffin, 1993). Taking advantage of this Schwann cells feature, Bunge and coworkers as well other teams, developed the Schwann cell guidance channel, a kind of graft between the two ends of an injured spinal cord (Kromer and Cornbrooks, 1985; Xu et al., 1997; Xu et al., 1995). In this system primary Schwann cells are encapsulated inside a tube in matrigel along which the regenerating axons can grow since these PNS glia make it a favorable environment. Nevertheless, the rate of successful regeneration is not complete, nor do the axons get much beyond the channel grafts (Xu et al., 1997). Recently another type of PNS glial cell, the ensheathing glia (EG) from the olfactory bulb, had been successfully used as grafts (Li et al., 1997). So when both pieces of information were put together, a much more successful guidance channel was developed,

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this time containing also the EG cells (Ramon-Cueto et al., 1998). In this last experiment the rate of regeneration beyond the graft was remarkable.

The data showing successful regeneration in the PNS after injury point to another very important factor for regeneration, the degenerative process that occurs after an injury. This process has been shown to be advantageous for axonal growth, for example, making repetitive lesions in a sciatic nerve will accelerate the process of regeneration (Lu and Richardson, 1991; Richardson and Verge, 1987). This improvement in regeneration is probably due to an array of several circumstances that take place because after repetitive injuries the responses of the Schwann cells are augmented. Some of these circumstances are: 1) the increase in the rate of neurofilament polymerization under these conditions, which leads to faster assembly of regrowing axons (McKerracher et al., 1990); 2) an increase in the amount of macrophage recruitment (Perry and Brown, 1992), which stimulates Schwann cell proliferation and upregulation of important markers (Saada, 1996); 3) increased secretion of NGF and other trophic factors by non-neuronal cells (Beuche, 1984; Lindholm, 1987), as well a change in the amount of phosphatases and proteases secreted at the injury site (George, 1995; Gold et al., 1995; Lasek and Katz, 1987). Last but not least, it has been shown that adult DRG neurons will extend neurites *in vitro* on pre-degenerated sciatic nerve, but not on normal peripheral nerves (Bedi, 1992). All these data show that the putative PNS permissiveness previously reported (Carbonetto et al., 1987; Shewan et al., 1995) can be explained by two conditions. First, the neurons that were used in those experiments were embryonic or early neonatal DRG neurons, which as explained above, are not responsive to the inhibitory factors of neurite growth in myelin. Second, the peripheral nerve grafts used in the transplant experiments had gone through the usual Wallerian degeneration of its myelin after grafting, and therefore these grafts are permissive.

Of all the processes that take place after an injury, Wallerian degeneration is fundamental (George, 1995). This is the mechanism by which the distal stump and myelin debris are removed by macrophages and Schwann cells. In the PNS, Wallerian degeneration is a normal process after an injury, but in the CNS this mechanism is nearly absent (Ramon y Cajal, 1968; Stoll et al., 1989), probably due to lack of accessibility to macrophages (David et al., 1990) and the presence of the blood brain barrier (Perry and Brown, 1992). Studies with the C57BL/Wld mice, a mutant line with delayed Wallerian degeneration, showed that these animals have impaired regeneration of

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their motor axons, even in the presence of NGF (Brown, 1992; Brown, 1994). This result underscores the hypothesis that the presence of myelin debris at the site of the injury and beyond can significantly impair regeneration (Berry, 1982). As the studies of Shimizu (1990) and Keirstead (1992, 1995) have shown, when myelination was disrupted regeneration of injured spinal cord tracts improved significantly (Keirstead, 1992; Keirstead et al., 1995; Shimizu, 1990).

After a lesion, therefore, an adult CNS neuron that is trying to regenerate very likely will face conditions that do not enhance its neurite outgrowth, for example: 1) a lack of sufficient or proper topographic clues to reach its target (Stretavan, 1994); 2) an array of CNS myelin substances that block their path (Mukhopadhyay et al., 1994; Schwab, 1993); 3) an inadequate battery of receptors to put out neurites in order to be able to regenerate its lost connections (Neugebauer, 1991).

All these data reinforce the suggestion that either astrocytes of the reactive type or lack of sufficient growth factors account only for part of the reason behind the lack of regeneration. The sole presence of myelin debris at an injury is sufficient to inhibit axonal regeneration. That is, the environment full of myelin debris after an injury in which CNS neurons regenerate is, as suggested earlier, one of the most important reasons for the lack of regeneration in the mammalian CNS.

During their early development and axonal pathfinding, neurons encounter a substratum very different from the adult CNS (Pini, 1993; Raper, 1990; Walter, 1987). It is the orchestration of axonal guidance proteins that is key for the correct development of the architecture of the nervous system (Drescher, 1995; Goodman et al., 1993; Hynes and Lander, 1992; Stretavan, 1994). The number of the axonal guidance proteins currently known is steadily growing; the more well-known are the cell adhesion molecules (CAMs) (Duband, 1987; Felsenfeld, 1994; Struyk, 1995), the collapsin/semaphorins (Kolodkin, 1993; Luo, 1993), the netrins (Serafini et al., 1994) and the Eph family (Davenport et al., 1998; Flanagan and Vanderhaeghen, 1998; Ghosh and Shatz, 1992). Their mode of action is by either simple adhesion (homophilic, heterophilic or both) as membrane attached molecules or by diffusion of chemoattractant/chemorepellent ligands in the surrounding area. The studies by Bonhoeffer and co-workers on how the expression of RAGS, helps define the retinotectal projections by the chemorepulsion of axons, are classical in the field (Drescher, 1995; Walter, 1987). Many of the members of these pathfinding proteins are expressed on cell surface

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via a transmembrane domain (N-CAM), GPI-anchor (neurotrimin), or both for example, Eph members and semaphorin. Others are diffusible proteins like the netrins.

The adult mammalian brain lacks this protein orchestration; moreover, the levels of some of these guidance molecules are very low. In addition to this limitation, CNS neurons after an injury encounter myelin molecules whose function is more structural or stabilizing, instead of a tropic type (with a function in guidance and architecture of the brain). An explanation for this inhibitory effect on neurite outgrowth is that as an undesired consequence of their interaction with some of these molecules, neurons respond by not growing back their axons to their targets. In this manner, those molecules present in the adult environment with a different physiological function become "inhibitors" of axonal regeneration, as a consequence of their precise function on the neurons and their axons.

The presence of these inhibitory or non-permissive substrates much more than the lack of positive signals from their targets in the mammalian CNS environment, have been shown to impair regeneration. These inhibitory compounds include substances as diverse as chondroitin sulphate-proteoglycans (CS-PG) and tenascin (Dou, 1994; Meiners, 1995) present in reactive astrocytes membranes, GP55 (Clarke and Moss, 1994), and myelin inhibitory proteins (Caroni, 1988; Caroni, 1988; McKerracher et al., 1994; Mukhopadhyay et al., 1994).

The process by which these molecules exert their effect is not completely understood. A clear distinction between permissive, instructive, promoting, and inhibitory substrates did not exist until recently (for reviews see: (Cunningham, 1995; Gumbiner, 1996; Hynes and Lander, 1992; Keynes and Cook, 1995); Lemmon and co-workers (1992) already observed that adhesiveness to laminin, L1 and N-cadherin did not correlate with the degree of neurite fasciculation or growth rates of DRG neurons on these substrates. They also found that neurites did not show much selectivity when offered to choose between CAM with very different adhesion capabilities as are L1, N-cadherin, or laminin, suggesting that relative substrate adhesiveness is not a chief factor in guiding and supporting neurite outgrowth (Lemmon, 1992).

A permissive substrate is the one that will induce or at least allow neurite outgrowth, irrespective of its concentration or gradient (i.e. laminin, poly-L-Lysine). An instructive substrate will make a neuron direct its axonal growth along a particular pathway because the neurons are especially sensitive to pathfinding gradients (e.g. RAGS, semaphorin, contactin, N-CAM)

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(Colamarino, 1995; Doherty et al., 1990). A promoting substrate (e.g. L1) allows neurons to grow better and faster when compared with a "classical" permissive substrate like collagen or poly-Ornithine. Finally, an inhibitory substrate causes growth cone collapse/arrest and inhibit neurite outgrowth (e.g. collapsin). Inhibitory substrates should not be confused with non-permissive substrates, for which growth is not sustained because of the poor adhesion of neurites or growth cones; that is, when bound to a surface, they do not support outgrowth from any neuron, and their apparent inhibitory effect depends on which permissive substrate with which it is combined (e.g. CS-PG, tenascin, janusin) (Luo, 1994). However, these concepts are not mutually exclusive; an instructive protein can exert its function by either being a promoting (L1, N-CAM) or an inhibitory (collapsin, RAGS) type of substrate.

Although inhibitory molecules were considered to be exclusively inhibitors (Schwab et al., 1993), recent observations with myelin associated glycoprotein (MAG), semaphorin and janusin/tenascin suggest that some elicit opposite effects under specific circumstances (for review (Filbin, 1995)). These opposing responses appear to depend on the type of neuron and/or its developmental stage (Colamarino, 1995; Mukhopadhyay et al., 1994). For example, the collapsin/semaphorin family of cell surface and secreted proteins was initially thought to function mainly as signal posts to guide pioneer neurons in the grasshopper limb bud. Nevertheless, more recently it has been shown to function: 1) as a selective inhibitor of motoneuron synaptic arborizations in *Drosophila* and 2) as a selective chemorepellent for DRG axons that terminate in the dorsal horn of the spinal cord (Kolodkin, 1993; Matthes et al., 1995; Messersmith, 1995). Tenascin and Janusin are related extracellular matrix glycoproteins that make the *in vitro* substrata unsuitable for neurite outgrowth from CNS neurons (Faissner, 1990; Pesheva, 1989). However, for sensory neurons neurite outgrowth may actually be stimulated by tenascin, provided that artificial means are used to ensure adequate attachment of the cell body to this substratum (Wehrle, 1990).

Myelin's powerful effect on regeneration is largely believed to come from its array of neurite inhibitors. To date, those identified are MAG, which is a cloned and well-characterized protein (Filbin, 1995; Quarles, 1992), and the NI-35 and NI-250 proteins identified by Schwab's group, which have not been cloned yet (Caroni, 1988; Spillmann et al., 1998). However, the NI-35 and NI-250 proteins have been partially characterized by the use of a monoclonal antibody (IN-1) that reverses the effect of both NI-35 and NI-250 and by observing the collapse of growth cones

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stimulated by them (Caroni, 1988; Caroni, 1988). Furthermore, when the IN-1 monoclonal antibody is administered by hybridoma transplantation after a spinal cord injury, it can induce long term growth of corticospinal axons, as well as partial recovery of function in adult rats (Bregman et al., 1995; Schnell, 1994; Schnell and Schwab, 1990).

The myelin associated glycoprotein (MAG), an Ig superfamily member with 5 Ig domains and a short cytoplasmic tail, is one of the first myelin-specific proteins to be expressed by the glial cells (Arquint et al., 1987; Salzer et al., 1987) during myelination. This glycoprotein has a somewhat different pattern of expression in the PNS and CNS. It is expressed at the periaxonal membrane loop of myelin in both tissues, but in the PNS it is also expressed in the outer and paranodal loops and the Schmidt-Lanterman incissures (Trapp et al., 1989; Trapp and Quarles, 1982). The temporal expression of this protein is also different for both tissues. Schwann cells in the PNS do not express MAG until axonal contact is established (Martini and Schachner, 1986), while it has been observed from *in vitro* cultures of oligodendrocytes that these cells can express MAG on their surface as soon as they have differentiated (Dubois-Dalcq et al., 1986). Another characteristic of this glycoprotein is that there are two isoforms developmentally regulated. These are referred as S-MAG (short) and L-MAG (long) for their distinct cytoplasmic tails, products of two transcripts (Frail et al., 1985; Inuzuka et al., 1991; Tropak et al., 1988). In the CNS L-MAG is the major form synthesized early in development, and it persists as a significant proportion of the MAG present in the adult. In the PNS, L-MAG is expressed at modest levels during development; but it is virtually absent in the adult. The expression of L-MAG is not limited to the CNS, as was formerly believed, suggesting that it plays a common role during the early stages of myelin formation by both oligodendrocytes and Schwann cells (Lai et al., 1987; Pedraza et al., 1991).

The subcellular location and high levels of MAG at the periaxonal membrane of myelin closest to the axon, led to the suggestion that this protein plays a role in the initiation of myelination and maintenance of myelin (Martini and Schachner, 1986; Trapp, 1990). In support of this hypothesis, Owens and co-workers showed by *in vitro* myelinating assays of DRG neurons and Schwann cells, that the presence of MAG antisense oligonucleotides in the Schwann cells was able to disrupt myelination (Owens and Bunge, 1991). Furthermore, when Schwann cells were engineered to express MAG constitutively, an earlier onset of myelination in similar neuronal

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cocultures was observed (Owens et al., 1990). These results reinforced the hypothesis that MAG function is involved in the initial investment of myelin by the glial cells. However, the early findings from MAG deficient mice did not uphold the hypothesis of an essential role for MAG in myelination (Li et al., 1994; Montag et al., 1994). In these animals there were no substantial abnormalities in their myelin besides loss of the cytoplasmic collar, widening of the periaxonal space, and higher incidence of redundant myelin in their CNS. However, later on under close study, some other observations were found that pointed indirectly to MAG as a key protein in myelination. First, in these MAG deficient mice, the onset of myelination is delayed in the CNS, while the PNS remains normal (Montag et al., 1994). Second, and very important, later on in adulthood these animals show evidence of demyelination and axonal degeneration of their PNS axons (Fruttiger et al., 1995). Third, in their CNS myelin some axons seem to have never been myelinated, or more peculiarly, are being slowly myelinated in adult animals (Bartsch et al., 1997). More recently, a mouse mutant line deficient only in L-MAG has been shown to display the same abnormalities in their CNS as the null MAG mice (Fujita et al., 1998). But in contrast to the CNS, the PNS axons and myelin of older animals do not degenerate as in the MAG deficient animals, indicating that S-MAG is sufficient for maintaining the integrity of PNS, but CNS myelin (Fujita et al., 1998). In addition, this putative function of MAG in myelination correlates with the non-receptor tyrosine kinase fyn in the fyn-deficient mice. One finding is that these mice have impaired myelination, as seen by a 50% reduction in total myelin basic protein, another major myelin protein, responsible for the compaction of myelin. The other important finding comes from Umemori and co-workers, who found that MAG coprecipitates fyn and that crosslinking of MAG with an antibody induces a rapid increase in the activity of fyn (Umemori et al., 1994). Therefore, the hypothesis that MAG plays a role in the initiation of myelination and in maintaining the myelin-axonal interface is still valid (Schachner, 1994).

Evidence for an additional role for MAG came with the finding that a recombinant form of MAG incorporated into liposomes can promote neurite outgrowth in neonatal DRG neurons (Johnson et al., 1989). Consequently, when MAG levels were down-regulated by INF-γ and TNF-α on immortalized Schwann cells, neurite outgrowth from neonatal DRG neurons was significantly reduced (Schneider-Schaulies et al., 1991). That is, MAG can bind to neurons and promote their

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neurite outgrowth as other CAM molecules have been found to do (Sadoul et al., 1990; Walsh and Doherty, 1997). Therefore, it was a new and unprecedented finding when it was shown that MAG can have another effect, as an inhibitor of axonal regeneration (McKerracher et al., 1994; Mukhopadhyay et al., 1994). Up to that time it was believed that all the molecules with a function in axonal growth were either promoting or inhibiting neurite outgrowth (Filbin, 1995).

The previous studies looked at the effect of liposomes with MAG on early neonatal DRG neurites (Johnson et al., 1989). When another type of postnatal neurons, cerebellar, were grown on a monolayer of MAG-expressing CHO cells the length of their neurites was strongly inhibited compared to the length on control transfected cells or cells expressing Po, another Ig superfamily member (Mukhopadhyay et al., 1994). In agreement with this finding, McKerracher and co-workers found that after immunodepletion of MAG from CNS myelin, neurite growth from the NG108 neuronal cell line was restored to about 63% of control. This result after immunodepletion of MAG of reduced inhibition of neurite outgrowth by myelin suggests that MAG can account for about 60% of the inhibitory effects of CNS myelin in vitro (McKerracher et al., 1994). In brief, MAG is a bifunctional molecule, it can either promote neurite outgrowth from early neonatal DRG neurons (Johnson et al., 1989; Mukhopadhyay et al., 1994) and can inhibit neurite outgrowth from postnatal cerebellar neurons (Mukhopadhyay et al., 1994).

When spinal cord injuries were carried out in MAG deficient mice, two opposing results on axonal regeneration were reported. Compared with wild type, a group did not find a major improvement in the MAG-deficient animals (Bartsch et al., 1995), while another group, found a significant number of axons that grew past the lesion (Li et al., 1996). Two possible explanations for these conflicting results are suggested. First, they used different types of analysis: the first group looked only at one axon per animal, the other at the average of axonal growth. Second, they used different background strains of mice; because there are still other potent inhibitors of axonal regeneration in the CNS the different strains may not respond similarly to an injury. The work by Schwab and co-workers in the past ten years has shown, using the monoclonal IN-1 antibody, which recognizes and blocks the effect of two myelin inhibitors (NI-35 and NI-250) that these inhibitors make a significant contribution to the lack of regeneration in the CNS (Schwab et al., 1993). However, when IN-1 is used in mice after injury, the number of regenerated axons is still very small. Obviously, MAG and the NI-35/NI-250 are not the only inhibitors present in

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oligodendrocytes; for example, collapsin-2 has been also localized in chicken oligodendrocytes (Luo, 1995). These results show that myelin and oligodendrocyte components are potent inhibitors of axonal regeneration *in vivo*.

In support of a role for MAG as an inhibitor of axonal regeneration *in vivo* are the studies with the C57BL/Wld^s mice, which display delayed Wallerian degeneration and a consequent impaired clearing of myelin debris in the sciatic nerve, and have a reduced regeneration of sensory and motor axons after a peripheral injury (Brown, 1992; Brown, 1994). Crossing C57BL/Wld^s with MAG-deficient mice, yields double mutant animals in which PNS regeneration is improved 100% (Schafer et al., 1996). That is, the absence of MAG in these animals was sufficient to enhance significantly PNS regeneration; the conclusion is that MAG is a powerful inhibitor of *in vivo* axonal regeneration in the PNS.

That MAG is only expressed postnatally, well after the neurons have established their connections in the CNS and PNS, is indicative that MAG does not play a role in axonal guidance. Why does it have a function as an inhibitor of axonal regeneration? The most likely answer is that this response of neurons is connected to another function, one that is relevant only in neurons that have already established their connections and are myelinated. From the work previously done on animals with delayed myelination came the suggestion that myelin may have a role as an axonal stabilizer and inhibitor of the spontaneous plasticity of normal CNS (Colello and Schwab, 1994; Schwegler et al., 1995). The finding in these animals of an increased number of collateral sprouts compared with the control group pointed to myelin and its components as inhibitors of this axonal sprouting. The reason for such a function, which can also oppose the regenerative process in the mammalian CNS, is obscure. It has been suggested that such a role is important for the stability and accuracy of the many connections present in the highly developed and complex nervous system of higher animals such as mammals (Schwegler et al., 1995). MAG, as the major component of the periaxonal membrane in myelin, is an ideal candidate to be one, if not the, component responsible for such a function.

There is abundant data supporting a role for MAG as an axonal stabilizer. The most prominent is the observation that MAG deficient mice undergo chronic atrophy of their myelinated PNS axons and eventually these axons degenerate (Fruttiger et al., 1995; Yin et al., 1998).

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Furthermore, the absence of MAG was correlated with reduced axonal calibers, decreased neurofilament spacing, and reduced neurofilament phosphorylation, all these pathologies have not been observed for other myelin deficient mice (Yin et al., 1998). Interestingly, all these happened in the absence of any inflammatory or hypomyelinating response, pointing to MAG as a molecule that can modulate the maturation and viability of axons (Yin et al., 1998). More recently, we have found that MAG has a powerful effect in neurite branching from postnatal DRG neurons in culture (Shen). When sensory neurons (DRG or SCG) were grown on Schwann cells expressing MAG their axonal branching was dramatically reduced in addition to inhibiting their length. This *in vitro* observation is reminiscent of the observation that myelin *in vivo* inhibits axonal sprouting, suggesting that MAG *in vivo* may carry a similar function and contribute to the control by myelin of axonal sprouting (Shen et al., 1998).

MAG, like many other Ig superfamily members, was initially thought to be capable of sustaining homophilic binding (Poltorak et al., 1987). But the location of MAG at the periaxonal membrane suggested that it should also interact in a heterotypic manner with a component in the axonal membrane. Several studies showed this to be the case. In one, Schachner and co-workers showed that liposomes expressing MAG can adhere specifically to DRG neurites in culture and that this adhesion can be partially inhibited by MAG-specific antibodies (Sadoul et al., 1990). By working with MAG transfected fibroblasts, another group found that MAG can function as a heterotypic cell adhesion molecule recognizing an unknown cell surface molecule expressed by the fibroblasts, and this binding was also blocked by MAG antibodies (Afar et al., 1991). For a long time the identification of this component(s) to which MAG binds has been the subject of intense research (Afar et al., 1991; Bachmann et al., 1995; Meyer-Franke et al., 1995; Sadoul et al., 1990). Only recently has there been substantial data on the nature of the receptor for MAG (Collins et al., 1997; Kelm et al., 1994; Tropak and Roder, 1997).

The question of what MAG is binding to on neurons to affect axonal regeneration found an answer when it was noticed that its first 4-Ig domains have considerable homology to the ones from members of the sialoadhesin family, sialoadhesin and CD22 (Kelm et al., 1994). These molecules are known to bind in a sialic acid-dependent manner and with a low affinity to their receptors (Crocker et al., 1994; Stamenkovic et al., 1991). When binding assays were carried out with a

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soluble form of MAG, composed of its extracellular domain fused to the Fc portion of the human IgG, it was found that MAG binds specifically to DRG and cerebellar neurons. Moreover, binding was completely abolished when neurons were desialylated prior to the assay (Kelm et al., 1994). This binding of MAG to a sialylated component on the neuronal membrane defines MAG as another member of the sialoadhesins. Each member of this family has specific affinity for certain sialic acid linkages. In order to dissect the specificity of MAG binding, sialic acid residues on sialidase-treated RBC, were reconstituted to give exclusively: 2,3-O-, 2,3-N- or 2,6-N-glycans; MAG preferentially binds sialic acid in a 2,3 O-linkage, but not 2,3 N-linked or 2,6 N-linked sialic acid sugars, while Sialoadhesin binds to 2,3 N- or 2,3 O-linked and CD22 to 2,6 N-linked sialic acid sugars (Kelm et al., 1994).

The findings that MAG binds to sialylated components on the neurons suggested that the receptor for MAG might be a ganglioside, molecules highly abundant in the CNS, especially axons (Yates, 1986; Yates et al., 1989). Indeed, MAG binds to GQ1bα and GT1b which have α2,3-O-linked sialic acids, but barely to other species such as GM3 which lack this linkage (Collins et al., 1997; Yang et al., 1996). This does not mean that GT1b or GQ1bα are the receptors or sole binding components for MAG, nor does it explain how the interaction of MAG with a ganglioside, would transmit an intracellular signal to affect neurite outgrowth. Nor has it been shown that by adding gangliosides (these or any kind) as competitors in the coculture media, or specific ganglioside antibodies, the inhibition by MAG can be reversed. On the contrary, the effect of ganglioside antibodies on neurite outgrowth has been linked to the carbohydrate moiety that these antibodies specifically recognize, more than the core molecule (Doherty et al., 1985). It appears to be that gangliosides have more of a cofactor role. In summary, the nature and identity of the putative receptor for MAG is still pending.

The aims of this project are: first, to get a more complete understanding of how MAG causes inhibition of axonal regeneration; second, to determine whether MAG contributes to the overall inhibition of neurite outgrowth by CNS myelin; third, to see if MAG can inhibit axonal regeneration *in vivo* as it does *in vitro*; fourth, to begin to characterize the component that MAG binds to on the neurons.

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Material and Methods

Expression of MAG by transfected CHO cells

CHO cells deficient in the *dhfr* gene (Urlaub and Chasin, 1980) were transfected with the pSJL plasmid containing the L-MAG cDNA in either a 5'-3' or, as a control, in a 3'-5' orientation as previously described (Mukhopadhyay et al., 1994). Expression of MAG protein was amplified and characterized as previously described (Filbin and Tennekoon, 1990). Transfected cells were maintained in DMEM supplemented with 10% dialyzed FCS, proline (40 mg/L), thymidine (0.73 mg/L) and glycine (7.5 mg/L) at 37°C in 7% CO2.

Isolation of Neurons

Neurons were isolated as previously described (Doherty et al., 1990; Kleitman et al., 1991). Briefly, for animals up to 9 days of age, the cerebellum, retina, hippocampus, and spinal cord were removed from two animals. Like tissue was combined and placed in 5ml of 0.025% trypsin in PBS, triturated, and incubated for a further 10min at 37°C. Trypsinization was stopped by addition of 5ml of DMEM containing 10% FCS and cells were centrifuged at 800rpm for 6 min. The cells were resuspended to a single-cell suspension in 2ml of SATO (Doherty et al., 1990) containing 2% FCS. For DRG and SCG neurons, ganglia were removed from 2 animals and incubated in 5ml of L-15 containing 0.025% trypsin and 0.3% collagenase type I (Worthington) for 30 min at 37oC. The ganglia were triturated with a fire polished Pasteur pipette. Trypsinization was stopped by adding 5ml of DMEM containing 10% FCS, centrifuged at 800rpm for 6min and resuspended in SATO. Cells were counted with a Coulter counter.

Desialylation of Neurons

Single cell suspension of different neurons were washed and resuspended in PBS, approximately 2x106 cells were incubated into 0.5ml of volume, with 50mU of Vibrio cholera sialidase (VCS, Calbiochem) for 2hr. at 37oC, after which they were washed with PBS and resuspended in SATO media for the neurite outgrowth coculture, or into PBS if for the solid binding assay. Where indicated, 20mU of VCS were added to the cocultures.

Neurite Outgrowth on Transfected CHO Cells

Confluent monolayers of control and MAG-expressing CHO cells were established over a 24h period in individual chambers of an 8-well tissue culture slide (Lab-Tek).

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Cocultures were established as described previously (Doherty et al., 1990; Mukhopadhyay et al., 1994) by adding 10,000 cerebellar, DRG and SCG neurons, retinal, hippocampal and spinal cord cells to the CHO cell monolayers. Culture medium was SATO containing 2% FCS. Where indicated, monolayers were incubated with sialic acid oligosaccarides for 1hr before adding the neuronal cell suspension, and throughout the coculture. After periods of time, as indicated, cocultures were fixed for 30min with 4% paraformaldehyde, and permeabilized with ice cold methanol for 2min. The cells were then blocked for 30min with DMEM containing 10% FCS and incubated for 2hr with a rabbit polyclonal antibody against GAP43 (1:4000, from R. Curtis & G. Wilkins). Cells were washed 3 times with PBS-BSA and then incubated for 30min at room temperature with a biotinylated donkey anti-rabbit Ig (1:500, Amersham), washed 3 times, and incubated with streptavidin-conjugated Texas Red (1:500, Amersham) for 30min. After 3 more washes, the slides were mounted in Permfluor (Baxter) and viewed with a Zeiss fluorescent microscope. The length of the longest neurite for each GAP43-positive neuron was determined using the Biological Detection System image analysis program (Pittsburgh).

Myelin and Membrane Preparation

Dissected sciatic nerves from adult rats and medulla from PND23 rats were homogenized separately in 0.85M sucrose with aprotinin, and layered on a sucrose gradient as described in Norton & Poduslo (Norton and Poduslo, 1973). After 20 h of high speed spinning, the interface between 0.25M and 0.85M sucrose was retrieved and resuspended in 10mM Hepes, protein assay on this suspension was carried out. Myelin (3 or 6ug/well) was dried overnight on 8-well chamber slides previously coated with poly-L-Lysine (PLL). In case of MAG deficient or wildtype mice, spinal cords were taken from adult animals.

Crude membrane preparations from neonatal rat liver and cortex, and CHO cells (control transfected or expressing MAG) were prepared by homogenizing these tissues in 0.32M sucrose. Nuclear fraction was discarded by low speed centrifugation, membrane pellets were collected by high speed centrifugation and resuspended in 10mM Hepes. Protein concentration of these membrane preparations was determined and 3ug/well of membranes were plated, dried overnight onto PLL coated wells and neurons were added in SATO media.

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Isolated neurons were incubated ($5x10^4$ cells) in these wells for 22 h, fixed and stained for GAP43. The longest neurite of 100 neurons was measured with an Image Analyzer.

Binding of Fc-chimeras to Neurons:

Plasmids containing the cDNA coding for MAG-Fc and MUC18-Fc were prepared as described previously (Kelm et al., 1994). The plasmids were transfected into COS cells and the Fc-chimeric proteins purified from the media as described previously (Crocker et al., 1994; Kelm et al., 1994). For the soluble binding assay MAG-Fc was iodinated with Iodogen. ^{125}I-MAG-Fc at 0.4mg/ml was incubated for 1 h at 4°C with various concentrations of anti-human IgG (Fc specific) to allow aggregates to form. Fresh cerebellar or DRG neurons, 1.5x105 cells/well, were then added to the aggregates. After one-hour incubation at 4°C, bound ^{125}I-MAG-Fc-aggregates were separated from free aggregates by centrifugation and washing with PBS. The bound radioactivity was then measured by a scintillation counter.

For the solid binding assay, Fc-chimeric proteins were adsorbed for 3 h at 37°C to wells of microtitre plates that had been coated for 2 h at 37°C with anti-human IgG at 15μg/ml in 0.1 M bicarbonate buffer, pH 9.6. Prior to the binding assay, neurons were vitally labeled with the fluorescent dye, 10 μM calcein AM (Molecular Probes) in PBS for 15 min at 37°C before being washed and resuspended in PBS. Labeled neurons in 100μl of a suspension containing 1 - 2 x 105 cells was added to each well and allowed to incubate for 1h at room temperature. The plates were washed 3 times with PBS applied to each well under gravity and the fluorescence measured in a FluorImager (Molecular Dynamics).

Construction of the p75-MAG transgene

A 4kb BamHI-SacII fragment was cut from pBRB51H3 (obtained from Dr. Moses Chao) and inserted into the pECE vector at HindIII site by blunt-ended ligation. L-MAG cDNA was previously subcloned at SmaI site, which is now immediately downstream the 4kb human p75 promoter and 5' regulatory sequence. An additional 200bp sequence including the SV40 untranslated and polyadenylation signals is just 3' to the L-MAG cDNA. The new construct, p4.0p75-LMAG, was used to test the expression competence in COS cells and in Schwann cells. A 6.2 kb BamHI fragment containing the p75 promoter, L-MAG cDNA, and polyA signals was derived from this construct, purified through

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electrophoreses in an 0.8% agarose gel and subsequent ultracentrifugation on a CsCl gradient. This fragment was used for microinjection into the male pronuclei of fertilized eggs. Oocyte surviving microinjection were implanted into the oviducts of pseudopregnant foster mothers following established protocol (Hogan, 1986). This step was done in collaboration with Dr. Carol Redhead, at Cedars Sinai, CA.

Transgenic mice identification

Transgenic founders were identified by PCR reaction using one primer (sense) corresponding to a sequence in the MAG cDNA spanning the first and second exon of the MAG gene (AGACTGAGGTGAGGGCCCTAGCTCG) and the other primer complementary to another sequence in the MAG cDNA which is in the third exon of the MAG gene (TGGGCAGTTGTCCGGCACCATGCAG). Transgene copy number will be determined by quantitative PCR and Southern blot.

Analyses of the expression of L-MAG in the transgenic mice

In order to determine that the rat L-MAG was on in the transgenic mice, we performed RT-PCR from total mRNA. Total mRNA was purified following the instructions for QuickPrep® Micro mRNA purification kit from Amersham-Pharmacia. Briefly, sciatic nerves were homogenized in Extraction buffer, diluted and centrifuged 1min to clear homogenate. Meanwhile 1ml of Oligo(dT)-Cellulose slurry was placed in microcentrifuge tube, spun 1min and the buffer aspirated. The cleared homogenate was added to the Oligo(dT)-Cellulose pellet, mixed 3min, spun few seconds, washed 5x 1ml of high salt buffer, 2x1ml with low salt buffer and resuspended in 0.3ml of low salt buffer. This was transferred to MicroSpin Column, centrifuged 5sec, washed 3x0.5ml of low salt buffer and eluted with 2x0.2ml of elution buffer at 65°C. RT-PCR was done following the Perkin-Elmer instructions, using AmpliTaq DNA polymerase with their standard cycle of 15min 42°C, followed by 5min 99°C, 5min 5°C.

Sciatic Nerve Injury

Adult mice from different transgenic lines were anesthetized with metofane, their sciatic nerves were exposed and crushed after the notch with a watchmaker's forceps or cut with fine scissors. After 7 or 14 days post injury (dpi) the animals were euthanized and their sciatic nerves carefully removed, encompassing a section proximal to the injury site and ending after the sciatic nerve trifurcation. These dissected nerves were either immediately frozen at –80°C or were fixed in 4% p-formaldehyde for 18hrs.

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Protein analysis

Protein was isolated from frozen sciatic nerves homogenized in Tris-buffered sodium dodecyl sulfate (SDS) lysis buffer (50mM Tris, pH 7.4, 2% SDS, antiproteases). Samples were incubated for 20min in 8-volumes of cold acetone, spun and the protein pellet was resuspended in SDS lysis buffer and quantitated by DC protein assay (BioRad, Hercules, CA). Samples were loaded onto a 9% SDS-PAGE gel, run at 35mAmp for 1.5hr and transferred to a PVDF membrane (Schleicher-Schluel). Proteins were analyzed by Western blot with primary antibodies diluted in 5% milk in PBS/0.5% tween solution. Primary antibodies included anti-MAG B11F7 monoclonal at a 1:3000 (kind donation by Dr. Quarles), anti-mouse HRP-conjugated (Sigma), and anti-mouse alkaline phosphatase-conjugated (Sigma) secondary (1:20,000). Membranes were developed by enhanced chemiluminiscence (Amersham) or by BCIPT/NBT (KPL) substrate for the alkaline phosphatase assay.

Immunohistochemistry

Fixed nerves were embedded in OCT, sectioned 5-7μm thickness with a microtome and mounted on a super-frost slide (Fisherbrand). After drying at room temperature the slides were either stored at –20°C or immediately used for immunocytochemistry. Slides with fixed sections were incubated for 10min in cold acetone, washed three times in PBS and blocked for 1hr in DMEM/10% FBS. Primary antibodies were added at a 1:500-1000 dilution in blocking buffer for 1hr. The antibodies used were a polyclonal anti-Neurofilament (Chemicon) 1:1000, a polyclonal anti-GAP43 (Curtis et al., 1993) 1:4000. The slides with the sections were washed three times in PBS and incubated for 30min in secondary antibodies (Molecular probes for Oregon green, Jackson Immunoresearch for FITC and Texas red) at a 1:1000 dilution. Slides were washed again three times in PBS and mounted in Permfluor for visualizing indirect Immunofluorescence in a Zeiss microscope.

Brains from adult mice were frozen and sectioned (20μm). The slides with the frozen sections were partially delipidated by 2min incubation in ethanol, blocked for 3hrs in DMEM/10% FBS and incubated with primary antibodies. These were monoclonal 513 (Boehringer-Manheim) 1:1000 and anti-MAP2 (Sigma) 1:500 for 1hr. Slides were washed three times in PBS and incubated with secondary antibodies as described above for 30min.

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Washed three times again and after a quick incubation with DAPI the slides were air dried and mounted in Permfluor for indirect Immunofluorescence.

Affinity purification of MAG-binding proteins

Isolated neonatal cerebellar and DRG or PC12 cells suspension in PBS were labeled with sulfo-NHS-biotin (200ug/ml) for 30min at room temperature washed 4 times with abundant PBS and lysed in buffer A (1% NP-40, 20mM Tris pH7.5, 150mM NaCl, 5mM EDTA, and a cocktail of antiproteases), for one hour on ice. The nuclei were removed by centrifugation, and the supernatant lysates were pre-cleared with human IgG and Protein A sepharose ON at 4°C in a rocker. The beads were spun down and the lysates were incubated with 10μg of the Fc-chimeras in a rocker overnight at 4°C. Protein A sepharose was added to trap the complexes and incubated for another 2 hours. The beads were washed 4 times with buffer B (Buffer A, but with 0.1% NP-40 and no antiproteases). After the final wash, the beads were resuspended in 30ul of sample buffer and β-mercaptoethanol, boiled and spun again. The supernatant was electrophoresed on SDS-PAGE, transferred onto PVDF membranes and blotted with streptavidin-HRP. The enzyme reaction was visualized by the ECL method from KPL.

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CHAPTER 1

The Role of Sialic Acid Binding in the Inhibition and Promotion of Neurite Outgrowth by MAG

Introduction

Myelin-associated glycoprotein (MAG), a well characterized component of CNS and PNS myelin, a potent inhibitory effect on axonal regeneration from cerebellar and adult DRG neurons (McKerracher et al., 1994; Mukhopadhyay et al., 1994). In contrast to its potent inhibitory effect, MAG can also promote neurite outgrowth from PND1 DRG neurons (Johnson et al., 1989; Mukhopadhyay et al., 1994); in other words, MAG is bifunctional, as other axonal guidance molecules (Kennedy et al., 1994; Serafini et al., 1994). That is to say, depending on the type and the age of the neuron, MAG can either promote or inhibit neurite outgrowth.

The neuronal component with which MAG interacts to effect inhibition is not known. The first clue to the identification of the MAG binding partner came from sequence alignment of Ig superfamily members. Fig. 1 shows that sialoadhesin, MAG and CD22 share a similar domain organization consisting of one N-terminal V-like domain and a variable number of C2-like domains. The highest homology between sialoadhesin and MAG (48.9% sequence similarity) or CD22 (46.8% sequence similarity) is found in the first 4 Ig domains (Kelm et al., 1994). These two other molecules are sialic acid-dependent binding proteins (Collins et al., 1997; Crocker et al., 1994). When binding assays were performed using a chimeric soluble form of MAG, MAG-Fc we found that MAG bound not only erythrocytes in a sialic acid-dependent manner, but this binding was preferential to α2,3-O-linked sialic acid (Kelm et al., 1994). From these observations, we were able to determine that MAG is a member of the sialoadhesin family.

Although it is well established that MAG can affect axonal outgrowth from postnatal cerebellar and adult DRG neurons, it is not known if other neurons respond to MAG and if so, whether their neurite outgrowth is promoted or inhibited by MAG. In addition, since MAG is a sialoadhesin, the question raised is whether or not MAG will bind in a sialic acid-dependent manner to neurons as do other sialoadhesins. If this is the case, is this interaction directly involved in the inhibition and/or promotion of axonal regeneration? To address these issues we

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will: 1) carry out binding assays to determine if MAG binds to neurons in a sialic acid-dependent manner; 2) assay neurite outgrowth to determine if neurons other than newborn DRG and postnatal cerebellar respond to MAG by being promoted or inhibited and if the sialic acid-dependent binding of MAG is necessary for such inhibition or promotion.

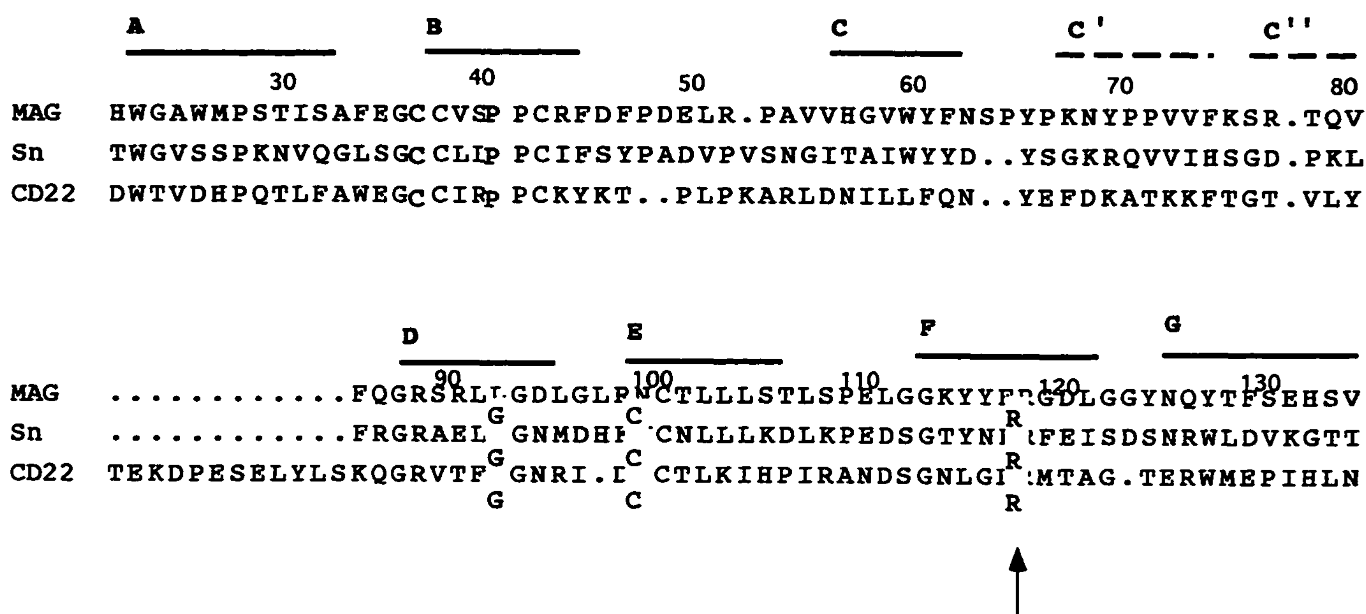

Fig.1. Sequence alignment of MAG, CD22 and sialoadhesin. Sialoadhesin, MAG and CD22 share similar domain organization consisting of one amino-terminal, V-like domain and a variable number of C2-like domains (V and C domains are named according to similarity to immunoglobulin domains). The most homology between sialoadhesin and MAG (48.9% sequence similarity) or sialoadhesin and CD22 (46.8% sequence similarity) is found in the first four extracellular domains.

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Results

MAG Binds to Different Types of Neurons

To establish whether MAG can bind in a sialic acid-dependent manner to neurons, purified MAG-Fc was radiolabelled and complexed with Fc specific, anti-human IgG. This soluble form of MAG, MAG-Fc, consists of the extracellular domain of MAG fused to the Fc portion of human IgG (Kelm et al., 1994). As a control, MUC18-Fc was used, which is another Ig superfamily member, and, like MAG, has 5-Ig domains (Hampel et al., 1997). Since MAG promotes neurite outgrowth from PND1 DRG neurons but inhibits neurite outgrowth from postnatal cerebellar neurons, we first measured MAG binding to these two types of neurons.

Dissociated postnatal cerebellar and DRG (PND1) neuronal suspensions were incubated with aggregated ^{125}I-MAG-Fc, washed and centrifugated, and the amount of Fc chimera bound to the cells was determined from the total radioactivity counted in the pellet (Fig. 2). We observed strong binding of MAG-Fc to cerebellar and DRG neurons, which was abolished by pre-treating the cells with a sialidase to remove sialic acid, or by including a MAG specific antibody in the binding assay (monoclonal 513). There was only background binding to MUC18-Fc for both types of neuron. These experiments provide the first evidence that MAG is a sialic acid binding protein for neurons regardless of whether neurite outgrowth is promoted (PND1 DRG neurons) or inhibited (cerebellar neurons).

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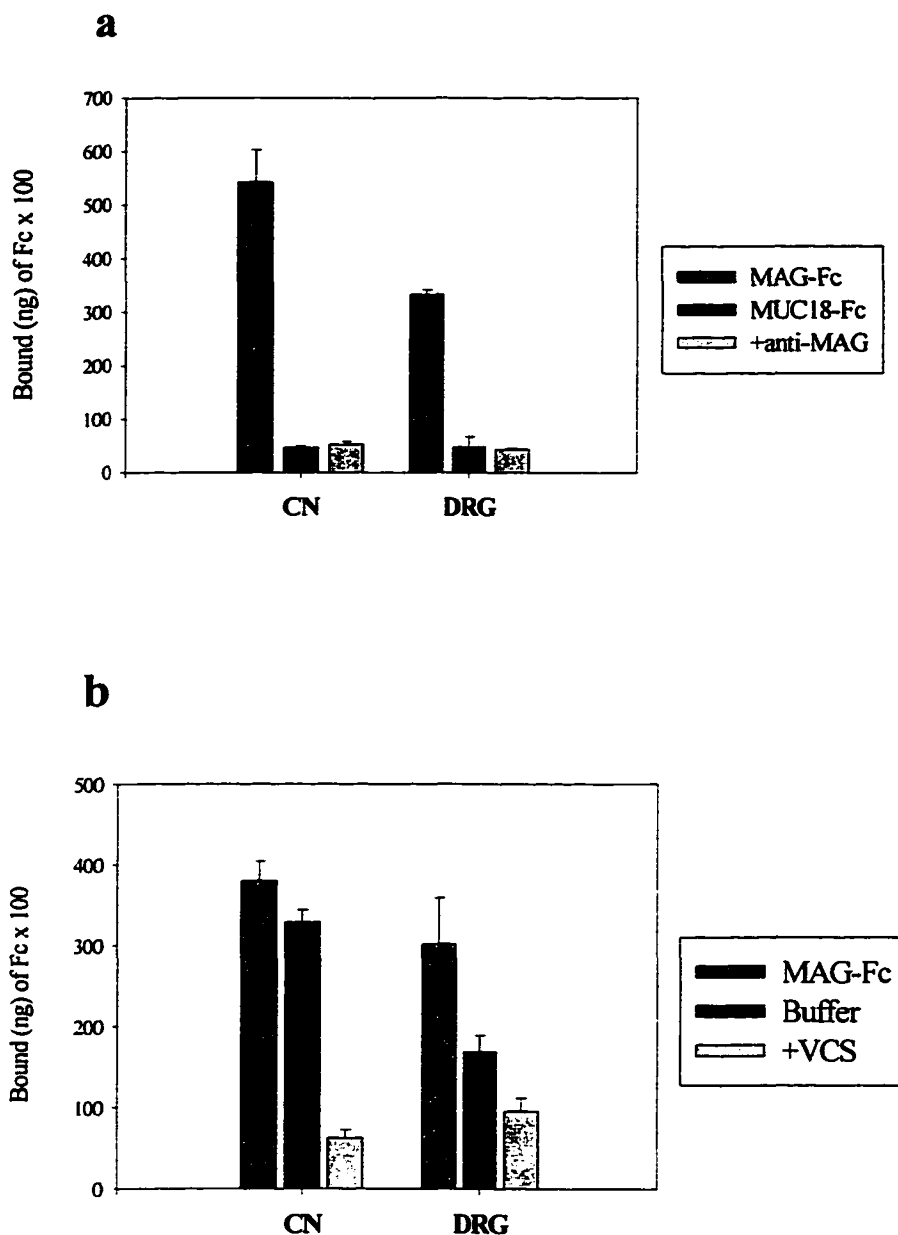

Fig. 2. Binding of ^{125}I-Fc chimeras to neurons. Cerebellar (CN) and DRG neurons were isolated and dissociated from PND5 and PND1 animals respectively. After incubation with aggregated Fc chimeras (1:10 ratio of Fc to anti-human-Fc) cells were washed, spun and bound cpm were counted. (a) Neurons were incubated with Fc-chimera (MAG-Fc or control MUC18-Fc) r in the presence of 33ug/ml of anti-MAG 513 antibody (+anti-MAG). (b) Neurons were incubated with MAG-Fc after treatment with 50mU of sialidase (+VCS), or buffer alone (Buffer), or without any treatment (MAG-Fc).

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To compare the sialic acid-dependent binding of neurons other than postnatal cerebellar and PND1 DRG neurons, superior cervical ganglia (SCG), spinal cord neurons, hippocampal neurons and retinal ganglion cells (RGC) were isolated from postnatal ages, PND2 and PND9. We already know that MAG promotes neurite outgrowth from PND1 DRG neurons, but inhibits outgrowth from these neurons from PND4 onwards (De Bellard et al., 1996; Mukhopadhyay et al., 1994), whereas we do not know if this change is reflected in differences in MAG binding as a function of the different postnatal ages.

MAG-Fc was immunoadsorbed to an ELISA 96-well dish, in a non-radiolabelled binding assay, by adsorbing anti-Fc first, then MAG-Fc, guaranteeing the correct orientation for MAG-Fc (Fig. 3). A single-cell suspension of neurons vitally labeled with the fluorescent dye Calcein AM was added to each well (100,000 cells/well), allowed to bind, washed, and the number of cells bound was determined with a FluorImager.

As shown in Fig. 4, SCG, spinal, hippocampal and RGC neurons all bind to MAG, as do cerebellar and DRG neurons at both postnatal ages tested. Only background binding was detected to MUC18-Fc. Moreover, the binding of MAG to all these types of neurons is sialic acid-dependent, as it is for cerebellar and DRG neurons, because when the neurons were desialylated before the assay, binding to MAG-Fc is abolished. However, there were differences in binding for the different types of neurons and differences within the same type of neuron at the two different ages used. The sensory neurons (DRG and SCG) and the CNS neurons, from cerebellum and RGC, gave the highest binding at the two postnatal ages tested. In contrast, hippocampal neurons had the lowest binding, followed by spinal neurons at both PND2 and PND9. These differences in binding among the different neuronal populations are most likely due to the characteristics of the solid phase binding assay, and not inherent to the neurons, and because the suspensions we used are not purely neuronal. This will be discussed later.

The total binding at PND9 was lower than binding at PND2 for all the neurons tested except for SCG. This is most likely to be because with SCG neurons (which are not myelinated) the ratio of neurons to glia will not vary much between the two postnatal ages used, while for DRG and the other CNS neurons used it will because their Schwann and satellite cells are dividing. This decrease in binding may be due to: a decrease in the amount of receptor per neuron or because at this older postnatal age, the increased amount of surface proteins and carbohydrates may impede access to the putative receptor binding site. In addition, it could be

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due to the greater number of non-neuronal cells at later postnatal ages, like astrocytes, fibroblasts, or microglia, which are also counted as part of the total cell suspension used in the binding assay. Of all these possibilities, the last one seems to be the most likely, because of previous data observed for cerebellar neurons. When we carried out a soluble binding assay with this type of neuron at different postnatal ages (PND2, 5, 7, 9, 12, 21 and 30), we found an increase in binding of MAG-Fc to this cell suspension with time. That is to say, binding of neurons to MAG increases with age.

These data show that MAG can bind in a sialic acid-dependent manner to other types of neurons besides cerebellar and dorsal root ganglia (DRG) neurons, irrespective of their age, of their type and how MAG affects their neurite outgrowth response.

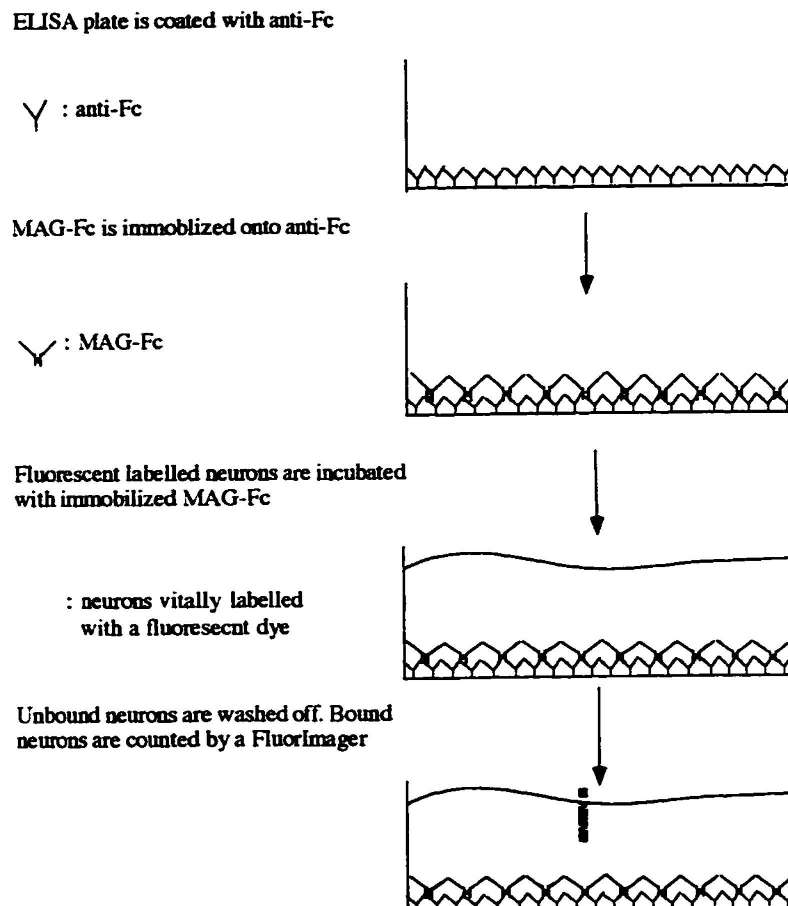

Fig. 3. Diagram of the Solid-Phase Binding Assay.

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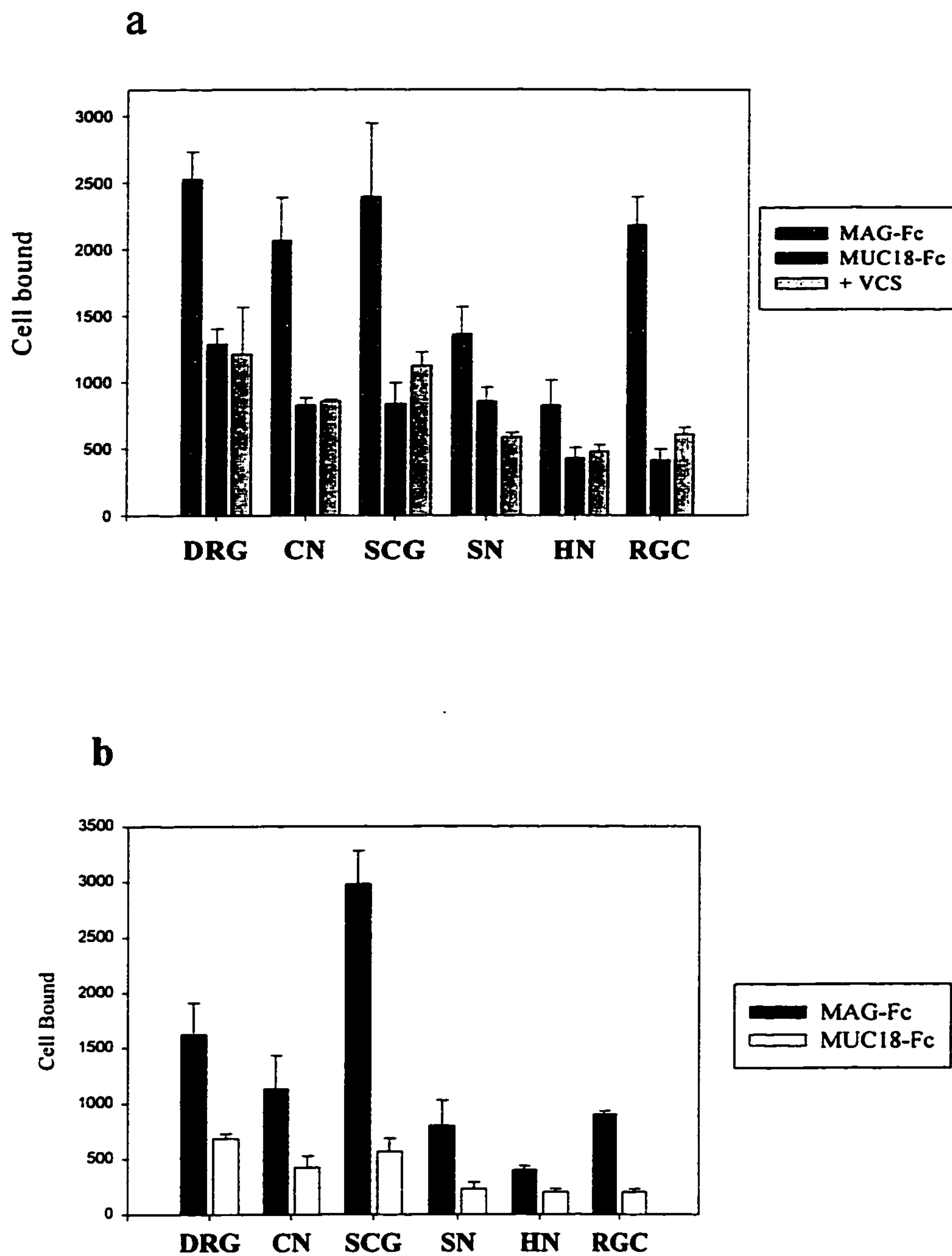

Fig. 4. Binding of a variety of neurons to MAG-Fc. Dissociated DRG, cerebellar (CN), SCG, spinal (SN), hippocampal (HN), and retinal ganglion (RGC) neurons vitally labeled with calcein AM, were isolated from (a) PND2 and (b) PND9 animals, allowed to bind to either MAG-Fc or MUC18-Fc immobilized at a concentration of 10ng/ml, on a 96-well dish. 100,000 cells were added to each well and after incubation and washing, the number of cells bound was quantified with a FluorImager. In (a) +VCS corresponds to binding after sialidase treatment.

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MAG interacts with a sialic acid glycoprotein

We have shown that MAG binds to a component on the neurons, and that because of this interaction the length of their neurites is affected. However, the nature and the identity of the receptor for MAG are unknown. In order to determine if this putative receptor is a glycoprotein or a glycolipid, an enzymatic assay was carried out. For this purpose, isolated cerebellar and DRG neurons (PND4-6) were treated with trypsin before the binding assay to test if the receptor is susceptible to this enzyme. When this assay was performed on both types of neurons, binding was reduced by about 50% after trypsinization (0.5%) for 45min (Fig.5). Control binding was taken as background binding to the wells or binding to MUC18-Fc. These results suggest that the putative receptor is a protein, or involves a protein component, since it was partly sensitive to trypsin.

We used a concentration of trypsin that is double that routinely used for cell isolation. Under these conditions, 90% of the cells were viable; they excluded trypan blue and were able to grow and extend neurites on laminin. Therefore, the reduction in binding to MAG that we observed is not due to damaging the cell membrane or absence of neurons because we disrupted them with the trypsin. The fact that binding was not completely abolished may come from lack of accessibility of the receptor to the trypsin, which can occur if these neurons have abundant surface carbohydrates (glyco-calyx) on their surface.

In summary, these results show that the putative receptor for MAG on DRG neurons and postnatal cerebellar neurons involves a sialoglycoprotein.

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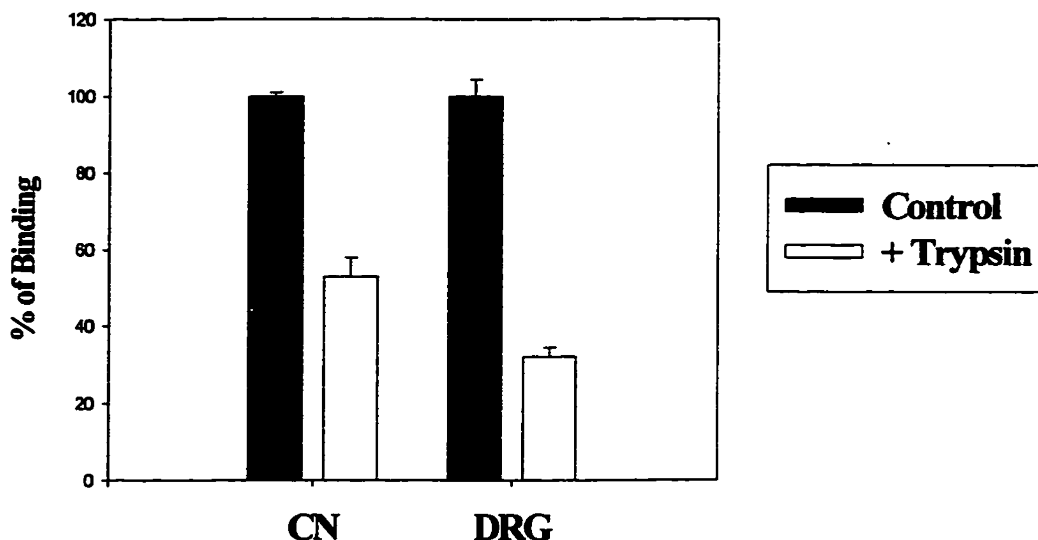

Fig. 5. Trypsin sensitivity of cerebellar (CN) and DRG neurons binding to MAG-Fc. Dissociated cerebellar and DRG neurons were vitally labeled with calcein AM and one-half of the population was treated with 0.5% for 45 min prior to binding assay. 100,000 cells were added to each well and after incubation and washing, the number of neurons was quantified with a FluorImager. Solid bars, untreated neurons binding to MAG-Fc; white bars, trypsin-treated neurons binding to MAG-Fc immobilized at a concentration of 10ug/ml. Results (±SEM) are from two experiments carried in quadruplicate

MAG Inhibits Neurite Outgrowth from Different Neuronal Types

It is known that MAG promotes neurite outgrowth from newborn DRG neurons and inhibits neurite outgrowth from all postnatal cerebellar neurons (Johnson et al., 1989; Mukhopadhyay et al., 1994). However, we do not know if other types of postnatal neurons respond to MAG, and if so, if their response is similar to cerebellar or DRG neurons. Hence, the same type of PND2 and PND9 neurons used in the binding assay were now used in a neurite

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outgrowth assay. This assay consists of coculturing postnatal neurons on transfected CHO cells expressing MAG (MAG) or control transfected CHO cells (Control). After overnight incubation, the cultures are fixed and the average length of GAP43-positive neurites from at least 120 neurons was determined.

The neuronal preparations used are not homogeneous populations, but include other types of cells that are neither neurons nor the primary type of neuron from the region. Because of this, the neurite measurements were carried out trying to adhere to one type of neuron by morphology for each of the populations used. This confinement to one kind of neurons was achieved as judged from being GAP43-positive and very importantly, their distinct morphology. For example, cerebellar granular cells are distinctly bipolar and have about 8um diameter; hippocampal pyramidal neurons are bigger, not bipolar, and have dendrites and a distinct axon; spinal neurons have morphologies somewhat similar to hippocampal pyramidal neurons.

The extent of inhibition by MAG ranged from 40% to 80% for all the neurons tested, at either postnatal age, compared to growth on control CHO cells (Fig. 6 and Fig. 7). These values are comparable to previous findings with postnatal cerebellar neurons (70%) and adult DRG neurons (40%) (Mukhopadhyay et al., 1994). The greatest inhibition was of RGC (80%) and the least was for spinal neurons (40%).

Another trend is apparent after comparing the same type of neuron at the two postnatal ages tested. The average length of neurites on the control CHO cells decreased at PND9 compared with the average length at PND2 for the same coculture time. That is to say, the neurites from PND9 neurons are shorter than the ones from PND2 neurons. However, the inhibition of neurite length by MAG was still within the same range, at both postnatal ages tested (Fig.6a & b). The reason for this general shortening of the total length of the neurites with age may be because the capacity of primary neurons to grow long neurites during *in vitro* culture decreases with age. This response of older neurons is a well-known phenomenon of *in vitro* culture of primary neurons (Goslin and Banker, 1989).

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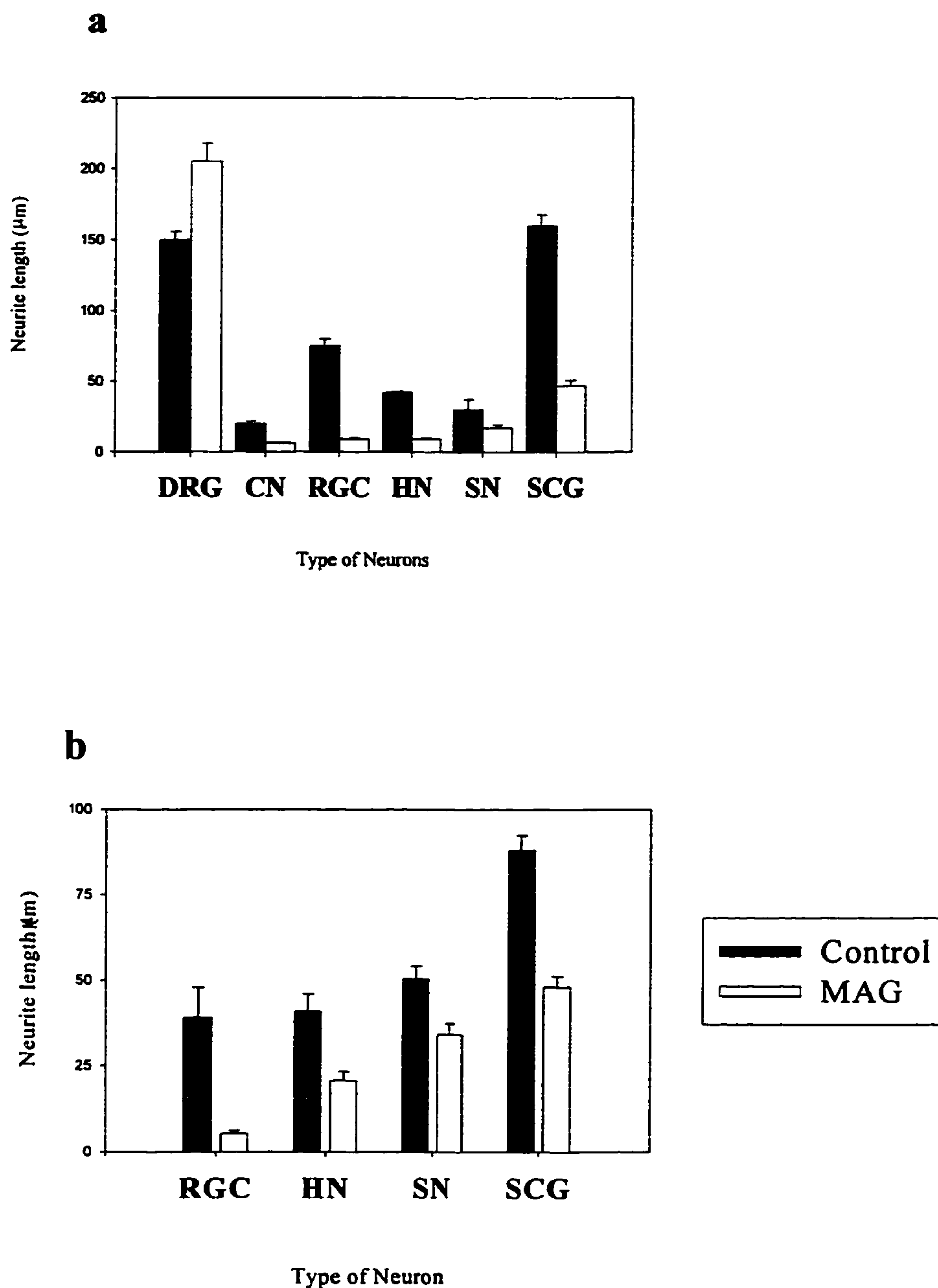

Fig. 6. The effect of MAG on neurite outgrowth from a variety of postnatal neurons. Dissociated DRG, cerebellar (CN), retinal (RGC), hippocampal (HN), spinal (SN) and SCG neurons from PND2 (a) or PND9 (b) were cultured for 18h on confluent monolayers of control or MAG-expressing CHO cells. After fixing and being immunostained for GAP43, the neurite length was measured. Results show the mean length of the longest neurite per cell (±SEM) for 120 individual neurons.

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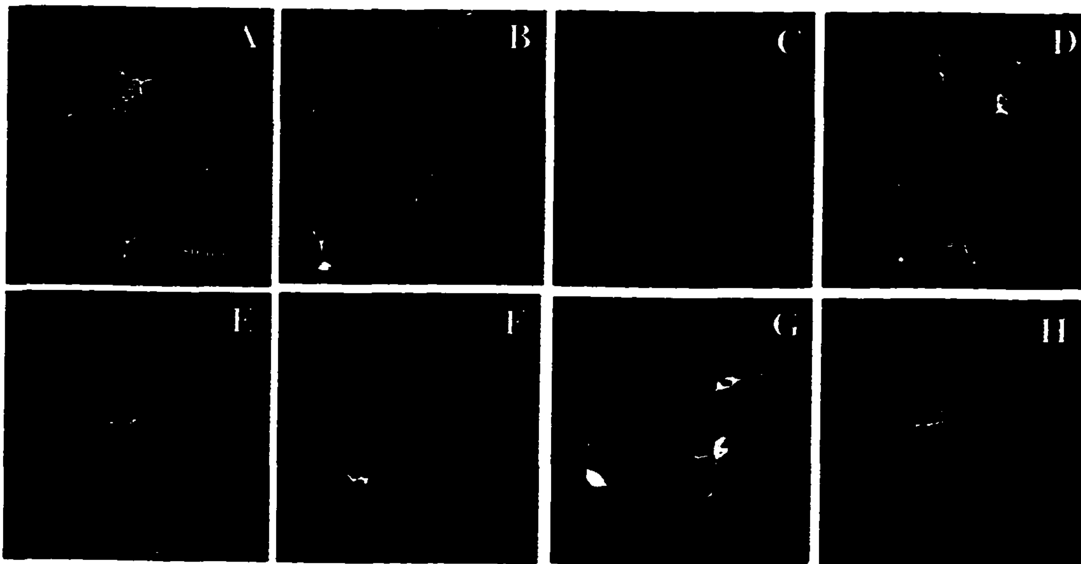

Fig. 7. The effect of MAG on neurite outgrowth from a variety of postnatal neurons. Dissociated retinal (A,E), hippocampal (B,F), spinal (C,G) and SCG (D,H) neurons from PND2 were cultured for 18h on confluent monolayers of control (A-D) or MAG-expressing CHO cells (E-H), before fixing and immunostained for GAP43.

To ensure that the effect of MAG-expressing CHO cells is not an artifact of transfection, a CHO cell line expressing another adhesion molecule and Ig-family member, Po, was used.

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When SCG, retinal, spinal and hippocampal neurons were cocultured on top of these Po-expressing CHO cells, there was no effect on the length of neurites from any of these neurons (Fig. 8). However, this lack of response from these neurons to Po is not because of its small size, since we have found that if DRG neurons are cocultured on Po-expressing CHO cells in the presence of NGF, their neurites are promoted by Po (results not shown).

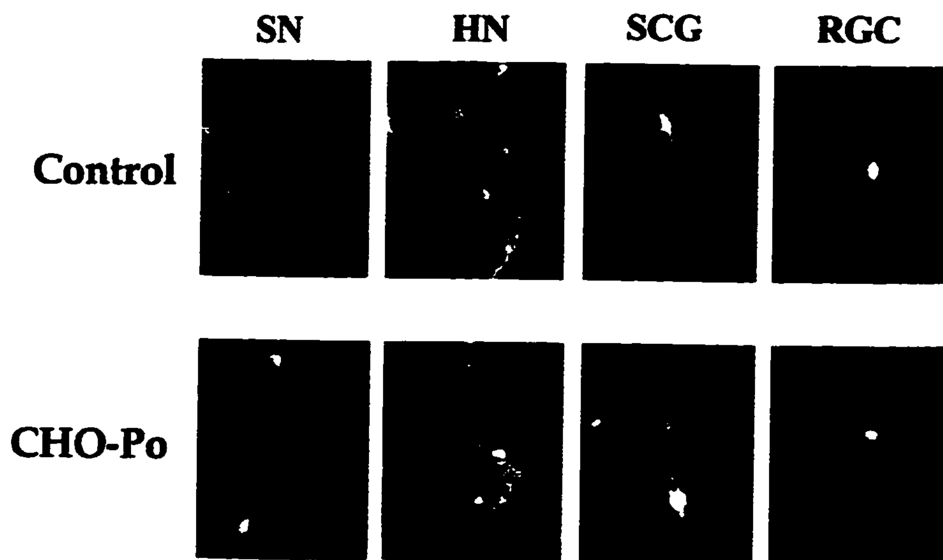

Fig. 8. The effect of myelin Po protein on neurite outgrowth from a variety of postnatal neurons. Dissociated retinal (RGC), hippocampal (HN), spinal (SN) and SCG neurons from PND2 animals were cultured for 18h on confluent monolayers of control or Po-expressing CHO cells. After fixing and immunostained for GAP43 the neurite length was measured. Results show the mean length of the longest neurite per cell (±SEM) for 120 individual neurons.

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To date, of all the postnatal neurons tested, only PND2 DRG neurons are promoted by MAG. For all other neurons tested (cerebellar, retinal, spinal, hippocampal and SCG neurons) MAG is a potent inhibitor of axonal regeneration.

These data and the results from the binding assay strongly suggest that the putative receptor for MAG is present on all the types of neurons tested.

MAG Inhibition of Neurite Outgrowth Involves a Sialic Acid-bearing Glycoconjugate

We have shown two distinct aspects of the interaction of MAG with neurons. First, we have shown that MAG binds to different types of neurons, of different postnatal ages, in a sialic acid-dependent manner and, second, that MAG can affect neurite outgrowth from different types of postnatal neurons. Nevertheless, we do not know if, or how, these results are linked. That is to say, is sialic acid-dependent binding involved in promotion and/or inhibition of axonal regeneration? To address this question, two approaches were taken. In one, neurons were desialylated to remove all the sialic acid on the surface of neurons before culturing them on MAG-expressing CHO cells. The other approach was to add small sialic acid sugar analogs to the media as competitors for MAG binding to the putative receptor on the neurons during the neurite outgrowth assay.

The results in Fig. 9a show that when cerebellar neurons are desialylated before the neurite outgrowth assay, the neurites on MAG-expressing cells were almost twice as long as when not desialylated. Moreover, when neurons were cultured in the presence of the sialidase enzyme to minimize sialic acid replacement, the neurites on the MAG-expressing CHO cells were even longer. A similar treatment of cerebellar neurons plated on control CHO cells showed

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no change on the length of extended neurites. From these results, we can conclude that the inhibition of neurite outgrowth by MAG can be reversed by about 50% by the removal of sialic acid residues from the neurons.

In contrast, when PND1 DRG neurons (Fig. 9b) were desialylated, the promotion of neurite outgrowth by MAG was completely reversed. That is to say, the length of neurites on MAG-expressing cells was the same as on control CHO cells. Addition of sialidase to the culture media during coculture had no further effect on the length of the neurites growing on MAG-expressing cells. This indicates that the background neurite outgrowth from PND1 DRG neurons on control cells does not depend on a sialic acid bearing component on their surface and shows also that the promotion is specific for MAG.

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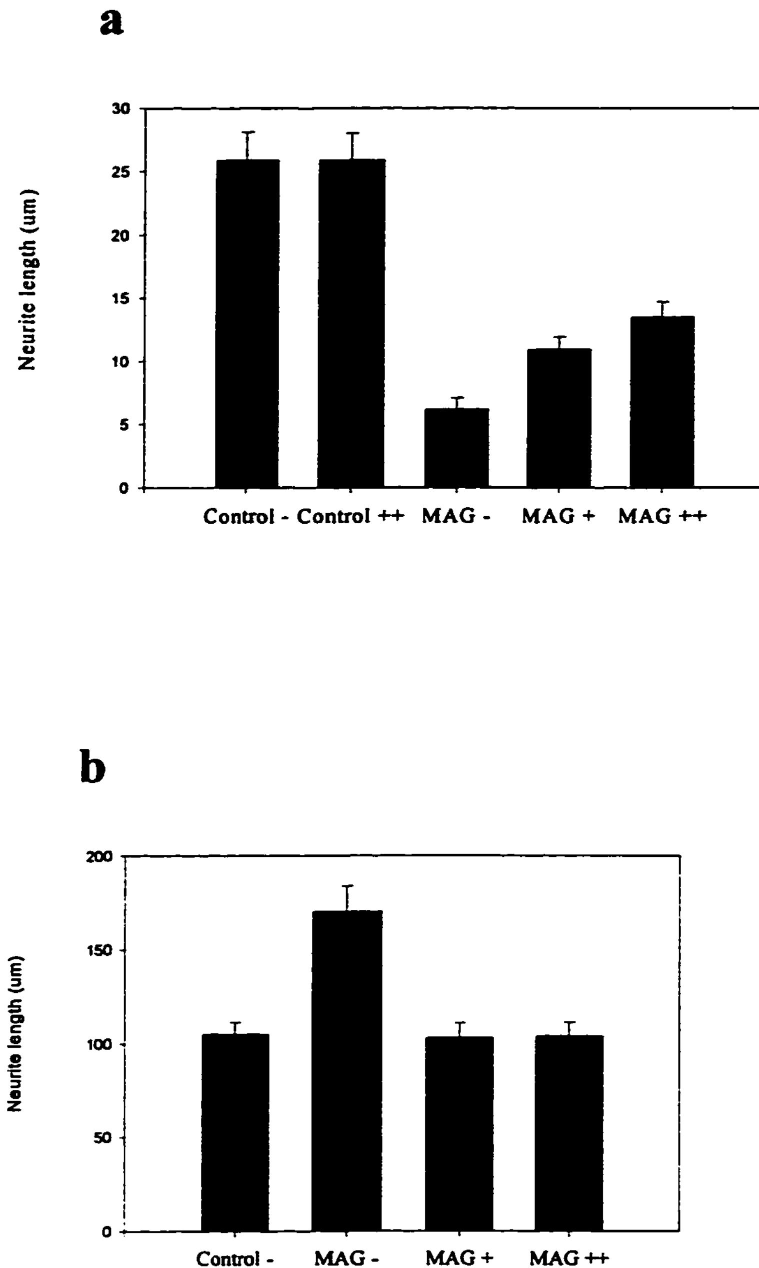

Fig. 9. The effect of MAG on neurite outgrowth from desialylated PND1 cerebellar (a) and DRG (b) neurons. Neurons from PND1 animals were treated with (+) or without sialidase (-) before being cultured for 18h on confluent monolayers of MAG-expressing (MAG) or control CHO cells. After fixing and immunostaining for GAP43 the neurite length was measured. Results show the mean length of the longest neurite per cell (±SEM) for 120 individual neurons. Where indicated (++) sialidase was also included in the cocultures.

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An alternative method of assessing if the inhibitory effects of MAG on neurite outgrowth are also sialic acid-dependent is to include small sialic acid sugars in the assay to block inhibition. Two sialic acid analogs were chosen. One, 2,3-dideoxy neuraminic acid (DDNANA), was used because it is a sialidase competitor, so it can compete for MAG with the sialic acid bearing component on the neuron. The other was α2,3-sialo-lactose (2,3-SL). 2,3-SL is a trisaccharide that has the same α2,3 sialic acid which is required for binding of MAG to neurons (Collins et al., 1997; Kelm et al., 1994) and is a substrate for sialidase. As controls, α2,6-sialo-lactose and lactose were used. The former is also a trisaccharide but with a different linkage (2,6 instead of 2,3) for MAG binding.

When each of these small sialic acid-bearing sugars (DDNANA and 2,3-SL) were included in the cerebellar cocultures, either was able to reverse inhibition of neurite outgrowth by MAG in a dose-dependent manner (Table I). At 20mM, DDNANA reversed inhibition by 64%, while 2,3-SL at 5mM reversed inhibition by 42%. When the 2,3 SL oligosaccharide was increased to 9mM, it had a toxic effect on the neurons, and the reversal of inhibition by MAG went from 42% to 26%. The control sugars, 2,6-SL (5mM) and lactose (9mM), did not have an effect on cerebellar neurite outgrowth on MAG-expressing or control CHO cells.

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TABLE I.

Neurite Outgrowth from Cerebellar Cells in the Presence of Small Sialic Acid-Sugars

Sugar	Conc. (mM)	Cell monolayer	Neurite length (μm)	% Reversal of Inhibition
DD NANA	0	control	20.1±1.7	0
DD NANA	20	control	21.7±2.4	0
DD NANA	0	MAG	6.0±0.6	0
DD NANA	1	MAG	8.2±0.8	15%
DD NANA	10	MAG	12.4±0.8	45%
DD NANA	20	MAG	15.0±0.9	64%
2,3 SL	0	control	21.9±2.5	0
2,3 SL	9	control	18.8±1.8	0
2,3 SL	0	MAG	6.4±0.6	0
2,3 SL	1	MAG	8.2±0.7	11%
2,3 SL	5	MAG	12.9±1.3	42%
2,3 SL	9	MAG	10.4±1.1	26%
Lactose	9	MAG	5.7±0.6	0
2,6 SL	5	MAG	8.9±±.9	0

DDNANA = 2,3 Dehydro-deoxy neuraminic acid
2,3 SL = 2,3 sialo lactose
2,6 SL = 2,6 sialo lactose

When PND1 DRG neurons were grown on MAG-expressing CHO cells in the presence of the sialic acid analogs, promotion of neurite outgrowth was blocked by 100% (Table II). DDNANA (20mM) and 2,3α SL (9mM) prevented promotion of neurite outgrowth by MAG completely. Promotion of neurite outgrowth by MAG was unaffected by the presence of the control sugar used, lactose. In addition, the length of DRG neurites grown on control CHO cells was not affected by 20mM DDNANA. In brief, the reversal of promotion of neurite outgrowth by MAG on PND1 DRG neurons is specific for the sialic acid sugar analogs used.

Throughout these experiments, we did not observe any change in the plating efficiency of either type of neurons on either MAG-expressing or control CHO cells.

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TABLE II.

Neurite Outgrowth from DRG neurons in the Presence of Small Sialic Acid-Sugars

Sugar	Conc. (mM)	Cell	Neurite length (μm)	% Reversal of Promotion
DD NANA	0	control	150.3±6.0	0
DD NANA	20	control	157.9±5.5	0
DD NANA	0	MAG	191.2±11.0	0
DD NANA	1	MAG	204.7±13.0	0
DD NANA	10	MAG	170.3±8.3	51%
DD NANA	20	MAG	151.5±8.0	97%
2,3 SL	0	control	91.0±6.3	0
2,3 SL	0	MAG	120.0±4.3	>100%
2,3 SL	1	MAG	132.0±3.8	>100%
2,3 SL	5	MAG	92.6±4.2	96%
2,3 SL	18	MAG	92.6±4.2	96%
Lactose	9	MAG	187.0±8.3	> 100%

DDNANA = 2,3 Dehydro-deoxy neuraminic acid
2,3 SL = 2,3 sialo lactose
2,6 SL = 2,6 sialo lactose

In order to determine if the reversal of inhibition of neurite outgrowth by MAG can be improved (more than the 60% obtained with simple sugars), we used sugar moieties from gangliosides because they are more physiological and are in a natural "aggregate" form. Dr. Ronald Schnaar (Johns Hopkins) provided the two carbohydrate moieties after enzymatic removal from the respective gangliosides. GM1 ganglioside was purified from brain, while GT1b was chemically synthesized *de novo* (Collins et al., 1997).

When postnatal cerebellar neurons were grown on MAG-expressing cells in the presence of either type of sugar moiety, the inhibition by MAG was reversed to about 50% (Table III). This reversal of neurite outgrowth inhibition was slightly better for the GT1b carbohydrate, which carries several α2,3-linked sialic acid sugars, while GM1 carries only one (Collins et al., 1997). Although we did not observe an improved reversal of inhibition of neurite outgrowth by MAG with these two types of carbohydrate beyond 50%, we were able to get the same reversal

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with a 1000 fold less (50μM) sugar in the medium than with DDNANA or 2,3 SL. Due to the difficulty in their preparation and small volumes available, we were not able to use them at higher concentrations. In brief, the complex carbohydrates from the gangliosides are more efficient in reversing inhibition by MAG, than the simple sugars used in the two previous assays.

TABLE III.

Neurite Outgrowth from DRG neurons in the Presence of Ganglioside Sugars

Oligosaccharide	CHO cell	Concentration (μM)	Neurite length (μm±SEM)
Gt1b	Control	0	31.2±2.7
	MAG	0	6.8±0.6
	MAG	0.5	9.9±0.9
	MAG	5	11.6±1.2
	MAG	10	14.6±1
	MAG	50	17.2±1.5
GM1	Control	0	33.2±2.9
	MAG	0.5	7.4±0.7
	MAG	5	10.9±1.3
	MAG	10	12.2±1

These results, together with those obtained when the neurons were desialylated, suggest that the effect of MAG on axonal regeneration depends on its interaction with a neuronal sialylated glycoprotein.

Sialic Acid-dependent Binding can be Dissociated from Inhibition of Neurite Outgrowth

To test further the role of the sialic acid-dependent binding to neurons in inhibition of neurite outgrowth another member of the sialoadhesins was used in the neurite outgrowth assay. Sialoadhesin, also an Ig superfamily member, was chosen because it binds to O- or N-linked 2,3-

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αsialic acids (MAG binds to a2,3-O-linked) (Crocker et al., 1994) and can bind to neurons such as MAG in a sialic acid-dependent manner (Fig. 10) (Tang et al., 1997). If the effect of MAG on neurite outgrowth depends solely on a 2,3-α linked sialic acid binding site, then sialoadhesin should also affect neurite outgrowth. Three lines of sialoadhesin-expressing CHO cells were used, one expressing the whole extracellular region Ig domains1-17, and the other two expressing a truncated form of this protein, the first 1-6 Ig domains. When postnatal cerebellar neurons were grown on the different sialoadhesin-expressing CHO cell lines, the length of the neurites (Fig. 11) was the same as on the control cells. Under the same conditions, the length of neurites on the MAG-expressing CHO cells was 70% shorter compared to their length on control CHO cells. The three sialoadhesin-expressing CHO cell lines used did not significantly affect the neurite length, which was always as long as on control CHO cells.

This result indicates that the binding of sialoadhesin expressed by CHO cells to sialic acid bearing molecules on the cerebellar membrane is not sufficient to induce inhibition of neurite outgrowth.

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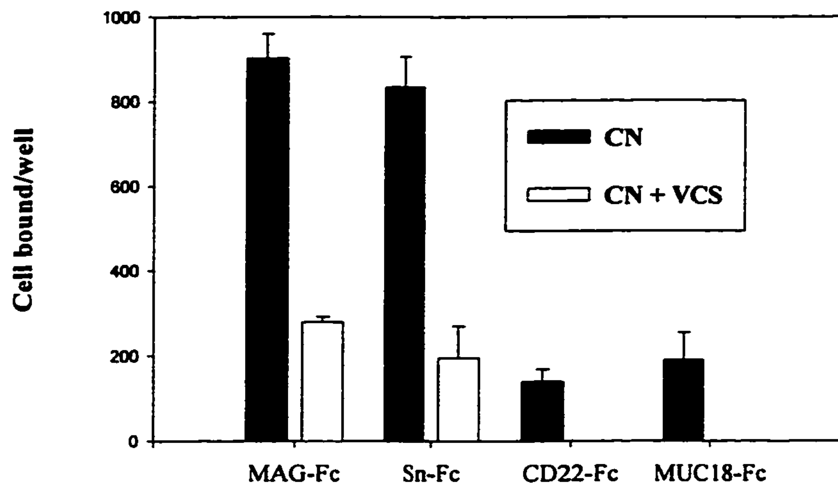

Fig. 10. Binding of cerebellar neurons to different sialoadhesins Fc-chimeras. Dissociated PND4 cerebellar neurons, vitally labeled calcein AM, were allowed to bind to MAG-Fc, sialoadhesin (Sn-Fc), CD22-Fc, or MUC18-Fc immobilized at a concentration of 10ug/ml, on a 96-well dish coated with anti-Fc antibody. 200,000 neurons, either pre-treated with sialidase (blank bars) or not (solid bars) were added to each well and after incubation and washing, the number of cells bound was quantitated with a FluorImager. Results are from 3 experiments each with 10 samples, and represents the mean±SEM.

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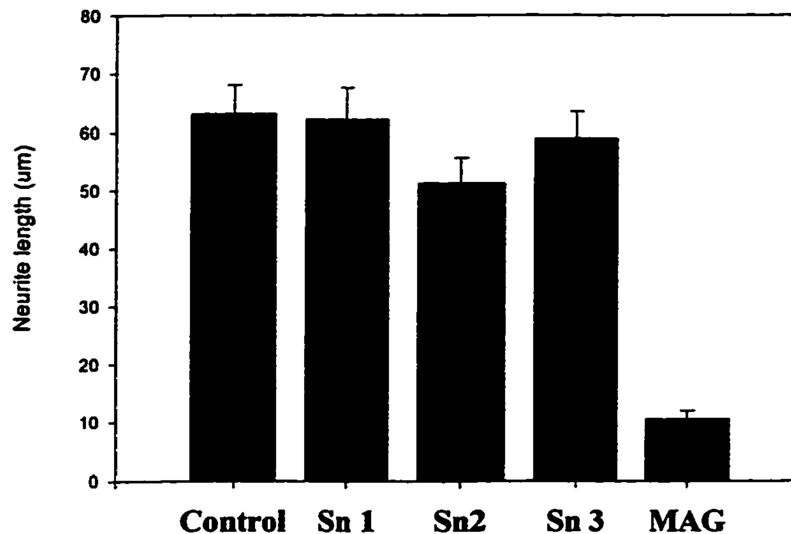

Fig. 11. The effect of sialoadhesin expressed by CHO cells on neurite outgrowth. Dissociated PND4 cerebellar neurons were cultured for 18h on confluent monolayers of MAG-expressing (MAG) or sialoadhesin-expressing (Sn) or control (Control) CHO cells. Sn1 and Sn2 correspond to Sn(d1-17), Sn3 corresponds to Sn(d1-6). After fixing and immunostained for GAP43 the neurite length was measured. Results show the mean length of the longest neurite per cell (±SEM) for 120 individual neurons.

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Discussion

The results presented here show that the inhibitory effects of MAG on neurite outgrowth are widespread for postnatal neurons from different regions of the nervous system. Neurite outgrowth from cerebellar, retinal, spinal, hippocampal, DRG and SCG neurons was inhibited by MAG between 40 and 90%. This inhibitory effect is specific for MAG as shown when CHO cells expressing another Ig-family member Po or sialoadhesin have no effect on the length of the neurites. That is to say, expression of a foreign protein in CHO cells is not "diluting" their ability to allow neurite extension. This specific effect on neurite outgrowth by MAG was further demonstrated when: 1) adding MAG antibodies reversed the inhibition (Mukhopadhyay et al., 1994); and 2) adding a soluble form of MAG, MAG-Fc was also capable of inhibiting neurite outgrowth (Tang et al., 1997).

We also show that MAG binds specifically to postnatal neurons, as do the sialoadhesins, via sialic acid, and that this binding occurs regardless of whether MAG causes promotion or inhibition of neurite outgrowth. In addition, when neurons were desialylated or when we added small sialic acid analogs to the coculture media, we were able to partially block inhibition and completely block promotion of neurite outgrowth by MAG.

We first tested if MAG, as a sialoadhesin family member, would also bind to neurons in a sialic acid-dependent manner. Two similar binding assays (a soluble and a solid) were used, with each taking advantage of a chimeric form of MAG, MAG-Fc. The results from both binding assays showed distinctly that MAG binds specifically to neurons in a sialic acid-dependent manner because if neurons were desialylated before the assay or a MAG antibody was included, binding was abolished. It should be noted that neither of these assays is a classical binding assay in which the Kd and Bmax of the receptor ligand:interaction can be measured. The reason for this limitation is that, in the soluble assay we do not know how much MAG-Fc is in the aggregates, and in the solid assay we only measure the number of neurons that bound to MAG.

We have shown that MAG binds specifically to a sialylated component on the surface of postnatal cerebellar, retinal, hippocampal, spinal, DRG and SCG neurons. Since this binding is also trypsin sensitive, a sialoglycoprotein rather than a sialoglycolipid, is most likely involved in

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the interaction of MAG with the neurons. Sialic acid-dependent binding occurred regardless of promotion or inhibition by MAG or age of the neurons (PND2 or PND9). We noticed that binding of the different neuronal types to MAG at PND2 was always higher than binding at PND9. This difference in binding for different neurons may be explained by the percentage of other cell types present in the cell suspensions used in the binding assay. The single cell suspensions we routinely used were prepared from particular brain regions, without any separation of neurons from other non-neuronal cells. At PND2 all the regions used for binding are comprised of about 90% neurons, but with age, the non-neuronal populations increases rapidly, thus reducing the proportion of neurons in the cell suspension at PND9. In particular in the spinal cord and the hippocampus, there could be up to 40% non-neuronal cells (Jacobson, 1991). Other regions, such as cerebellum, remain largely neuronal, where approximately 90% of the cells are of granular type at PND9 (Jacobson, 1991). In addition, for the solid-phase assay, the size of the neurons can make a difference in the total cells that fit as a monolayer in the Elisa wells. Consequently, this limits the number of cells of a particular type of neuron that can be added. In other words, measurements from the solid-phase binding assay are more a reflection of the size of the neuron rather than the affinity of a particular neuron for MAG. However, these differences between both binding assays do not affect the affinity of the neurons for MAG. In our studies the biggest cells used were DRG and SCG neurons, of which only 100,000 made a cell monolayer in the Elisa wells.

To date, of the postnatal neurons tested (cerebellar, DRG, retinal, spinal, hippocampal and SCG neurons), only PND1-3 DRG neurons are promoted by MAG (De Bellard et al., 1996). All the other postnatal neurons tested are inhibited by 40-90%when growing on MAG-expressing cells. However, it is possible, that these neurons which at postnatal ages are only inhibited by MAG, at embryonic ages respond differently, as it has been found for hippocampal neurons, response to NCAM and N-Cadherin (Doherty et al., 1992). Indeed, it has been reported, that embryonic retinal ganglion (not quantitated) and spinal neurons are promoted by MAG (Salzer et al., 1990; Turnley and Bartlett, 1998). It is also possible that subpopulations within any neuronal type will respond to MAG differently, while the overall response is inhibition.

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Since MAG can either promote or inhibit neurite outgrowth, we conclude that MAG is a bifunctional molecule. Recently, another family of molecules, the netrins, has been shown to have a bifunctional effect on axonal outgrowth. Netrin I can be a chemoattractant for commisural axons (Kennedy et al., 1994; Serafini et al., 1994) but a repulsive molecule for trochlear motor axons (Colamarino, 1995). There are two possible explanations for this bifunctionality: 1) there is a separate receptor on the neurons for promotion and inhibition of neurite outgrowth; or 2) the receptor is the same but has two different downstream pathways that occur at different times or in different neurons. How this change in response to MAG or netrin takes place has recently begun to be elucidated when it was shown that elevating the intracellular cAMP levels, for both netrin (Ming et al., 1997; Song et al., 1997) and MAG (Cai et al, 1998) was able to reverse the chemoattractant or inhibitory effects of these molecules respectively. In other words, these findings suggest that the receptor for MAG is the same at both ages, however the downstream pathway would be different at different ages and/or neurons.

There are other inhibitors of axonal regeneration in the adult brain (Davies et al., 1997; Faissner, 1990; Ghosh and David, 1997; McKeon et al., 1991), in the developing brain (Drescher, 1995; Puschel et al., 1995) and especially in myelin (Schwab et al., 1993). To date MAG is one of the best characterized of all those inhibitors present in myelin (Filbin, 1996; Filbin, 1995). MAG, like other myelin-specific proteins, has been observed to be expressed postnatally after the axon has reached its target and myelination has begun (Martini and Schachner, 1986; Quarles, 1983). This would suggest that MAG does not have a role in axonal guidance during development. However, in apparent contradiction to the common dogma that myelin proteins are only expressed after an axon has reached its target, a number of myelin-specific proteins have now been shown to be expressed by migrating, immature oligodendrocytes and Schwann cells (Jessen et al., 1994; Yu et al., 1994). It remains to be determined whether MAG is present during embryonic development as are Po, CNP and PLP, and therefore may be capable of playing a role in axonal guidance. To date, the studies we report here have focused on MAG as an inhibitor of axonal regeneration. Nonetheless, in view of the finding that there are oligodendrocytes in the developing nervous system expressing myelin proteins (Jordan et al., 1989), together with knowing that these cells may express MAG, makes it interesting to investigate a possible role for MAG as an axonal guidance molecule during development.

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We have shown that MAG binds to a sialoglycoprotein on the surface of postnatal cerebellar, DRG, spinal, hippocampal, retinal and SCG neurons in a sialic acid-dependent manner. This binding was regardless of whether neurite outgrowth from these neurons was inhibited or promoted by MAG. More important, desialylation or adding small sialic-bearing sugars reversed the promoting effect of MAG by 100%, and the inhibition by up to 50%. This finding strongly suggests that the binding we observed of MAG to a sialoglycoprotein on the neurons is involved directly or indirectly in the signaling that affects axonal regeneration.

It is unexpected that the monomeric analog of sialic acid DDNANA, is able to reverse MAG's effect on neurite outgrowth, while sialic acid in the wrong linkage (α2,6 instead of α2,3) has no effect. A possible explanation for this finding emerges if we consider another sialoadhesin family member, CD22, which has been suggested to carry a "pocket" where the sialic acid-dependent binding site is located (Powell and Varki, 1995). Since MAG and CD22 have a strong homology in their first 4 Ig-domains, a similar binding pocket is likely for MAG. In this way a monomeric sialic acid may fit well into the pocket, while a sialic acid with an α2,6-linkage adds steric hindrance and prevents sialic acid from reaching its site of interaction. Furthermore, the effects of these sialic acid sugars are specific for the inhibition of neurite outgrowth by MAG, as they have no effect on background neurite outgrowth from either cerebellar or DRG neurons growing on control cells.

The results from binding assays together with the reversal of the effect of MAG by oligosaccharides suggest several points about the type of interaction of MAG with its putative receptor. The need to aggregate MAG-Fc before binding to increase its avidity supports the suggestion that MAG like other sialoadhesins binds to its receptor with low affinity. The same conclusion is drawn from the requirement for high concentrations (high millimolar) of small sugars needed to observe an effect on inhibition or promotion by MAG. Another suggestion that comes from the results shown so far is that this low affinity interaction of MAG with its receptor likely reflects the *in vivo* situation. That is to say, MAG may need to cluster within the membrane before stable interaction with its receptor is achieved. A similar need to cluster has been found for Eph receptor activation (Davies et al., 1994). These two points lead to the suggestion that a glycoconjugate with many sialic acid residues attached would be more effective at much lower concentrations than the simple sugars. This suggestion was strengthened when we observed that the carbohydrate moieties from GT1b or GM1 gangliosides were as effective as

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DDNANA or 2,3-SL but at a 1000-fold lower concentration. However, the fact that we never achieved full reversal of inhibition of neurite outgrowth by MAG after desialylation of neurons or by adding small sialic acid sugars, further suggests that the sialic acid binding site of MAG has a low affinity for the receptor. Since we never fully reversed inhibition with those two approaches as it did promotion, we conclude that the sialic acid binding site alone is not sufficient for inhibition of axonal growth. However, it may be enough to confer promotion of neurite outgrowth, because we were able to reverse completely promotion by desialylation of neurons or adding small sialic acid sugars. There are several possible explanations for this difference. First, the replacement of sialic acid on the receptor for MAG in cerebellar neurons might be faster and more efficient than in DRG neurons and/or because cerebellar neurons might be harder to desialylate completely than the DRG neurons. Second, the sialoglycoprotein with which MAG interacts with on newborn DRG neurons may be different from the one it interacts with later in development, when it causes inhibition. Third, that another sialic acid-independent site/component is involved in the inhibition. Each of these possibilities does not exclude the other as any of them can be true, and the three can apply simultaneously. Nevertheless, the first explanation is the least probable, because the effect on neurite outgrowth inhibition of adding the sugars was dose dependent but never reached full reversal as they did on the promotion. For the second we do not yet have any information as to how many proteins bind and respond to MAG. The third one has been shown definitely to take place.

The conclusion that the sialic acid binding site is neither necessary nor sufficient for neurite outgrowth inhibition by MAG came from several experiments. The first one was to assess if sialoadhesin, which binds to neurons in a sialic acid-dependent manner, has any effect on neurite outgrowth. When cerebellar neurons were grown on sialoadhesin-expressing CHO cells, their neurite length was unaffected. That is to say, sialic acid binding of a protein homologous to MAG to cerebellar neurons is not sufficient to inhibit axonal regeneration. However, we do not know if sialoadhesin is indeed binding to the putative receptor for MAG. One way to test this possibility would be to determine if the binding of sialoadhesin to neurons is reversed with a specific antibody for the receptor. This experiment is not feasible yet, as the receptor is unknown and no antibody to it exists. A second clue on the nature of the sialic acid site came after mutations of CD22 and sialoadhesin mapped their sialic acid binding site to a specific arginine (Vinson et al., 1996), and that MAG also shares this arginine at the R118 site. This hypothesis

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was confirmed when we found that MAG mutated at R118 did not bind to neurons, showing that R118 site is the sialic acid binding site. However, when the mutated MAG was expressed in CHO cell lines (R118A to alanine, or R118D to aspartate), neurite outgrowth from cerebellar neurons was still inhibited up to 70% as with wildtype MAG (Tang et al., 1997). These two findings together implied the existence of yet another site on MAG that causes inhibition of neurite outgrowth. This suggestion was further supported when: 1) a soluble MAG-Fc, mutated at the R118 site could neither bind nor inhibit neurite outgrowth; 2) a truncated form of MAG-Fc (carrying only domains 1-3), which binds to neurons in a sialic acid-dependent manner, did not inhibit neurite outgrowth (Tang et al., 1997). A model to explain this distinctions by the different forms of MAG is shown in Fig. 12.

Since the sialic acid binding site of MAG is neither necessary nor sufficient for neurite outgrowth inhibition and the reversal of inhibition and promotion after sialidase treatment or adding sialic acid analogs to the media can be due to a charge effect, we cannot strictly affirm that the binding of neurons to MAG-Fc that we measured, corresponds to its physiological receptor.

To date, the inhibition or promotion of neurite outgrowth by MAG can be partially reversed by desialylation of the neurons or competition with small oligosaccharides. This observation suggests that the receptor on the neuronal surface depends on its sialic acid to interact with MAG on the glial membrane, but not in order to cause inhibition of neurite growth of all postnatal neurons tested.

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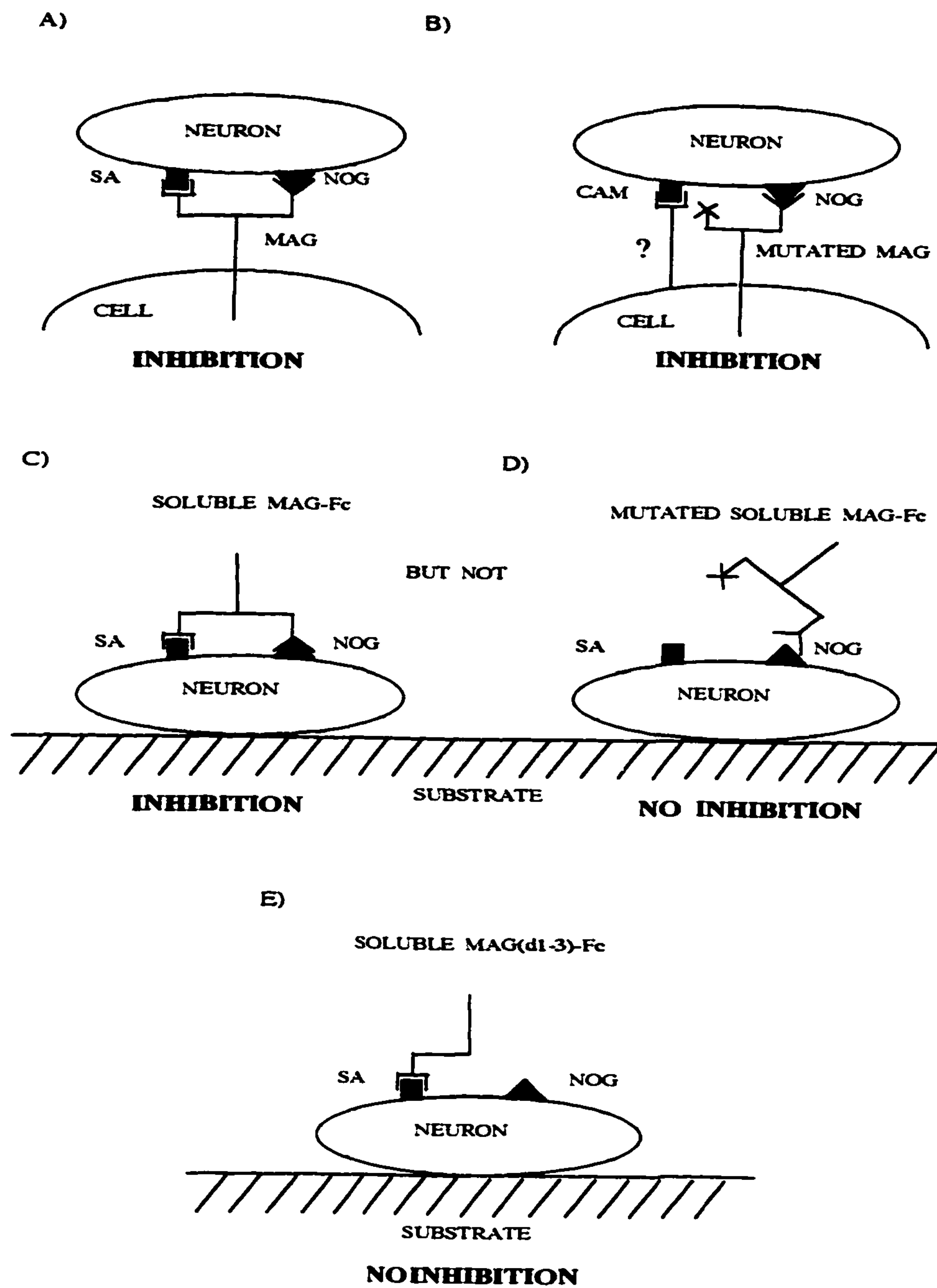

Fig. 12. Model for MAG on neurite outgrowth inhibition

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CHAPTER 2

Inhibition of Neurite Outgrowth by MAG in Myelin

Introduction

From the early studies on regeneration by Ramon y Cajal CNS white matter was suggested to be an unfavorable substrate for the growth of axons (Ramon y Cajal, 1968). However, it was not until the 1980s that several laboratories were able to test this hypothesis. In a key paper, Carbonetto showed that neurons will not grow on sections of white matter from the CNS and if possible avoid it when it is offered as a substrate (Carbonetto et al., 1987). Another study demonstrated that the inhibitory effect of CNS myelin on axonal regeneration can be reversed with an antibody to some of its components (Caroni, 1988; Caroni, 1988).

Recently, we (De Bellard et al., 1996; Mukhopadhyay et al., 1994) and others (McKerracher et al., 1994) have shown that MAG, a well-characterized component of myelin, is a potent inhibitor of axonal regeneration for a variety of neurons when expressed on CHO cells and is a sialic acid-dependent binding molecule (Kelm et al., 1994). Interestingly, the effect of MAG on inhibition of axonal regeneration can be partially reversed if neurons are desialylated before the outgrowth assay (Chapter 1 and (De Bellard et al., 1996)).

However, the contribution that MAG makes to the overall inhibition of regeneration by myelin, *in vivo* or *in vitro,* has yet to be determined. This is because previous studies with the MAG-deficient mice (MAG-/-) reported opposite results. One group found an improvement in the number of axons that grew after a spinal cord injury, as well an increase in the mean length of axonal regrowth (Li et al., 1996). The other group did not find any improvement in the extent of regeneration in the animals after injury (Bartsch et al., 1995). These conflicting results can be reconciled if we look closely at the data, and are mindful of the fact that the use of knockout animals has certain flaws when studying the function of any protein (Filbin, 1996; Keverne, 1997). First, the lines of mice used for each study are different, which may account for some difference in response after injury. Second, the method of analysis was different for both groups. Li and co-workers measured the total number and length of all regenerating axons, while Bartsch's group measured only the length of the longest axon per animal. In other words, they

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measured one axon per mouse. The more rigorous analysis of the results by Li may have exposed subtle differences in regeneration between MAG-/- and MAG+/+. Third, it is possible, as for other knockout mice (Orioli et al., 1996), that another molecule is compensating for the inhibitory properties of MAG on axonal regeneration. Fourth, there is also the possibility still unexplored, that the combined effects of inhibitory molecules may not be additive *in vivo*. That is to say, the presence of any one inhibitor may be sufficient to prevent most regeneration. On the other hand when studied *in vitro,* individual inhibitors may be more easily neutralized, because they are now more accessible. What we are suggesting is that it is not the same studying axonal regeneration in MAG-deficient mice and studying regeneration after adding an antibody to a specific myelin inhibitor, as shown by the following findings. For example, Schwab and co-workers have observed no more than 5-10% of all the axons regenerating after adding IN-1 antibody (Bartsch et al., 1995; Bregman et al., 1995; Schnell, 1994; Schnell and Schwab, 1990), suggesting that there must be some other inhibitors in myelin besides IN-1. Another example of the difference between studying molecules *in vivo* rather than *in vitro* is provided by these same groups that carried out the *in vivo* studies with the MAG deficient mice. When myelin was isolated from MAG-deficient mice and used as a substrate to grow NG108 cells, one group observed a decrease in inhibition by myelin in the MAG-/- wells compared with the wildtype myelin (Bartsch et al., 1995). The other group also observed a difference in NG108 neurite outgrowth, but only after fractionation of myelin (Li et al., 1996). In addition to these inconsistencies, a third group reported no significant difference in the length of the neurites with the same neuronal cell line (Ng et al., 1996). In summary, these discrepancies in results between *in vivo* and *in vitro* assays suggest that the analysis of myelin inhibitors is a very complex issue, and underscores the extreme care that must be taken in defining the conditions for assessing the inhibitory characteristics of myelin.

The studies by McKerracher *et al.* (McKerracher et al., 1994) and Tang *et al.* (Tang et al., 1997) support of the role of MAG as an inhibitor of axonal regeneration in CNS myelin. In the first study, they showed that when MAG was immunodepleted from myelin, the inhibition of neurites from NG108 cells was reduced by 60%. The second study showed that a soluble form of MAG (dMAG), which is released from myelin preparations by proteolysis, is a potent inhibitor of neurite outgrowth from cerebellar neurons. In addition, there are the results from double mutant mice, MAG-/- mice crossed with the C57BL/Wld^s mice. These C57BL/Wld^s

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mice have a mutation that causes them to have delayed Wallerian degeneration, this makes them have impaired PNS regeneration from lack of myelin debris clearance after an injury (Brown, 1994). In these double mutants for MAG and C57BL/Wld^s, axonal regeneration after a PNS injury was significantly improved (Schafer et al., 1996).

Frequently PNS myelin has been referred as a permissive substrate for axonal regeneration (Carbonetto et al., 1987; Caroni, 1988). However, this conclusion was drawn because neuronal growth on PNS myelin was compared with CNS alone or because regeneration does take place in the PNS, not in the CNS. Furthermore, there is evidence to the contrary from the mutant C57BL/Wld^s mice, which because of their delayed Wallerian degeneration, PNS axons have to regenerate along intact myelin. In these animals there is very poor regeneration after an injury (Brown, 1994). In other words, PNS does not show permissiveness as a substrate for axonal regeneration. Here we would like to test if PNS myelin, which has MAG, is such a permissive substrate by comparing growth on PNS myelin with other kind of substrates.

Many inhibitory molecules have been assessed by measuring neurite extension on membrane carpets prepared from cells or tissue which express the molecule under study (Drescher, 1995; Walter, 1987; Wang and Anderson, 1997). Here we will try to clarify the role of MAG in myelin on axonal regeneration. For this purpose, we decided to use the same assay as was performed in the studies mentioned before, but with some modifications. One change is to use controls that are more stringent in the membrane preparation, like liver or other nervous system tissue, instead of poly-L-Lysine or any purified extracellular matrix protein for control growth. The *in vitro* studies done with the MAG-deficient mice were mostly carried out using transformed cell lines. However, it would be more informative if these same experiments were performed with primary neurons that are known to bind and to be inhibited by MAG.

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Results

Inhibition of Neurite Outgrowth by Different Membrane Substrates Containing MAG

We have already shown that MAG expressed by a monolayer of CHO cells inhibits neurite outgrowth from a variety of neurons relative to outgrowth on control transfected CHO cells (De Bellard et al., 1996; Mukhopadhyay et al., 1994). It has also been shown that MAG, when added to growing neurons in a soluble form, can inhibit neurite outgrowth (Tang et al., 1997). In other words, MAG inhibits axonal regeneration when incorporated into membranes of live cells and as a soluble molecule in a neuronal culture. Since measuring neurite extension on membranes containing the molecule was used to study the effect of other putative inhibitory molecules, the question was raised whether MAG, under similar conditions could inhibit neurite outgrowth (Drescher, 1995).

In order to test this possibility, primary cerebellar neurons were isolated as in the previous experiments and were cultured on top of different membrane carpets that were freshly prepared for each experiment. The wells were coated with membrane preparations from myelin purified from rat CNS or PNS, or with crude membrane preparations from rat's liver or newborn cortex (gray matter) or CHO cell lines. In order to ensure an even and homogeneous membrane carpet throughout the well, the membrane suspension (in a 200μl volume/well) was dried overnight under vacuum. As shown in Fig.13, neurite outgrowth on membranes from MAG-expressing CHO cells is reduced by 45% compared to the length of neurites on membranes from control CHO cells, liver or gray matter. This level of inhibition is less than that routinely seen (about 70-90% depending on neurons type) with neurites growing on live, MAG-expressing CHO cell monolayer, but is still significant.

The neurites from cerebellar neurons were also inhibited when grown on CNS (70%) or PNS (60%) myelin relative to control liver and gray matter membranes. That is, under these conditions PNS myelin, like CNS myelin, is inhibitory for neurite outgrowth. We observed also that, overall, the plating efficiency of cerebellar neurons on PNS myelin was lower than on CNS myelin. This phenomenon usually happens from lack of adhesiveness of a substrate or viability of the neurons. It may be that in our PNS preparations there is a higher proportion of non-

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permissive molecules compared with the other membrane preparations. Carbonetto observed that sciatic nerves have heparan sulfate proteoglycan and fibronectin immunoreactivity, while CNS white matter did not, (Carbonetto et al., 1987). Both of these molecules are known to be non-permissive for CNS neurons, that is, they do not support neurite extension nor do they sustain binding of neurons (Snow and Letourneau, 1992).

These data together show: 1) that MAG can inhibit neurite outgrowth, either when expressed by a monolayer of live cells, as a soluble molecule and when presented as part of an immobilized substrate over which the neurites grow; 2) that under our *in vitro* conditions PNS myelin like CNS myelin can inhibit axonal growth. That is to say, PNS myelin is not as permissive substrate as has been suggested before (Aguayo et al., 1991; Carbonetto et al., 1987). If this were the case, the neurons would have grown neurites as long as on liver or control-CHO cell-membranes.

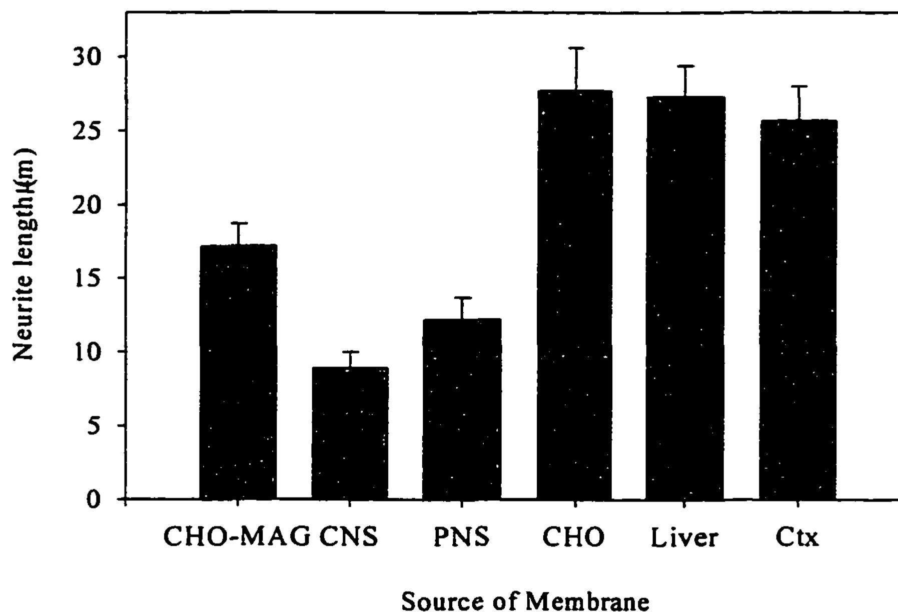

Fig. 13. Neurite outgrowth from cerebellar neurons on various isolated membranes. Dissociated neonatal cerebellar neurons were cultured overnight on wells coated with membranes (3ug total protein/well) prepared from MAG-expressing CHO cells (CHO-MAG), CNS myelin (CNS), PNS myelin (PNS), control CHO cells not expressing MAG (CHO), liver or gray matter (Ctx) before being fixed and immunostained for GAP43. Neurite length was then measured and the results show the mean length of the longest neurite per cell (+- SEM) for 120 individual neurons.

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Inhibition of Neurite Outgrowth by CNS Myelin can be Partially Reversed by Small Sialic Acid Sugars or by Desialylation of Neurons

We have shown that the inhibition of neurite outgrowth by MAG on cell monolayers can be partially reversed by desialylation of the neurons or by adding small sialic acid sugar analogs (De Bellard et al., 1996). In addition, it has been reported that inhibition of neurite outgrowth by a soluble form of MAG can be completely reversed by desialylation (Tang et al., 1997). In order to see if CNS myelin also inhibits neurite outgrowth in a sialic acid-dependent manner, neurons were desialylated prior to the assay on myelin carpets or small sialic acid sugars were added to the culture. After desialylation, sialidase was also included in the media to minimize resialylation during culture period.

When postnatal cerebellar neurons were pre-treated with sialidase and cultured on CNS myelin, the mean neurite length was significantly increased by about 70% compared to untreated neurons (Table IV). Similarly when the sialic acid NANA was included in the cultures, the neurites were almost twice as long as in its absence. Neither of these treatments had a significant effect on the neurite outgrowth length of the cerebellar neurons growing on liver or gray matter control membranes (Table IV). This is consistent with our previous results of neurite outgrowth on CHO cells, and suggests that the inhibition by CNS myelin involves a sialic acid component on the neurons.

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TABLE IV.

Neurite Outgrowth on Purified Membranes

Tissue (3ug/well)	Control	VCS treated	+ NANA 20mM	% Change
CNS myelin	8.37± 0.9	14.62 ±1.6 *	15.21±1.5 *	+77%*
Liver	17.89±2.3	19.06±2.3	21.21±2.2	+10%
Cortex	13.88±1.4	17.44±2.4	16.48±1.8	+21%

* $p < 0.03$ by a Student T-test.

These findings suggest an active role for MAG in the inhibition of axonal regeneration by CNS myelin and that when MAG is present in a solid substrate it can still interact with a sialylated component on neurons.

Inhibition of Neurite Outgrowth by CNS Myelin when MAG is Absent

To assess if MAG contributes to the inhibition of neurite outgrowth by CNS myelin, purified CNS myelin from MAG-deficient (MAG-/-) mice was prepared as in the previous experiments, and used as a substrate for neurons. Slides were coated with 1.5, 3 or 6 μg of total myelin protein/well. When postnatal cerebellar neurons were grown on MAG-/- myelin carpets, their neurite outgrowth was significantly longer compared to growth on MAG+/+ myelin (Fig.14a). This difference in the length of the neurites was higher (twice as long or more) at 3 and 6 μg of total protein/well of MAG-/-, than at the lowest concentration of myelin (1.5 μg protein/well).

Similarly, when DRG neurons older than PND3 were isolated and grown in similar cultures, their neurites were significantly longer (25-35%) on the myelin from the MAG-/- mice (Fig.14b). This increase in neurite length is not as great as for cerebellar neurons, but the

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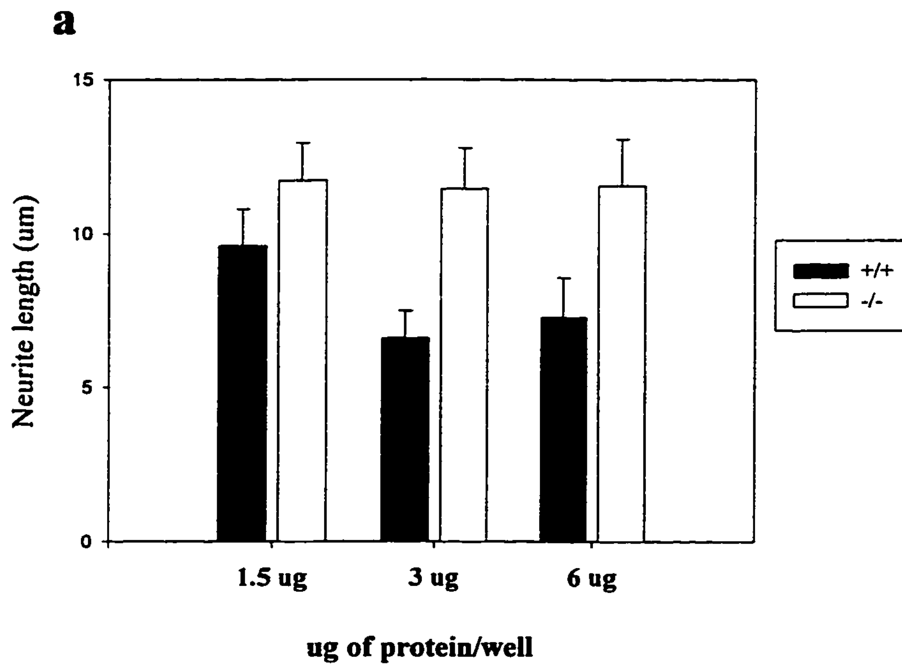
increase was always significant. When DRG neurons were grown on CNS myelin at 6 μg protein/well or higher, their plating efficiency and neurite outgrowth was dramatically reduced.

In brief, neonatal cerebellar and older DRG neurons grow longer neurites when cultured on CNS myelin carpets from MAG-deficient mice than when grown on wildtype myelin, containing MAG. In addition, the extent of the improvement in growth is different for each type of neuron and this may be due to their intrinsic responsiveness to other myelin inhibitors as well as to MAG.



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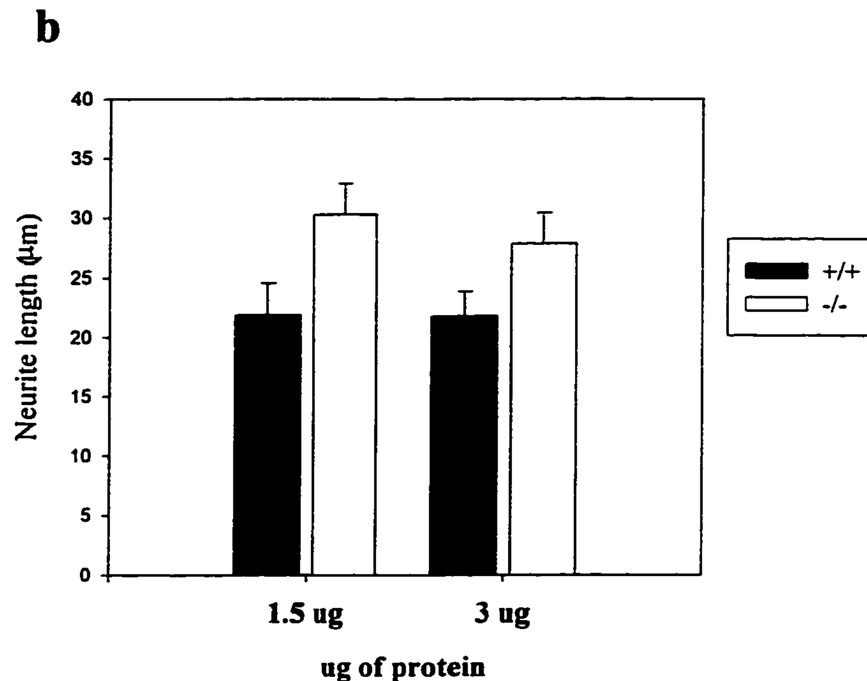

Fig. 14. Neurite outgrowth on MAG-/- and MAG+/+ myelin. CNS myelin was isolated from MAG+/+ (black bars) and MAG -/- (blank bars) and coated onto wells at a concentration of 1.5, 3 and 6ug total myelin protein/well. Dissociated (a) neonatal cerebellar or older DRG (b) neurons were cultured overnight on the myelin substrate before being fixed, immunostained for GAP43 and neurite length measured. Results show the mean length of the longest neurite per cell (±SEM) for at least 120 individual neurons.

In contrast to neonatal CN and older DRG neurons, when PND1 DRG neurons, which are promoted by MAG, are grown on the myelin carpets, neurites are longer on myelin from the MAG+/+ mice than on myelin from the MAG-/- mice (Fig.15). This is consistent with the previous findings which showed that PND1 DRG neurons are promoted by MAG (Johnson et al., 1989; Mukhopadhyay et al., 1994). The difference in the neurite length from PND1 DRG neurons between both types of myelin membranes increases with myelin concentration. That is to say, the higher the concentration of myelin coated onto the dish, the bigger the difference between the neurites on MAG-/- compared to MAG+/+. We noticed that these differences between MAG+/+ and MAG-/- myelin because the neurite length on MAG+/+ myelin remains

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constant, regardless of the concentration of myelin, while the neurites on the MAG-/- myelin are shorter with increasing myelin concentration.

These results show that the presence of, or absence from, MAG in myelin can make a difference in the neurite outgrowth of cerebellar and DRG neurons. These results are consistent with what was previously found for these same neurons when growing on MAG expressing cells (Johnson et al., 1989; Mukhopadhyay et al., 1994). In other words, MAG promotes neurite outgrowth from PND1 DRG neurons and inhibits neurite outgrowth from postnatal cerebellar and older DRG neurons even when presented as an immobilized substrate.

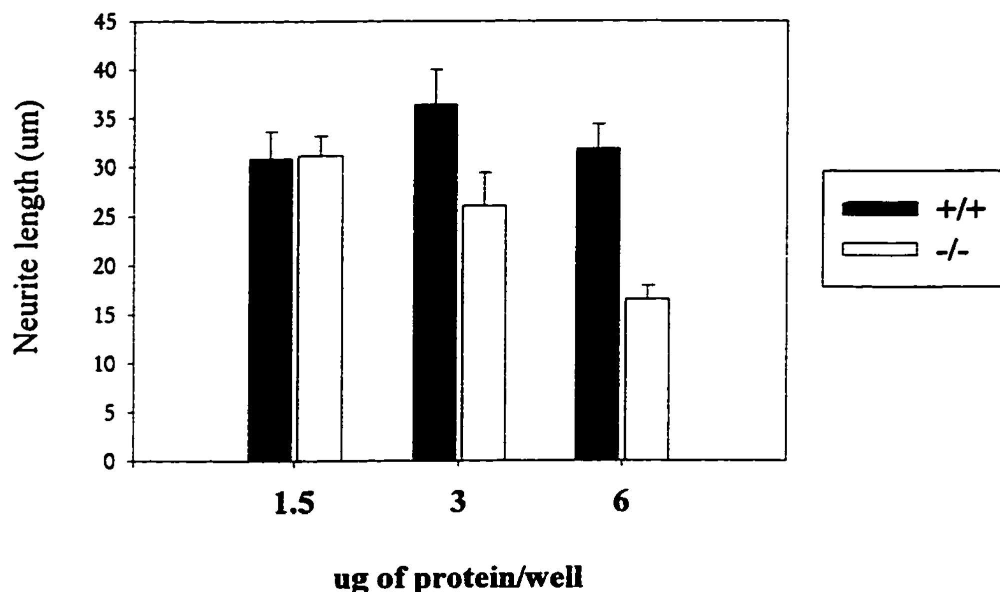

Fig. 15. Neurite outgrowth from PND1 DRG neurons on MAG-/- CNS myelin. CNS myelin was isolated from MAG+/+ (black bars) and MAG -/- (blank bars) and coated onto wells at a concentration of 1.5, 3 and 6ug total myelin protein/well. Dissociated PND1 DRG neurons were cultured overnight on the myelin substrate before being fixed, immunostained for GAP43 and neurite length measured. Results show the mean length of the longest neurite per cell (±SEM) for at least 120 individual neurons.

An alternative approach to determine if MAG has a role in the overall inhibition by CNS myelin is to assess the effect on neurite outgrowth inhibition by CNS myelin in the presence of

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an antibody specific for MAG. For this purpose, we raised a rabbit polyclonal antibody to MAG, using the MAG-Fc chimera as an antigen. This serum was used after we determined that it recognized MAG on both Western blots of myelin and immunofluorescence of cells transfected with MAG (data not shown). When this serum was added to cultures of cerebellar neurons on 1.5 and 6ug total protein/well of rat CNS myelin, the neurites of these cells were longer by about 100% (Fig.16). There was no difference in the length of the neurites when we used a rabbit antiserum raised against another myelin protein, Po (results not shown). When this same MAG antiserum was used on neurons growing on liver membranes, we did not observe any effect, which indicates the effect on neurite outgrowth by this MAG anti-serum is specific.

These results, together with those presented in Fig.13 support the hypothesis that MAG plays a role in the overall inhibition of axonal regeneration caused by CNS myelin.

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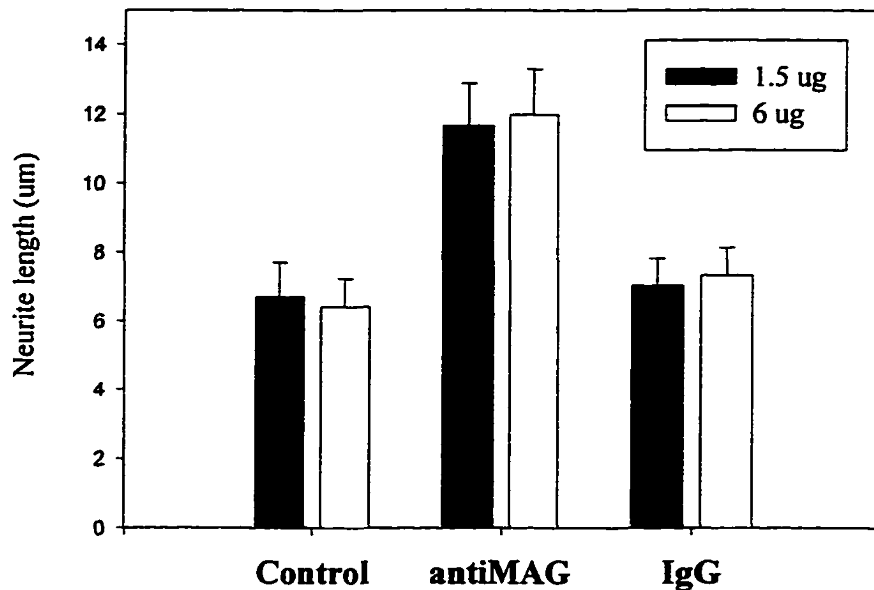

Fig. 16. Neurite outgrowth on CNS myelin in presence of MAG antibody. CNS myelin was isolated from rat CNS and coated onto wells at a concentration of 1.5ug (black bars) and 6ug (blank bars) total myelin protein/well. Dissociated neonatal cerebellar neurons were cultured overnight on the myelin substrate in the presence of a polyclonal anti-MAG (1:200) or of rabbit serum (1:200) before being fixed, immunostained for GAP43 and neurite length measured. Results show the mean length of the longest neurite per cell (±SEM) for at least 120 individual neurons.

Role of MAG in the Inhibition of Neurite Outgrowth by PNS Myelin

The observation that MAG in CNS myelin inhibits axonal regeneration from cerebellar and DRG neurons poses the question of its role as an inhibitor in PNS myelin. As discussed

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above, it has been shown that PNS myelin is inhibitory for axonal regeneration *in vivo* and *in vitro* (Bedi, 1992; Brown, 1992; David et al., 1995) and that MAG has a role *in vivo* in this inhibition (Schafer et al., 1996; Torigoe and Lundborg, 1998). Nevertheless, we do not know if MAG can contribute *in vitro* to the inhibition of neurite outgrowth of PNS myelin observed under the same conditions as our previous experiments.

As mentioned above, we observed that cerebellar neurons did not grow well on rat PNS myelin membranes. Whereas DRG neurons plated on the same PNS preparations were capable of growing abundant neurites on this substrate and their plating efficiency was not significantly affected. Therefore, we decided to use DRG neurons instead of cerebellar neurons to test the effect on neurite outgrowth of MAG in PNS myelin carpets. Table V shows the results when DRG neurons are grown on rat PNS myelin in the presence of a polyclonal MAG antibody or after desialylation. We observed a modest but significant ($p<0.03$) increase in the length of the DRG neurites in the presence of a MAG polyclonal antibody and after desialylation. This increase after treatment with an antibody to MAG or desialylation (25%) is not as great as that observed for cerebellar neurons under the same conditions, on CNS myelin (100%), but is still significant.

TABLE V.

Neurite Outgrowth from DRG Neurons on PNS Myelin

Treatment	Mean Neurite length (um)
Control	20.28+1.5
+ anti-MAG	25.74+1.9 *
+ VCS	26.32+1.5 *

$p< 0.03$
+VCS = desialylated neurons plus 20mM of more VCS on the culture medium.

The inability of PNS myelin to support survival and growth of cerebellar neurons appeared to be even stronger when we prepared myelin from mice, either MAG-/- or MAG+/+, rather than to rat. In contrast to its effect on cerebellar neurons, PNS myelin from mice was able

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to support neurite outgrowth from older DRG neurons (Fig.17). Furthermore, when older postnatal DRG neurons are plated on PNS myelin from the MAG-/- mice, their neurites are about 30% longer compared to those on MAG+/+ PNS myelin. Although this difference is small, it is significant ($p<0.0001$). This demonstrates that MAG in PNS myelin, as in CNS myelin, is contributing to the inhibitory properties of this membrane *in vitro*.

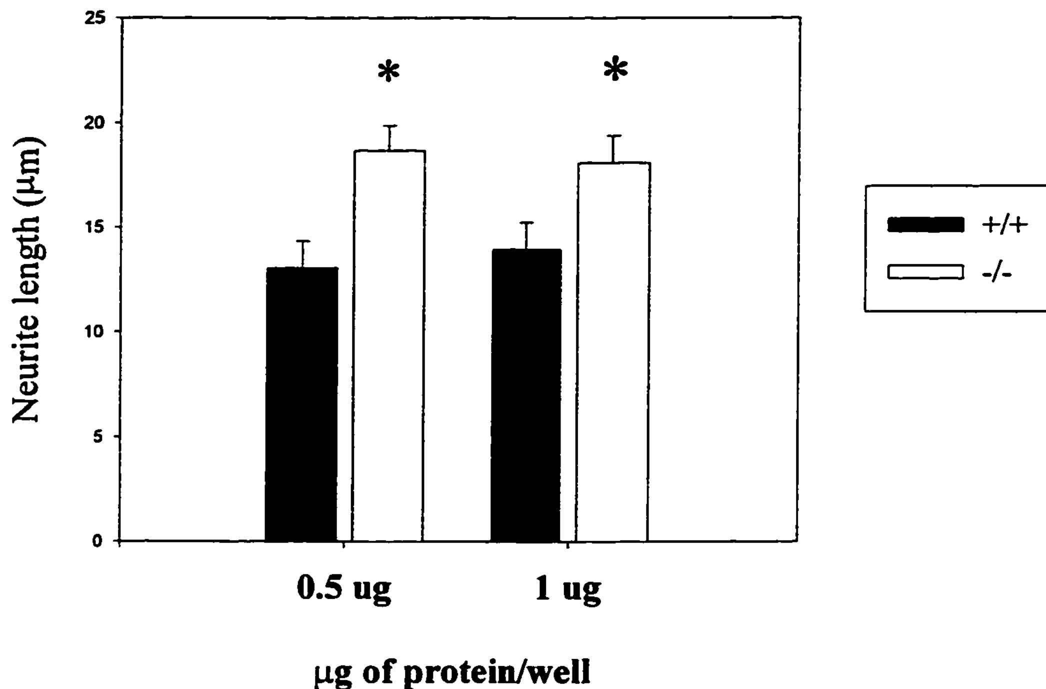

Fig. 17. Neurite outgrowth on MAG-/- and MAG+/+ PNS myelin. PNS myelin was isolated from MAG+/+ (black bars) and MAG -/- (blank bars) and coated onto wells at a concentration of 0.5 and 1ug total myelin protein/well. Dissociated older DRG neurons were cultured overnight on the myelin substrate before being fixed, immunostained for GAP43 and neurite length measured. Results show the mean length of the longest neurite per cell (±SEM) for at least 120 individual neurons.

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In summary, our results suggest that MAG contributes *in vitro* to the inhibition of axonal regeneration by PNS myelin and that this inhibition can be partially reversed by adding a MAG polyclonal antibody, desialylating the neurons or growing neurons on PNS myelin from MAG deficient mice.

Discussion

It is well established that MAG is a potent inhibitor of axonal regeneration when encountered by neurons in culture (De Bellard et al., 1996; McKerracher et al., 1994; Mukhopadhyay et al., 1994; Tang et al., 1997; Tang et al., 1997). However, the contribution that MAG has *in vivo* to the overall inhibition by CNS myelin after injury is still controversial. The results presented here strongly suggest that MAG when used as a substrate makes a significant contribution to the inhibition of axonal regeneration by myelin.

Before addressing the inhibition of neurite outgrowth by MAG in myelin, we first had to assess if MAG is capable of inhibiting axonal growth when immobilized in purified membranes. Here we show that MAG does inhibit neurite outgrowth when presented to cerebellar neurons as a substrate prepared from MAG-expressing CHO cell membranes. Although our studies are similar and our results agree with the group of McKerracher, there are two key differences: 1) McKerracher and co-workers used a transformed neuronal cell line, NG108 cells; and they grew these cells on a purified, recombinant form of MAG as a substrate. Our use of primary neurons growing on crude cell membranes, with or without MAG, is a more accurate substrate to assess its effect on neurite outgrowth. The reason being that, very frequently purified proteins, and even more after detergent extraction, are not suitable substrates nor mirror accurately the physiological milieu. Despite these differences, our results confirm and extend the previous finding of McKerracher, by showing that primary neurons can be inhibited by MAG in an immobilized membrane as has been shown for other inhibitory molecules (Drescher, 1995).

Although inhibitory, these MAG-containing membranes are less effective inhibitors of neurite outgrowth from cerebellar neurons (50%) than live MAG-expressing CHO cells (70%).

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This difference is most likely because in the membrane carpets MAG is immobile, while within the membranes of live cells MAG is in a more fluid form. Therefore, when expressed within the fluid cell membrane MAG can actively cluster on the surface, and so in turn it can cluster and very likely activate its neuronal receptor. These results are consistent with our observations that a purified soluble form of MAG can effectively inhibit neurite outgrowth from cerebellar neurons, but when immobilized and used as a substrate is less potent (Tang et al., 1997).

Cerebellar and older than PND3 DRG neurons growing on MAG-/- myelin carpets (CNS and PNS) showed a significant increase in the length of their neurites compared to growth on MAG+/+ myelin. Nevertheless, this percentage of neurite outgrowth increase was never as dramatic as the percentage of inhibition we have seen in neurite outgrowth assays on MAG-expressing CHO cells. This observation is most likely due to the reasons mentioned above in reference to MAG in membranes. That MAG is a less effective inhibitor of neurite outgrow when immobilized as a solid substrate than on the membrane of a live cell, and because we cannot compare inhibition of neurite outgrowth on live cells with promotion of neurite outgrowth on immobilized myelin membranes, with or without MAG present.

Throughout these experiments, we have found consistent and significant increases in neurite outgrowth length from cerebellar and DRG neurons on MAG-/- myelin compared with MAG+/+ myelin. Other studies have reported small, significant improvements in the neurite outgrowth of NG108 neurons (Bartsch et al., 1995; Li et al., 1996), but not for PC12 cells or adult DRG neurons (Bartsch et al., 1995) growing on MAG-/- myelin. The disparity between these *in vitro* results and ours, most likely stem from differences in the way myelin was prepared, stored and used in these studies. It has been shown that MAG is readily lost from purified myelin preparations (Tang et al., 1997). Therefore, depending on how myelin is prepared and stored the content of MAG may vary considerably. This eventually will reduce or even can mask any effect of myelin on neurite outgrowth from the MAG-deficient mice compared with myelin from wildtype animals. Very importantly, the type of neuron used can also make a big difference. We have shown that not all the neurons are equally inhibited by MAG (see Ch.1 and (De Bellard et al., 1996). Most of the *in vitro* experiments cared out by others with myelin from MAG-/- mice were with transformed neuronal cell lines that may be less responsive to MAG and to other inhibitors. For example, we have observed that PC12 cells are inhibited by MAG-expressing CHO cells only about 20% (results not shown). Furthermore, studies in our lab have

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shown that neurite outgrowth inhibition by MAG as well as myelin, can be reversed if DRG neurons are primed with NGF (Cai et al., 1998). The studies mentioned above used NGF during culture of adult DRG neurons and of PC12 cells on myelin, which may have allowed these neurons to grow on the MAG+/+ myelin as long as on the MAG-/- myelin carpets.

We have shown that axonal regeneration is substantially improved for both cerebellar and older DRG neurons grown on myelin carpets from MAG-/- mice compared to myelin from MAG+/+ mice. The converse was true for neonatal DRG neurons; their neurites grew longer on myelin from MAG+/+ mice compared with myelin from MAG-/- mice. These results support our previous findings which show that MAG is a bifunctional molecule, as it inhibits neurite outgrowth from cerebellar and older DRG neurons but promotes it from neonatal DRG neurons (De Bellard et al., 1996). The switch in response that we observed from DRG neurons on MAG-expressing CHO cells was also observed on myelin from the MAG-/- mice. These results argue strongly that MAG is directly responsible for these effects.

When DRG neurons were grown on CNS myelin carpets, we observed that there was significant reduction in the plating efficiency and neurite outgrowth at 6ug protein/well or higher. There are two possible explanations for this: 1) DRG neurons are more sensitive to the other inhibitors present in CNS myelin than cerebellar neurons; and 2) because the CNS myelin substrate is less adhesive for the cells (the plating efficiency was significantly reduced for DRG neurons on CNS myelin compared to cerebellar neurons at 6ug/well), their neurites cannot grow as well as CNS neurons do on CNS myelin.

Under the same conditions of membrane coating, neurite outgrowth from cerebellar neurons on PNS myelin carpets is also inhibited compared to growth on liver or gray matter membranes, suggesting that PNS myelin may also potently inhibit axonal regeneration. In addition to cerebellar neurons, older DRG neurons also grew longer neurites on PNS myelin from MAG-deficient mice, indicating that MAG contributes to the inhibitory properties of PNS myelin. Until recently, PNS myelin was described as permissive for axonal growth (Carbonetto et al., 1987; Caroni, 1988). This difference between CNS and PNS myelin was suggested as one reason why regeneration was successful in the PNS but not in the CNS. Possible explanations for the apparent permissiveness of PNS myelin are 1) the age and type of neurons used in previous studies were embryonic retinal or neonatal DRG neurons. These embryonic retinal neurons do not respond to the inhibitors of myelin, similar to many other embryonic neurons

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(Shewan et al., 1995). Moreover, it has been observed though not quantitated, that embryonic retinal neurons are promoted by MAG, but after birth they are strongly inhibited by MAG (De Bellard et al., 1996; Salzer et al., 1990). Likewise neonatal DRG neurons used by others are promoted by MAG (Mukhopadhyay et al., 1994). This change in response has now been observed also for embryonic retinal and DRG neurons for the myelin inhibitors NI-35 and NI-250 (Bandtlow and Loschinger, 1997). 2) As mentioned before, PNS myelin may appear slightly permissive if we compare the growth of neurons on CNS versus PNS myelin alone, instead of using a more comparable membrane for control growth. 3) The preparation of myelin is a crucial step in these experiments and can make a dramatic difference in the outcome of the experiment. Recently David and co-workers showed that the method of myelin preparation could significantly alter the "permissiveness" of PNS myelin (David et al., 1995). The reason being that there were substantial amounts of laminin in the myelin depending on the method of preparation. In addition to this finding, we have shown that MAG can be readily lost from purified myelin as a proteolytic fragment (Tang et al., 1997). 4) The coating of myelin or any membrane can make a difference in the capacity of the neurons to put out neurites. When myelin is left to dry overnight, the coating is never homogeneous but follows the drying meniscus. When we used such technique and stained the myelin for MAG we observed that the coating was in a gradient distributed concentrically. That is to say, the protein coating was not uniform in the well. Under these conditions, it is hard to determine the levels of protein per well necessary to inhibit.

The most convincing demonstration of the inhibitory properties of PNS myelin comes from *in vivo* studies with the C57BL/Wld^s mice that have delayed Wallerian degeneration (Brown, 1992; Brown, 1994). In these animals, because myelin and myelin debris are not quickly removed, regeneration in the PNS is retarded compared to control mice. Importantly, when these mice are crossed with the MAG-deficient mice, regeneration proceeds at a faster pace, now that MAG is not present (Schafer et al., 1996). In summary, axonal regeneration in the PNS takes place because myelin, and hence MAG, is removed, not because PNS myelin is permissive for growth.

An important observation from the neurite outgrowth experiments on PNS myelin carpets was that, overall, the cerebellar neurons did not grow well on PNS myelin. This is not because of the myelin preparation itself, because when postnatal DRG neurons are plated on the PNS

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myelin carpets, these cells are able to attach and grow neurites. It appears that cerebellar neurons cannot attach and grow on PNS myelin, while they can on CNS myelin, liver and cortex. The reason may be in the intrinsic growing properties of cerebellar neurons and their responsiveness to certain substances peculiar to PNS myelin.

We have shown that MAG binds to neurons in a sialic acid-dependent manner. In addition to this, we have shown that desialylating neurons or adding small sialic acid sugars before the neurite outgrowth assay on MAG-expressing CHO cells reverses the inhibition by MAG up to 40% (Ch.1 and (De Bellard et al., 1996). Here we also show that, as on live CHO cells, desialylation of cerebellar neurons or adding small sialic acid sugars to the culture media partially reverses the inhibition of neurite outgrowth by CNS myelin. Similarly, when older postnatal DRG neurons are desialylated before plating them on PNS myelin, their neurites are longer compared to those growing without any treatment. That is to say, desialylation can partially reverse the inhibition caused by CNS or PNS myelin. This in turn suggests that there are neuronal sialic acid-bearing components involved in the inhibition of neurite outgrowth by myelin, from both the CNS and the PNS.

The reversal after treatment with sialidase or after adding a MAG antibody of neurite outgrowth inhibition from DRG neurons on PNS myelin carpets was never as great as that for cerebellar neurons on CNS myelin. The cerebellar neurites were nearly twice as long after sialidase or after anti-MAG antibody, while the DRG neurites were about 30% longer after those same treatments. This difference in response may be due, first, to less MAG being present in PNS myelin compared with CNS myelin (10 times less); second, DRG neurons do not grow as robustly as they usually do, probably because of higher amounts of non-permissive substrates (e.g. proteoglycans) in PNS myelin that can lower their plating and growing efficiency. That the DRG neurites never grew longer than 30μm, was unexpected, because generally they can grow much longer neurites on other substrates like live CHO cells or laminin (at least 150μm) (Bartsch et al., 1995; De Bellard et al., 1996). These small increases in neurite outgrowth observed from DRG neurons on PNS myelin after the various treatments also mirrored the small increase that was observed for the same cells growing on CNS myelin from the MAG-/- mice. In other words, the increases in neurite outgrowth for DRG neurons were consistent throughout our cultures on myelin carpets, of either CNS or PNS. Taken together, the shorter neurites on PNS myelin and the lower plating efficiency on CNS membranes suggest that DRG neurons do not grow well on

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myelin, probably because they are more sensitive to the non-permissive substrates present in these preparations.

The results presented here show that when neurons were grown on myelin from MAG-/- mice their neurites were twice as long compared with neurites on MAG+/+ myelin. These findings are comparable to the *in vivo* results with the MAG-/- doublecrossed with the C57BL/Wld^{s} mice with impair Wallerian degeneration (Schafer et al., 1996). In these experiments, the percentage of intact myelin sheaths with growing axons in the $^{MAG-/-}$C57BL/Wld^{s} was twice as much as on the $^{MAG+/+}$C57BL/Wld^{s}. Although they measured number of axons in contact with myelin and we measured the length of their neurites growing on myelin, their results and ours are similar. They found that twice as many axons regenerated when MAG was not present in the undegenerated myelinated axons; we found that cerebellar and DRG neurons grew neurites twice as long when MAG was not in our purified membranes. Probably, if they had scored the length achieved by e.g., half the axons a set time after injury, they would have reached similar results to ours: the average neurite length was twice on the $^{MAG-/-}$C57BL/Wld^{s} than on $^{MAG+/+}$C57BL/Wld^{s}. That is to say, when MAG is absent from myelin, there is improved regeneration. These results also suggest that *in vitro* analyses of axonal regeneration can mirror somehow what happens *in vivo* when studying regeneration.

In summary, we have shown, first, that MAG in membrane preparations can inhibit axonal regeneration. Second, that MAG in myelin can promote growth from early neonatal DRG neurons and its absence from myelin results in an increase in the length of neurites for postnatal cerebellar neurons on MAG-/- myelin. Third, that the inhibition of neurite outgrowth by CNS and PNS myelin is somewhat dependent on neuronal sialic acid-bearing moieties. It is very likely that MAG *in vivo* carries out a similar function and contributes to the overall inhibition of axonal regeneration by CNS myelin.

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CHAPTER 3

Effect of MAG in vivo

Introduction

We have shown that MAG *in vitro* affects axonal regeneration from a variety of neurons when presented on transfected CHO cells or in immobilized membranes. However, it remains to be shown definitely that MAG can have an inhibitory effect on axonal elongation when encountered *in vivo* during regeneration.

This question remains unanswered because the data provided by the existing transgenic mice is inconclusive. First, after injury in the MAG-deficient mice only a negative environment is provided for the regenerating neurons: that is to say, no MAG is present (Bartsch et al., 1995; Li et al., 1996). Second, the results from crossing the C57BL/Wld^s mice with the MAG-deficient mice showed an improvement of regeneration in the PNS when MAG was not present (Schafer et al., 1996). In addition, we do not have an effective MAG neutralizing monoclonal antibody, that would allow us to conduct similar experiments to those carried out with IN-1 after CNS injury (Filbin, 1996).

The ideal way of showing that MAG can be an inhibitor of axonal regeneration *in vivo* would be to express it in a system where the presence of other inhibitors during regeneration is minimal. Since this is not possible in the CNS, we chose the PNS. In the PNS, regeneration does take place due to Wallerian degeneration. This process involves the removal of myelin after injury by macrophages and Schwann cells, as well as the removal of the distal part of the injured axons. Therefore, the inhibitors provided by myelin are not present when the axons are growing through the injury site and distal to it. Also very importantly, regeneration takes place because the distal part of the nerve contains the Büngner bands, composed of the basal lamina and proliferating Schwann cells with their interlaced cytoplasmic processes within the space occupied by each former internode (Griffin, 1993). Together, Wallerian degeneration and the bands of Büngner provide a permissive environment along which the regenerating axons can grow. In addition to these changes, the Schwann cells, before they start to myelinate, express a variety of permissive molecules, like NCAM and L1 (Bunge, 1993; Ide, 1996). This permissive environment is

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an essential requirement for regeneration in the PNS, as shown by culturing neurons on PNS sections before and after injury. It has been shown that neurites will grow inside the Schwann cell endoneurium (basal lamina), on pre-degenerated nerve sections, whereas on intact nerve sections, neurites extended only along the endoneurial basal laminae, but never in direct contact with the Schwann cells (Agius and Cochard, 1998). That is to say, neurites would regenerate along pre-degenerated nerves, but if these were intact, neurites never grew wherever there was intact myelin. It should be noted that in the PNS, MAG is present in the outer loop of myelin that the neurites are actively avoiding.

Throughout regeneration in the PNS, Schwann cells play a crucial role. These cells will secrete growth factors that help guide regenerating axons along the Büngner bands (Griffin, 1993). Moreover, it has been observed that Schwann cells play a primary role in initiating sprouting and in guiding the growth of the resulting sprouts during regeneration (Son and Thompson, 1995). After an injury in the PNS, Schwann cells de-differentiate, stop expressing myelin markers and start expressing other markers, required for proliferation (Scherer et al., 1993; Scherer and Salzer, 1995; Scherer et al., 1994; Scherer et al., 1995; Shy et al., 1996; Trapp et al., 1988). Our approach was to take advantage of this feature in the Schwann cell and express MAG under the control of a specific promoter that will be turned on precisely when the process of regeneration is taking place. Among the molecules with an increased expression during this period is the p75 receptor, previously known as the p75 low affinity NGF receptor. This receptor is expressed during Schwann cell division, and is likely to be important for their survival in the absence of axonal contact (Taniuchi et al., 1986). We decided to use the promoter from the p75 receptor because of its somewhat restricted expression to glial cells, its expression by undifferentiated Schwann cells and because expression of p75 increases after injury (Huber and Chao, 1995).

The p75 neurotrophin receptor is referred to as the low affinity NGF receptor because it binds NGF, as well BDNF, NT-3 and NT-4 neurotrophins with lower affinity than the *trk* receptors (Enfors et al., 1990). The specific role of the p75 receptor in the physiological function of NGF has yet to be unequivocally defined. Transfection experiments with full length or EGFR-chimeras of this receptor have been shown to induce several signal transduction pathways (Berg et al., 1991). It has also been proposed that the presence of the p75 receptor is necessary for high affinity binding of neurotrophins for their

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trk receptors (Hempstead et al., 1991). In support of these suggestions, mice deficient in p75 receptor show a reduced number of DRG neurons, and have severe defects in their sympathetic innervations (Lee et al., 1992). In general, these animals show deficits in their sensory system, suggesting that the p75 receptor plays a key role in the development of that system.

The aim of the experiments described here is to provide a simple model to demonstrate that MAG could affect axonal regeneration *in vivo*. In order to do this several lines of mice expressing rat L-MAG under the p75 receptor promoter were engineered, and peripheral nerve injuries were carried out and rates of axonal regeneration in the transgenic mice compared to that in the wildtype mice.

Results

Construction of Transgenic Mice Ectopically Expressing MAG

We subcloned rat L-MAG into pECE (Fig.17), downstream from the human p75 receptor promoter region kindly supplied by Dr. Moses Chao (Huber and Chao, 1995). The Cedars Sinai Transgenic facility created the mouse lines used in the following experiments. Briefly, a 4kb upstream sequence of the human p75 receptor gene, previously shown to activate gene expression in the Schwann cells after peripheral nerve injury, was used to direct the ectopic expression of rat L-MAG cDNA. A 4kb BamHI-SacII fragment containing the p75 promoter (generated from the pBRB51H3 plasmid obtained from Dr. Moses Chao) was inserted immediately upstream from the L-MAG cDNA in pECE vector, which is followed by the SV40 3' untranslated and polyadenylation signals. The new construct was referred to as p4.0p75-LMAG (Fig.18). A regulatory sequence, promoter, rat L-MAG cDNA, and the polyA signals was derived from this construct and used for microinjection into the eggs.

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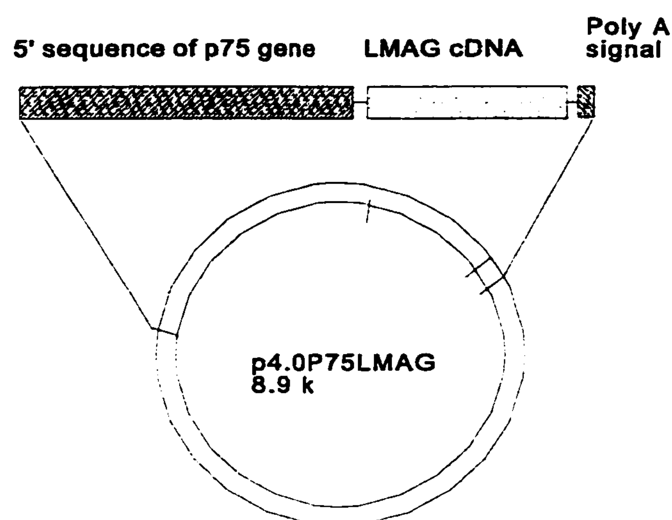

Fig. 18. Construction of the transgene. Subcloning strategy is described in experimental methods.

Expression of MAG in the Transgenic Mouse Lines

Several transgenic founder mice were generated and analyzed for expression of the transgene. The presence of p75-MAG in the founder mice was indicated by the appearance of the appropriately sized fragment after PCR of tail DNA using a primer specific for rat L-MAG cDNA. Several mouse lines carried the transgene and passed it to their offspring. Other lines never passed the transgene to their offspring. These lines were not used. Of the positive lines (Fig.19), we selected lines 3, 6 and 8 and used these in our experiments. These three lines had the best rate of passing the transgene to their offspring, while line 14 was inconsistent. The number of transgene copies present in founder animals and their offspring was estimated from densitometry of Southern blots (Fig.20). As seen in Fig.20, line 6 had the lowest copy number of p75-MAG (about 1 per genome) and line 8 the highest (20 copies) while line 3 was intermediary between those two.

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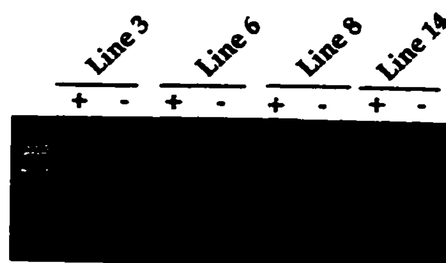

Fig. 19. Tail PCR for rat L-MAG in the mouse lines. DNA analysis was carried out on –ug of tail DNA isolated from lines 3, 6, 8 and 14. As seen in the agarose electrophoresis, all four lines express the transgene. The + correspond to a positive animal, the – to a sibling lacking the p75-MAG transgene.

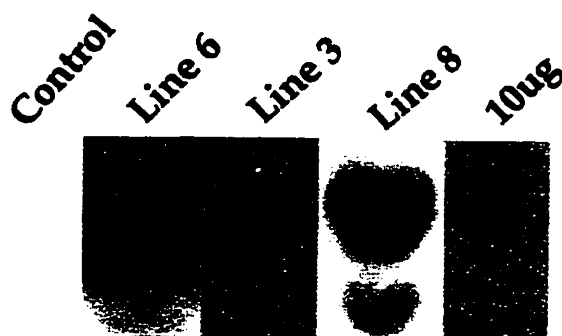

Fig. 20. Southern blot analysis of mice that harbor the p75 promoter-MAG transgene. DNA analysis was carried out on 20ug of tail DNA isolated from Line 3, 6 and 8 mice and plasmid DNA control reflecting hybridization to the integrated minigene (3kb)

In order to assess the expression of rat L-MAG in these transgenic mice we took two approaches. One used RT-PCR of total mRNA for rat L-MAG on crushed or permanently transected sciatic nerves. The other was to culture Schwann cells for few days and then immunostain them for MAG. As already mentioned, when Schwann cells are kept in culture their myelin genes are turned off, and the p75 receptor promoter along with other proliferating markers, is switched on (Scherer and Salzer, 1995; Shy et al., 1996). Hence, in our transgenic mice we should be able to detect the expression of MAG under these conditions if the transgenic p75 promoter is turned on at the same time as that of the endogenous gene.

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Sciatic nerves of adult animals from the mouse line 8 (highest copy number) and line 6 (lowest copy number) were dissected and cultured in the presence of NGF (50ng/ml) for 3-5 days after allowing Schwann cells to migrate away from the tissue (Heumann et al., 1987). These cells were passed directly onto laminin coated slides and the next day stained for MAG, p75 receptor, NCAM, L1 or tenascin. It is well established that dedifferentiated Schwann cells will not express MAG, but will express NCAM, L1 and tenascin (Martini, 1994). From these cultures, we were able to establish that the p75-MAG Schwann cells express p75 receptor and MAG on their surface (Fig.21). Cells from control animals, under the same conditions, did not express MAG but expressed p75 receptor. In addition, we found that the transgenic Schwann cells had significantly higher amounts of NCAM and tenascin than control cells, as was apparent from their more intense immunostaining for these proteins (Fig.22). However, L1 levels in control and p75-MAG Schwann cells did not show any major difference after injury (results not shown). When these experiments were repeated with Schwann cells from the low copy number mouse line 6, the results were the same as with the high copy number line 8. The line 6 cells express MAG, as well as higher amounts of NCAM, and tenascin than control cells (Fig22). However, overall the levels of expression of the same proteins in line 6 were generally lower compared to line 8. In summary, mice positive for the transgene are expressing rat L-MAG under the p75 receptor promoter as well as the proteins that usually are turned on during dedifferentiation. The control Schwann cells only express the corresponding markers of dedifferentiated Schwann cells.

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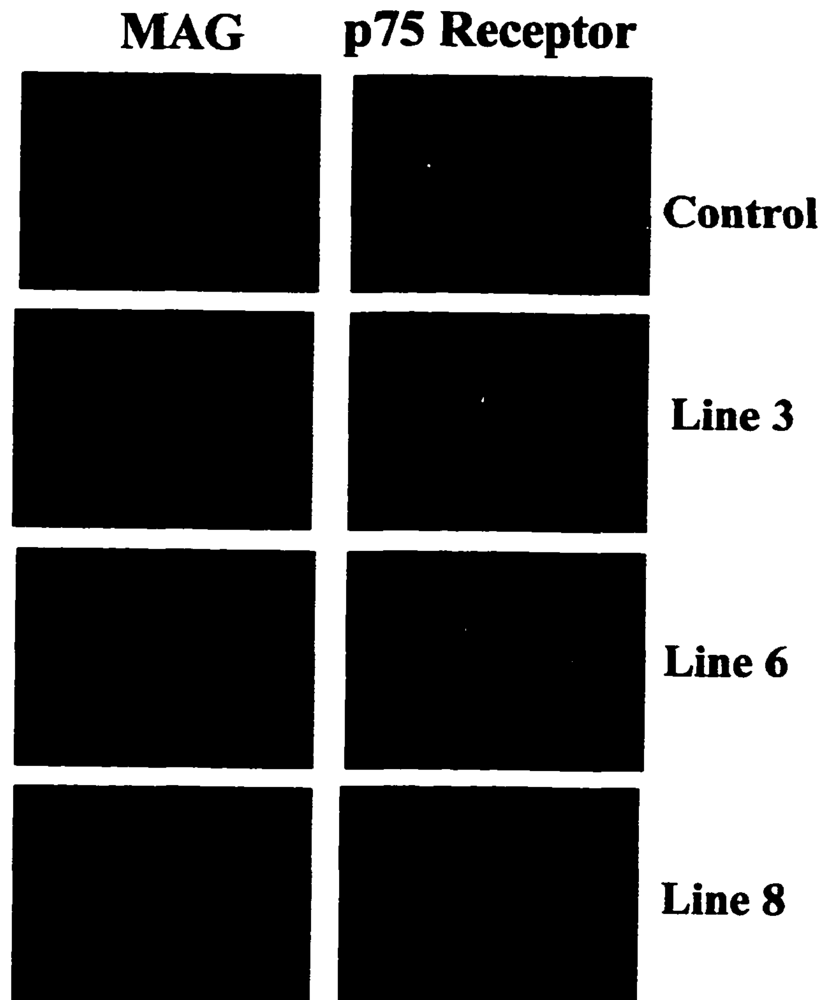

Fig. 21. Surface detection of MAG and p75 receptor on p75-MAG transgenic Schwann cells. Live Schwann cells from Lines 3, 6 and 8 p75-MAG or from control mouse were incubated with antibodies to MAG then with an Alexa red-conjugated goat anti-mouse IgG (1:1000). The p75 receptor was detected by post-fixing the cells, permeabilizing with cold methanol and incubating them wit a polyclonal anti-p75 receptor (M. Chao), then with an anti-rabbit Oregon green 1:1000.

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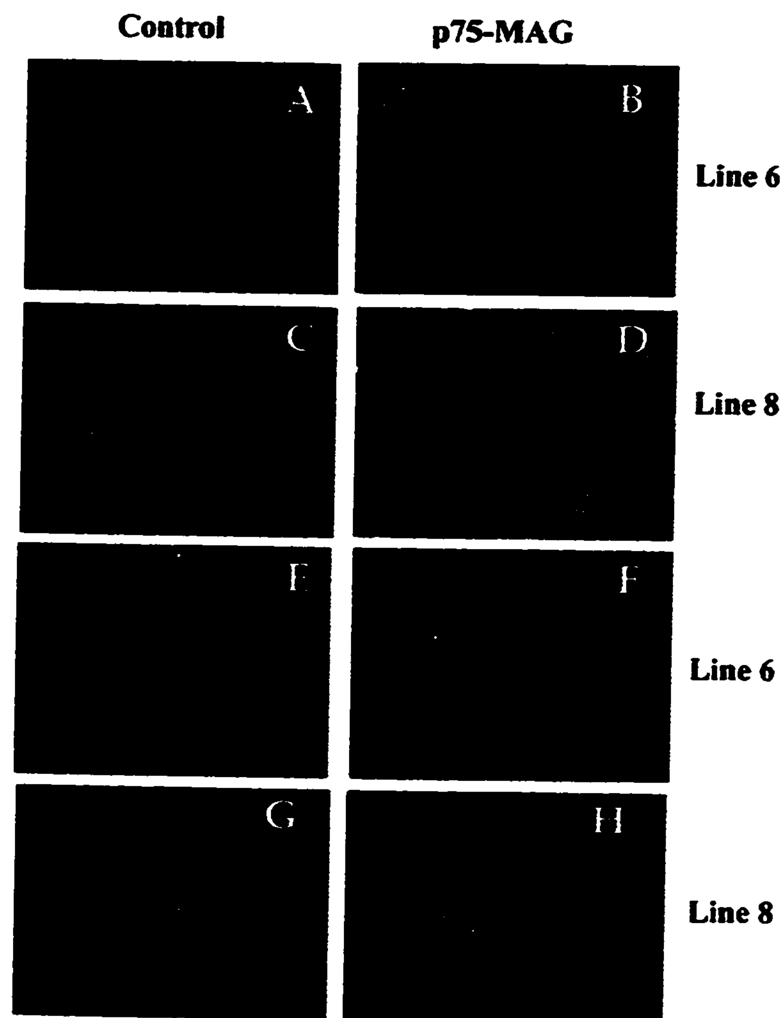

Fig. 22. Surface detection of NCAM and Tenascin on p75-MAG transgenic Schwann cells. Live Schwann cells from Lines 6 and 8 p75-MAG or from control sibling mouse were incubated with antibodies to NCAM (A-D), and tenascin (E-H) then with an Oregon green-conjugated goat anti-mouse IgG (1:1000).

It is well established that Schwann cells upregulate p75 receptor shortly after crush or transection of the nerve (Heumann et al., 1987). The expression of p75 receptor mRNA peaks at 4 days post injury (dpi), and starts to decline with increasing re-myelination of

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newly formed axons (Heumann et al., 1987; Shy et al., 1996). Correspondingly, when mRNA was extracted from 7dpi crushed sciatic nerves and RT-PCR carried out for rat L-MAG, we were able to see that the transgene (rat L-MAG) is turned on in the p75-MAG positive animals (Fig.23A). There was no signal for rat L-MAG in the crushed or uninjured sciatic nerves from control animals. However, the uninjured contralateral nerves, for all positive mice also showed rat L-MAG expression. In addition, although this is not a strictly quantitative method, we observed that there were distinctions in the expression of rat L-MAG among the three mouse lines. Line 6, the one with the lowest copy number, had barely any detectable levels of L-MAG in the contralateral side, while the ipsilateral had low but detectable levels. Line 3, on the contrary had very high levels of rat L-MAG in both nerves, injured and uninjured. Finally, line 8, with the highest copy number, had high levels in the injured nerve and very low levels in the contralateral uninjured side. When sciatic nerves were removed 7 days after transection, and the proteins separated on a PAGE gel, a Western blot for MAG showed that there was twice as much MAG in the p75-MAG transgenic nerve as in the control (Fig.23B). Finally, and very important, when sciatic nerve sections were stained for MAG after injury (14dpi) we were able to observe that the expression of MAG increased after injury, locating to the Schwann cell tubes (Fig.23C). We did not observe such a pattern after immunostaining for MAG in nerves from control animals.

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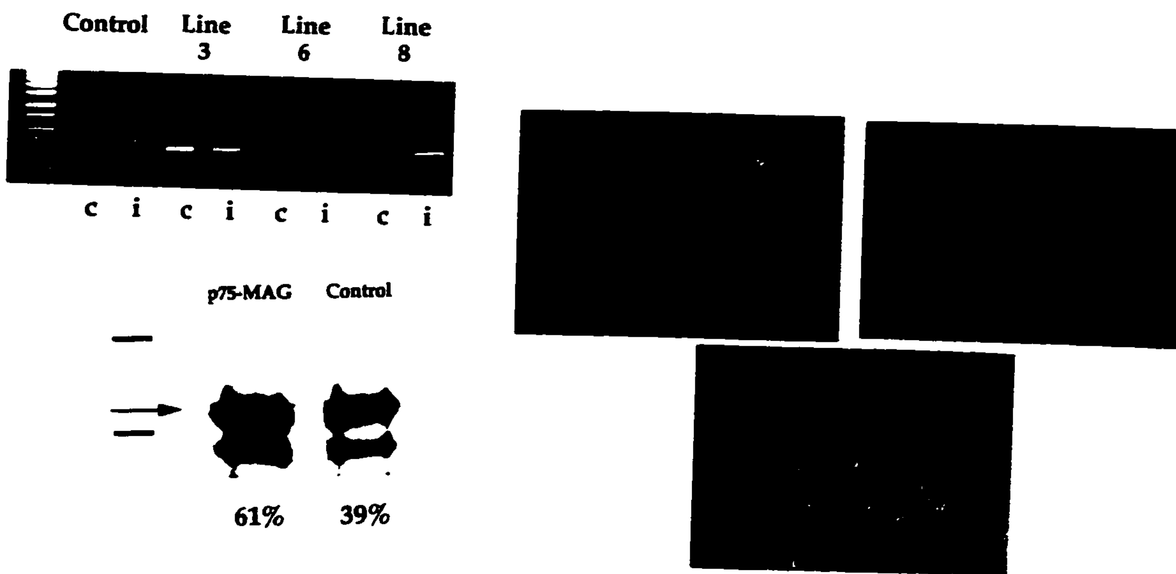

Fig. 23. Expression of rat L-MAG in transgenic mice after an injury. **A)** mRNA RT-PCR from sciatic nerves 7dpi for the different mouse lines. After extraction of mRNA with a Pharmacia kit, total mRNA was RT-PCR using a probe specific for rat L-MAG. I= ipsilateral, C= contralateral crushed nerve. Only crushed sciatic nerves from the transgenic mice have MAG mRNA, the control nerves did not express L-MAG. **B)** Western blot analyses of sciatic nerve proteins from wildtype and p75-MAG transgenic mice. Samples containing 10ug of total protein from line 8 after permanent transection were electrophoresed in 8% SDS-PAGE and transferred. The filter was probed with monoclonal anti-MAG and detected by ECL (Amersham). **C)** Control (a) and transgenic (b and c) crushed sciatic nerves after 14dpi from line 6 were immunostained for MAG, (c) corresponds to higher magnification.

All these results above show that the p75-promoter was able to direct the expression of L-MAG in the p75-MAG mice. In addition, this expression in the Schwann cells coincides with the upregulation reported for the p75 receptor after an injury or after de-differentiation of Schwann cells (Huber and Chao, 1995; Taniuchi et al., 1986).

The p75 receptor gene is also known to be expressed in other neural tissue such as cortical neurons, oligodendrocytes, and granular cells (Huber and Chao, 1995). In order to determine if MAG was also expressed in other tissue, frozen sections from the positive transgenic mice were immunostained for MAG. In sections stained for MAG we observed that there were areas positive for MAG that were not so in the control mice (Fig.24). In this figure, the cerebral pre-frontal cortex, hippocampal granular layer (CA3 area) and striatum, which are regions rich in neurons, are positive for MAG, while the same areas in the control

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mice are stained for MAG only where the myelinated fibers are found. We also observed that the meninges are positive for MAG. As mentioned before, the previous characterization of the p75 receptor promoter-expression showed that this promoter is also active in mesenchymal tissue such as meninges (Huber and Chao, 1995). In summary, the transgenic mice express MAG in areas that usually express the p75 receptor, such as cortical and striatal neurons and the mesenchymal cells, as well as in dedifferentiated Schwann cells (Huber and Chao, 1995).

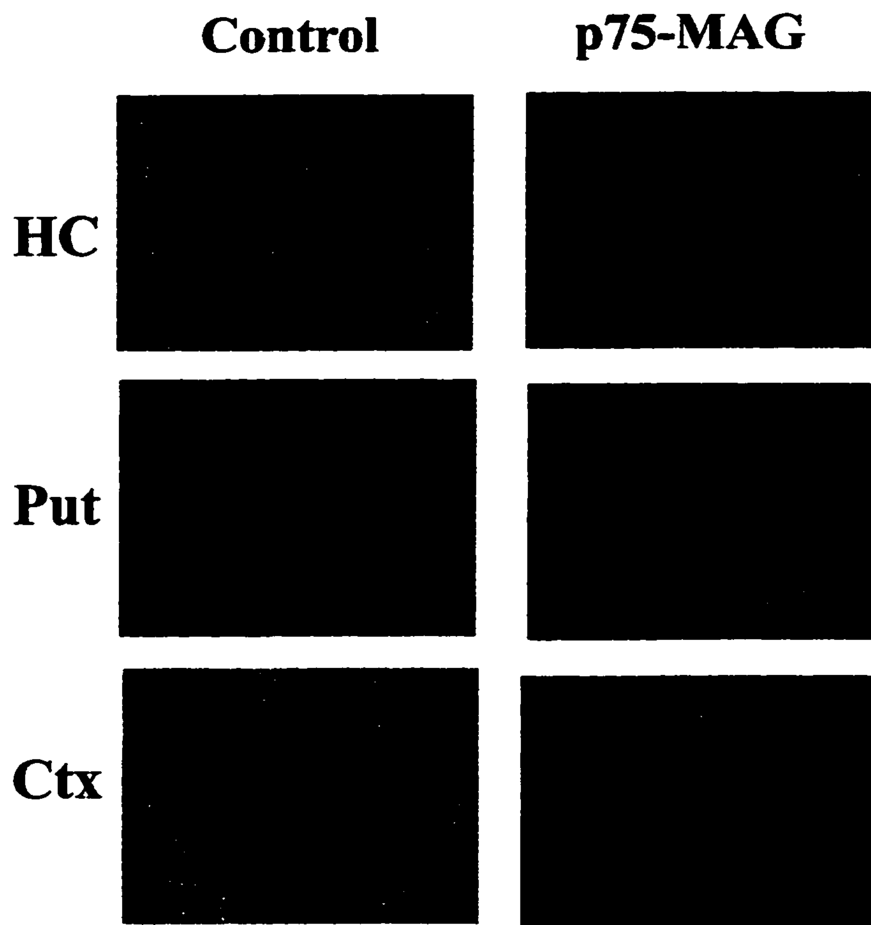

Fig. 24. Immunocytochemical localization of MAG in transgenic brains. Frozen brain sections from line 8 adult mice were probed with 513 anti-MAG, and anti-mouse Oregon green (Molecular Probes). Pictures were taken from prefrontal cortex (Ctx), hippocampus (HC) and striatum-putamen (Put). a) MAG stain in control and transgenic brains. b) Frozen spinal cord sections and trigeminal nerve from line 8 adult mice were probed with 513 anti-MAG, and anti-mouse Oregon green (Molecular Probes).

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Sciatic Nerve Injury

We have shown that the p75 receptor promoter in the transgenic mouse lines 3, 6 and 8 is turning on expression of MAG at the same time that Schwann cells dedifferentiate and come in contact with growing axons. However, we do not know if this timed expression of rat L-MAG is sufficient to inhibit axonal regeneration after an injury. To assess the effect of MAG on axonal regeneration we crushed sciatic nerves from the three different mouse lines positive for the transgene. After either 7 or 14 dpi, the nerves were removed, fixed and sectioned for data analysis. We chose these two time points because by that time there should be extensive regeneration in the control animals and because the p75 receptor expression is at its peak during those days. That is to say, if MAG is capable of inhibiting axonal regeneration, it is during these days that we should be able to observe the biggest difference in regeneration between control and transgenic mice after an injury.

There are several ways to analyze regeneration in the PNS. The most commonly used technique is electron microscopy of the injured nerves, to score regenerating fibers. Another method is by immunofluorescence of neuronal markers, specific for regenerating axons. One example is to stain for different cytoskeleton components like neurofilament, GAP43, microtubule-associated glycoprotein (MAP), or tubulin. Of these markers, we chose neurofilament and GAP43 for two reasons. Neurofilament was chosen because they constitute a large portion of the axonal cytoskeleton and they correspond to that portion of the axon that has already become stabilized. That is to say, the regenerated axon that has already grown beyond the injury site and is ready to be myelinated. GAP43 was chosen because it is a protein with an almost exclusive neuronal distribution and is associated with actively growing axons, however, dedifferentiated Schwann cells have low levels of this protein (Campbell et al., 1991; Curtis et al., 1993). Importantly, GAP43 is greatly enriched in the growth cones of the regenerating axons; mature axons do not express this protein.

In order to assess the extent of regeneration by immunostaining for neuronal cytoskeletal markers, the number of positive fibers perpendicular to the length of the sciatic nerve were counted. In this manner, we can score the number of axons that have

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successfully grown in that sciatic nerve section at different distances beyond (distal) to the crush site. We decided to score at 30 and 100mm distal to the injury site because that way we could determine if there are differences in axonal regeneration closer to the injury site or further down the nerve, close to its branching into smaller nerves.

When the sciatic nerve sections from crush injuries were stained for neurofilament positive fibers by immunofluorescence, it was obvious that the number of positive axons was greatly reduced in the transgenic mice compared to control mice (Fig.25). In the sections from the transgenic mice, we observed a smaller number of thin, brightly stained neurofilament positive fibers, and higher number of large, faintly stained neurofilament fibers. The former corresponds to the regenerated axons, while the latter correspond mostly to the degenerating axons from the distal stumps that have not yet been cleared. This reduction in the number of neurofilament positive fibers was observed: 1) when counting closer (30mm) as well farther (100mm) from the injury site; 2) at both times, 7 and 14dpi; and 3) for all mouse lines, irrespective of their copy number. These results indicate that, regardless of distance to regenerate and time post-injury, the transgenic animals show a dramatic reduction in axonal regeneration.

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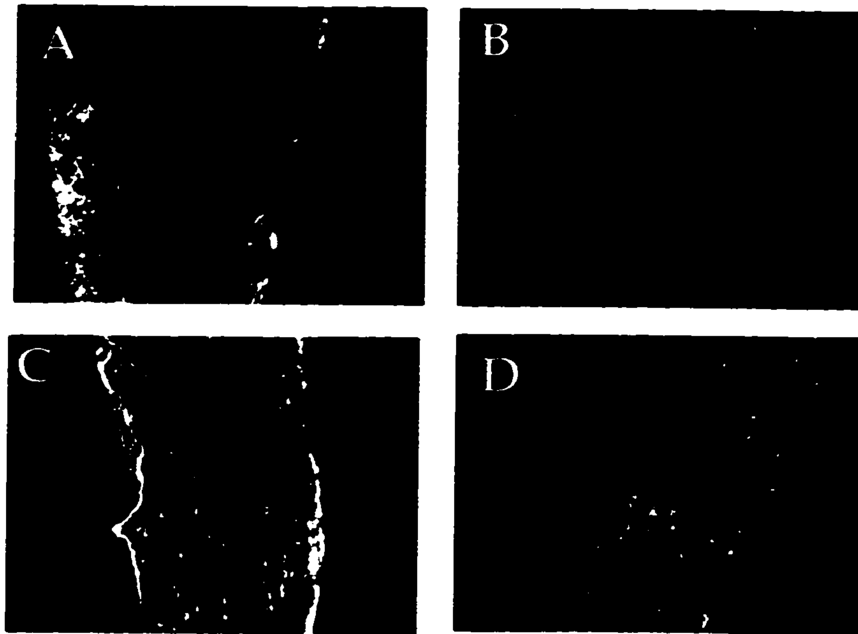

Fig. 25. Immunofluorescence stain for Neurofilament of Sciatic Nerve after crush. Fixed sections from lines 6 and 8 sciatic nerves 7 days after crush were stained with a polyclonal to neurofilament and detected with anti-rabbit Oregon green (Molecular Probes). **A)** Control line 6, **B)** p75-MAG line 8, proximal area, before injury site, notice the uninjured axons, **C)** p75-MAG line 6 distal to injury, **D)** p75-MAG line 8 distal to injury.

When we quantitated these differences by counting the number of positive fibers across the section, we observed a significant reduction in the p75-MAG lines compared to the control animals (Table VI). The reduction in number of regenerated axons in the transgenic mice was about 50% less than nerves from the control mice when we estimated the number of neurofilament positive fibers. These differences between control and transgenic positive fibers were found consistently, in all the lines expressing the transgene with different copy numbers. Moreover, this 50% reduction in regenerated fibers was observed at both 30 and 100mm from the injury site as well as at both post-injury times, 7

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and 14dpi. Hence, these results corroborate our previous observations by immunostaining, that there is a marked reduction in axonal regeneration in the p75-MAG mice after injury. Together these observations indicate that the presence of MAG in the Schwann cells during regeneration in the sciatic nerve results in a reduced number of axons growing into the distal stump.

TABLE VI.

Neurofilament Positive Fibers in Sciatic Nerves after a Crush Injury

Mouse line (Copy number*)	dpi	Distance from injury	Mean of Neurofilament positive fibers ± SD		Average of fibers
C57 control	7	(30mm)	14.6±1.3		19
8 control	7	(30mm)	24.1±7.3		
6 p75-MAG (l)	7	(30mm)	9.3±1.3	**	6.5
3 p75-MAG (m)	7	(30mm)	2.6±0.3	**	
8 p75-MAG (h)	7	(30mm)	7.6±0.3	**	
C57 control	7	(1cm)	17.0±0.5		19
8 control	7	(1cm)	21.3±17		
6 p75-MAG (l)	7	(1cm)	9.6±0.3	**	10.5
6 p75-MAG (l)	7	(1cm)	7.3±0.3	**	
3 p75-MAG (m)	7	(1cm)	16.3±1.4	**	
8 p75-MAG (h)	7	(1cm)	9.1±1.0	**	
3 control	14	(1cm)	6.5±0.3		6.5
3 p75-MAG (m)	14	(1cm)	3.5±0.3	**	3.5

* Copy number = refers to amount of copies in the genome of the mouse line. They are: h for high, m for medium and l for low copy number.
** The means are different by a one-way ANOVA, $p<0.0005$
Neurofilament count was 30mm or 1cm after the injury site.

As for the neurofilament stained fibers, when crushed sciatic nerves were analyzed for GAP43-positive fibers, the number of regenerated axons in the distal stump of the transgenic animals was reduced compared to the controls (Fig.26). Figure 25 shows the GAP43-positive fibers that grow parallel to the length of the nerve as thin, bright fibers, representing the distal portion of the regenerating nerves. As previously observed when using an anti-neurofilament antibody, there are fewer GAP43-positive fibers in transgenic nerves than in control nerves. This was so for all the three mouse lines. There is no stain in

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the contralateral side of the transgenic nerve as was expected, because fully-grown and mature axons do not express GAP43; only actively growing axons express high levels of GAP43.

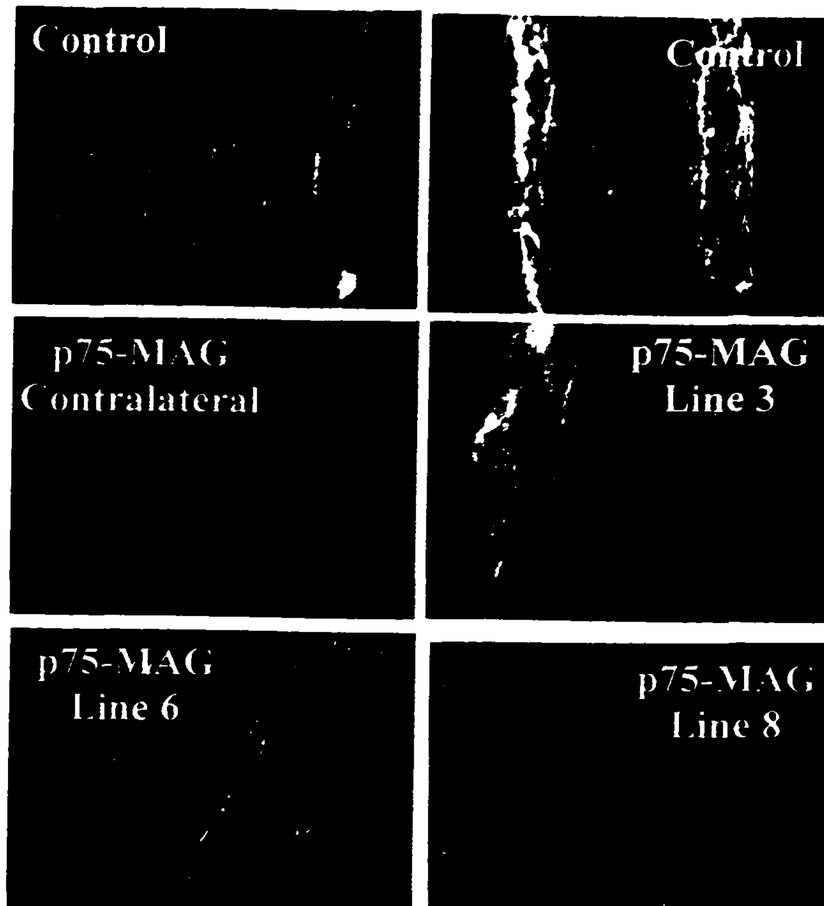

Fig. 26.. Immunofluorescence for GAP43 of sciatic nerve after crush. Fixed sections from line 8 mice sciatic nerves 7 days after crush were stained with a polyclonal antibody to GAP43 and detected with anti-rabbit Oregon green (Molecular Probes). The nerves were from two different control line 8 or from the ipsilateral and contralateral (p75-MAG contralateral) side of a transgenic line 8 (p75-MAG). GAP43 stain reveals abundant regenerating axons in both controls and none in the transgenic, also there is no GAP43 in the uninjured nerve, because mature axons do not express GAP43.

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When the number of GAP43-positive fibers was quantitated (Table VII), the number of regenerated axons varied, but there was always a significant decrease in the number of regenerated fibers in nerves from the transgenic compared to control mice. There are more fibers in the area close to the injury site, at about 30mm, than farther away, at about 100mm distally, for all the lines studied, both transgenic and control nerves. We also observed that the number of GAP43-positive fibers is higher after 7 than after 14 days post injury for both control and transgenic mice. In contrast to the observations from counting neurofilament-positive fibers, the number of GAP43-positive fibers decreased even more with distance and time, especially in the transgenic nerves. This finding suggests that many axons still have not been able to regenerate in the transgenic mice. Probably they are stalled at the boundary of the injury site. We are currently testing this hypothesis by checking the injured nerves under electron microscopy at different time intervals.

In summary, the control animals had between twice and six times more GAP43-positive fibers compared with the transgenic mice at both distances and times post-injury. We observed two exceptions in this pattern of regenerating axons. One was a transgenic animal from line 3 that had normal regeneration compared to the controls; the other was a control animal that had a significant reduction in the number of GAP43-positive fibers compared to the other control. These exceptions are most likely due to an error in tagging a transgenic as control and vice-versa.

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TABLE VII.

GAP43 Positive Fibers in Sciatic Nerves after a Crush Injury

Mouse line (Copy number*)	dpi	Distance from injury	Mean of GAP43 positive fibers ± SD	Average of fibers
C57 control	7	30mm	14.5±1.2	14.5
6 p75-MAG (l)	7	30mm	9.3±1.1 **	5.1
3 p75-MAG (m)	7	30mm	2.6±0.5 **	
C57 control	7	1cm	3.6±1.3	
8 control	7	1cm	10.7±3.8	10.7
6 p75-MAG (l)	7	1cm	0.5±0.5 **	2.3
6 p75-MAG (l)	7	1cm	4.6±2.6 **	
3 p75-MAG (m)	7	1cm	9.2±1.0	
8 p75-MAG (h)	7	1cm	2.0±1.0 **	
6 control	14	30mm	7.6±2.0	7.6
6 p75-MAG (l)	14	30mm	4.0±1.0 $	4
6 control	14	1cm	6.8± 2.3	6.8
6 p75-MAG (l)	14	1cm	1.6±1.8 **	1.2
8 p75-MAG (h)	14	1cm	0.8±0.8 **	

* Copy number = refers to amount of copies in the genome of the mouse line. They are as h for high, m for medium and l for low copy number.
** The means are different by a one-way ANOVA, $p<0.0005$
$ $p<0.05$ by T-test
Neurofilament count was 30mm after the injury site or 1cm distal to it.

Another approach to assess the extent of regeneration is by electron microscopy (EM). This method, which is the most commonly used technique for evaluating regeneration, is different from using immunostaining in several ways. The most important distinction is that it gives a more accurate picture of the process of regeneration itself in that it allows a closer and more precise image of the conditions in the distal stump of the nerve. One of the parameters used to score successful regeneration in EM sections is to count the number of axons present in the section. This is carried out in transverse sections of the injured nerves, and the axons are seen as clearings in the sections, because of their high content of neurofilament, which is less refractory by EM, or because they are already myelinated.

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When the crushed sciatic nerves at 7dpi, were examined by electron microscopy, the results were consistent with those obtained after immunostaining (Fig.27). That is to say, there were many fibers growing past the injury site in the control mice, which were already myelinated, and there were very few regenerated fibers in the transgenic mice (Fig.27 D, E and F). Another observation made from the EM pictures was the large amount of "debris" in the transgenic nerves. This increased amount of "debris" can be observed as a greater amount of space between growing axons filled with what looks as incompletely degenerated axons. The presence of this partly degenerated axon and its debris in the distal stump can explain the higher background staining observed when carrying out immunostaining in the p75-MAG crushed nerves.

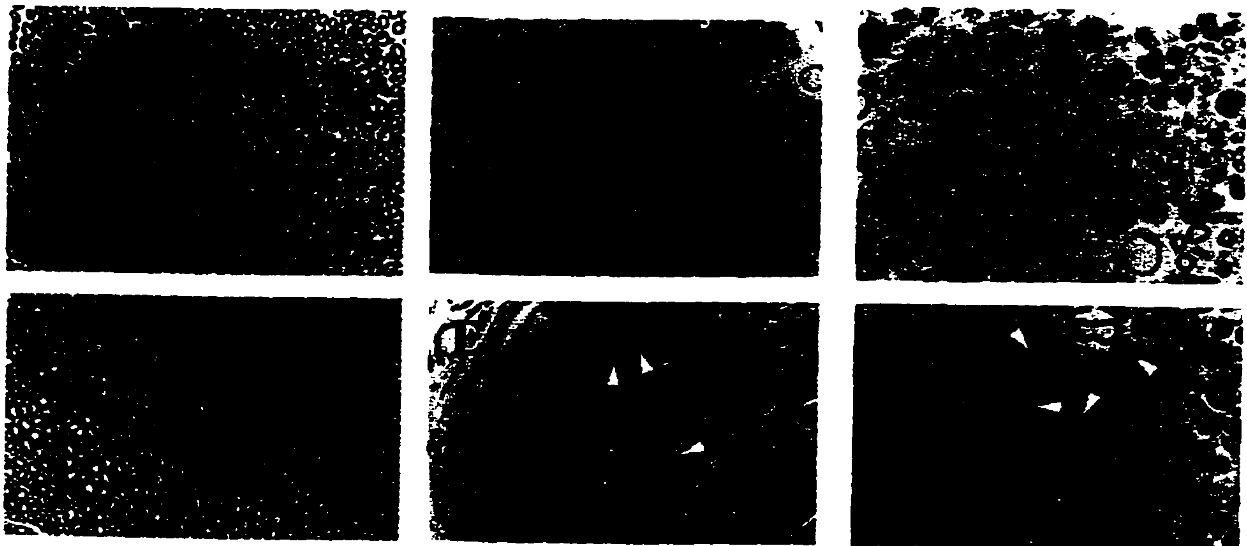

Fig. 27. Electron microscopy of injured control and p75-MAG sciatic nerves. Sciatic nerves from control (A, B, C) and p75-MAG transgenic (E,F,F) from the high copy number line 8, were sectioned and visualized under EM. A,D) Proximal section of control and p75-MAG nerves. B, E) First distal section of nerves 7dpi. C,F) Higher magnification of second distal segment of the injured nerves. Arrows indicated the few axons that regenerated into the distal stump of the p75-MAG nerve. Arrowheads indicate partly degenerated axons present in the p75-MAG nerve.

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Axons regenerating into the distal stump quickly become ensheathed and myelinated by the neighboring Schwann cells. However, we observed that in the transgenic nerves there was a higher proportion of unmyelinated axons compared with the control nerves. On a closer examination, we found that these axons were ensheathed by Schwann cells and were started to be myelinated. Most likely because of the delay in axonal regeneration in the p75-MAG mice, there is consequently a delay in myelination of the few, newly regenerated axons as they are not large enough to be myelinated.

Myelinated axons tend to degenerate retrogradely from the site of axotomy injury, and this retrograde degeneration is proportional to the size (caliber) of axons (Griffin et al., 1977). This process, which depends on the neurofilaments and is observed as a decrease in the caliber of the axons, gives indirect indication of successful regeneration in the injured axons (Griffin, 1993). When we took a closer look at the proximal stump of the crushed sciatic nerves from Fig.26, we observed that there were more axons of smaller caliber in the transgenic nerve compared with the control. When we counted one set, there were 492 small axons in the p75-MAG nerve and 342 in the control, that is 143% more than in control. That is to say, there is an increase in retrograde degeneration in the transgenic mice compared with the control animals, suggesting once more that there is a dramatic lack of axonal regeneration in the p75-MAG mice.

Regeneration in the PNS can be influenced by different effects. In the C57BL/Wld^s mice, there is a delay in regeneration due to impaired clearance of myelin (Brown, 1992; Brown, 1994), or this delay in this mice can be changed to enhanced regeneration by the absence of MAG (Schafer et al., 1996). In order to assess if the transgenic mice we have generated have any alteration in their PNS proteins after sciatic nerve injury, we compared the levels of various neuronal and glial proteins in control and transgenic mice. Westerns blots and/or immunofluorescence stain was carried out for L1, NCAM, Po, MBP, tubulin, neurofilament, MAPs, laminin and tenascin from 7dpi crushed or transected sciatic nerves (Fig.28). When we compared the levels of the extracellular matrix proteins, laminin and tenascin, between control and transgenic animals we observed that laminin was the same and there was about twice as much tenascin in the transgenic mice as in control animals. These findings and the observations from the EM suggest that basal lamina is mostly the

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same in both mouse lines after the injury. However, the neurite promoting cell adhesion molecule NCAM was more abundant in the p75-MAG nerves compared to the control nerves, while L1 was not different. When we examined the myelin proteins, MBP and Po, we observed that their levels did not change in the transgenic compared with the control. The neurofilament medium subunit (NF-M), tubulin and MAP (results not shown) protein levels showed a small increase in the transgenic mice. In addition, when crushed nerves were checked by immunostaining for MBP and L1, we did not observe any major difference in the distribution or levels for these two proteins between lines 6 and 8, neither between control and transgenic nerves (Fig.28). In summary, in the p75-MAG mice after injury, there are significant increases in the amounts of tenascin, NCAM, NF-M, tubulin and MAPs, but no detectable changes in laminin, L1, MBP and Po proteins when compared to control injured nerves.

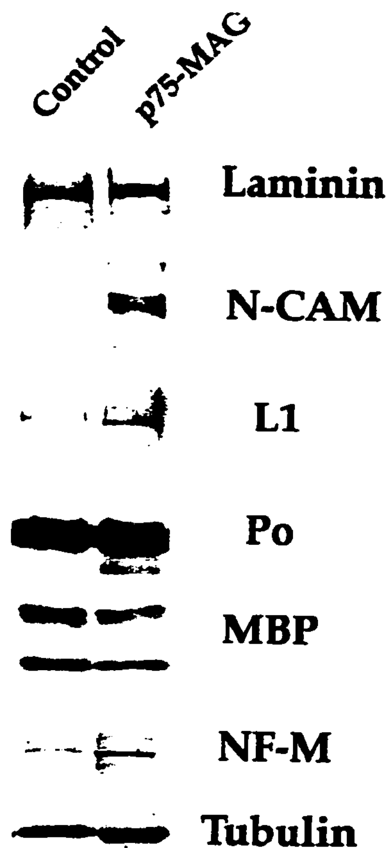

Fig. 28. Western blot of ECM, CAM, myelin and cytoskeletal proteins. Proteins (5 or 15ug) from permanent transected sciatic nerves (7 dpi) from control and p75-MAG mice (line 8), were run on a PAGE and blotted onto PVDF membranes. Westerns for laminin, NCAM, L1, Po, MBP, NF-M and tubulin were performed and detected with secondary antibodies conjugated to alkaline phosphatase or HRP and developed following manufacturers instructions (KPL).

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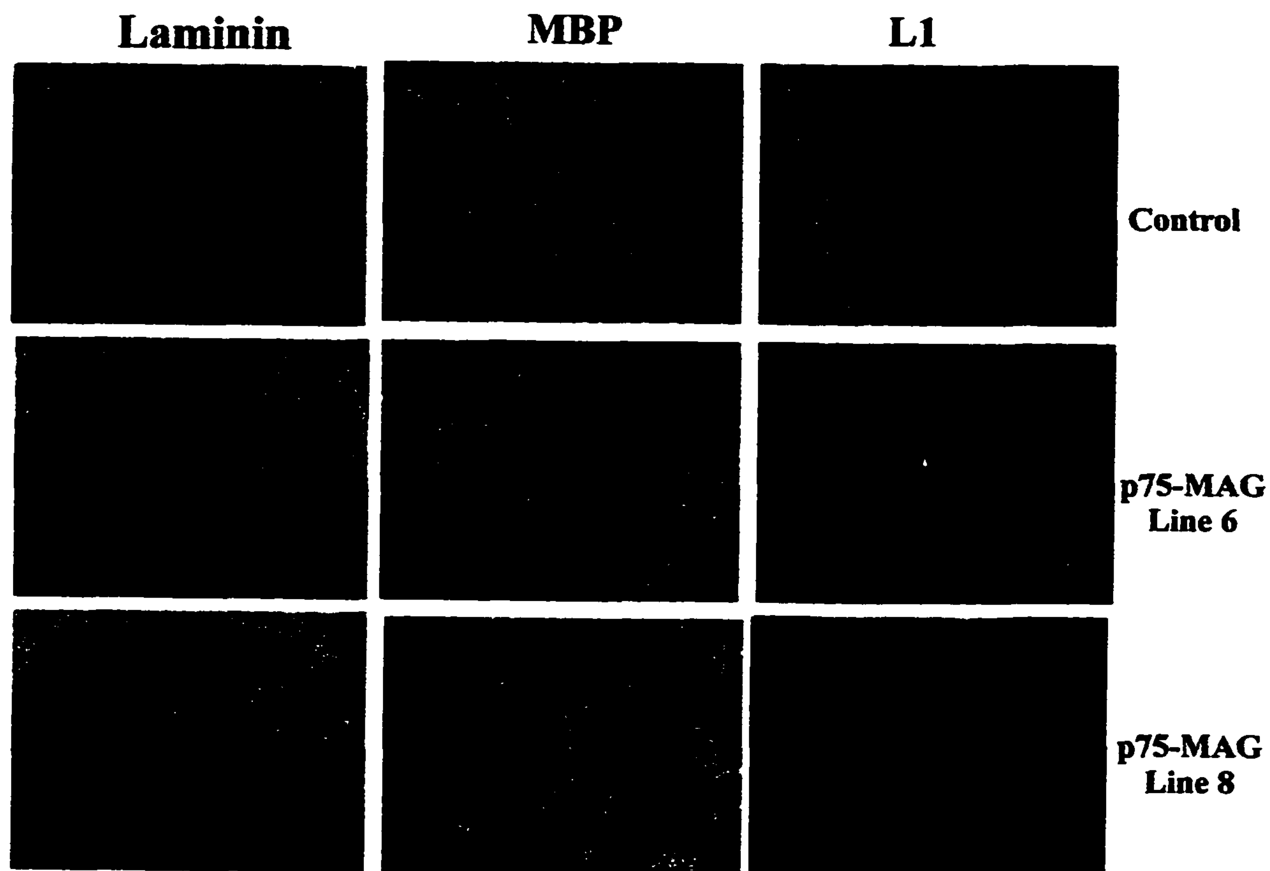

Fig. 29. Immunofluorescence of laminin, MBP, and L1. Indirect immunofluorescence of crushed sciatic nerves 14dpi was done for Laminin, MBP and L1 in control and p75-MAG (line 6 and 8).

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Discussion

The results presented here show that MAG can inhibit axonal regeneration *in vivo*. When PNS nerves from mice genetically engineered to express rat L-MAG under the control of the p75 receptor promoter are injured, regeneration of their axons is strongly inhibited. These results show directly for the first time that MAG can affect axonal regeneration *in vivo*. Previous studies were carried out by crossing MAG-deficient mice with the C57BL/Wld^s mice, with impaired clearance of myelin, showing that in the absence of MAG regeneration in this double mutant is enhanced now that MAG is absent (Schafer et al., 1996), but not that MAG can actively inhibit regeneration.

After an injury in the PNS, there are several processes that take place. One is the invasion of macrophages to the injury site and with it the beginning of Wallerian degeneration. This entails degeneration and clearance of damaged myelin and axons by the glia and macrophages, as well as the proliferation of Schwann cells that will first provide a permissive environment for regeneration and then will eventually remyelinate the regenerated axons (Griffin, 1993). These processes begin 24 hrs after injury (Stoll et al., 1989). Following close behind Wallerian degeneration, at 1-2 days post injury, regeneration begins with the first growth cone sprouts emanating from the node of Ranvier (McQuarrie, 1978).

Axonal regeneration starts with the first sprouts that enter the injury site and go beyond it. It has been shown that axons regenerate along the Büngner bands, which are the basal lamina tubes containing the proliferating and de-differentiated Schwann cells, as well their interlaced cytoplasmic processes (Griffin, 1993). Hence, when we engineered mice to express MAG in Schwann cells when they are dividing we targeted the expression of MAG to the time of axonal regeneration along the Schwann cells paths. This specific expression was achieved by using the human p75 receptor promoter because it is expressed in Schwann cells only at the time when they are normally permissive for regeneration, while dedifferentiated, not during myelination. Also because the complete sequence of the human p75 promoter is known and has been used successfully by others in transgenic mice (Huber and Chao, 1995).

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In our present studies we created several lines of transgenic mice, each expressing rat L-MAG in Schwann cells at the same time that regeneration is proceeding. Because in the different lines of mice regeneration is strongly inhibited by the presence of MAG, the lack of axonal regeneration in these mice is unlikely to result from disruption of an endogenous gene at the transgene insertion site. If this were so, we would have observed that lack of regeneration correlated either with the transgene copy number (because of different levels of expression) or with a specific mouse line (because of different insertion sites). However, what we observed was that in all the mouse lines, there was a significant reduction in regeneration, regardless of their copy number. In other words, the different amounts of MAG expressed after the injury by the three different lines did not make a difference in their extent of regeneration. This finding is not surprising, because we already knew that *in vitro,* different amounts of MAG will not have a proportionately stronger inhibitory effect on neurite outgrowth (Mukhopadhyay et al., 1994). That is to say, the concentration threshold at which MAG can potently inhibit axonal regeneration *in vivo* and *in vitro* must be very low.

When Schwann cells from the transgenic mice were cultured for a few days, the level of MAG expression was so low that we could not detect it by immunostaining. This was expected for the endogenous mouse MAG but not for the p75-MAG in our transgenic lines (Heumann et al., 1987; Jessen et al., 1994; Lemke and Chao, 1988). It was only after 3-5 days in culture in the presence of NGF that we were able to see high levels of MAG expressed by Schwann cells from the transgenic mice. Under the same culture conditions, the Schwann cells from the control animals showed no expression of MAG. The use of NGF in Schwann cell culture is consistent with what takes place in the distal stump of the sciatic nerve after injury. The initial studies on the effect of NGF on Schwann cells after transection showed that endogenous mRNA NGF increased and decreased rapidly in the Schwann cells. However, the mRNA for the p75 receptor increased steadily, only to decrease once regeneration occurred and the Schwann cells were ensheathing the newly formed axons (Funakoshi et al., 1993; Heumann et al., 1987). These findings implied that in order to turn on expression of the p75 promoter in Schwann cells, an initial boost of NGF is required, but NGF is not needed to sustain subsequent high levels of p75 receptor expression. In support of this suggestion of a need for the presence of NGF before p75

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receptor goes up, is the observation that the expression of p75 receptor is increased only after Schwann cells have secreted NGF (Monuki et al., 1993). In a system comparable to what takes place in the distal nerve after an injury, we found increased *in vitro* expression of MAG after we added NGF to the culture media. These results indicate that the p75-MAG Schwann cells express detectable amounts of MAG when they are dedifferentiated and under similar conditions to that present in a nerve after injury.

The aim of these experiments was to show that MAG can affect axonal regeneration *in vivo* when expressed by Schwann cells when they are in close contact with growing neurites. Hence, the demonstration that MAG is indeed expressed by the glial cells of the PNS at the right time is fundamental. We have shown data that support this statement by several approaches. As mentioned above, one of them was showing that the de-differentiated transgenic Schwann cells would express MAG *in vitro* under the control of the p75 receptor promoter. The second and most important method was to show that *in vivo* the transgenic mice express MAG after an injury. This expression of MAG *in vivo* was shown by RT-PCR of crushed nerves, western blot for MAG of transected nerves, and very important, by staining for MAG after injury. These findings show that p75-MAG transgenic mice express MAG after injury, at the same time that regeneration is taking place. However, it is also known that regeneration can also be affected if other molecules are altered (up-regulated or down-regulated) in transgenic mice compared with control non-transgenic mice (Gondre et al., 1998; Schneider-Schaulies et al., 1990). In order to assess if this is the case in our transgenic mice, we carried out western blots and immunostaining for several molecules known to be either promoting or non-permissive for neurite outgrowth, which are important for PNS regeneration. The molecules we studied after PNS injury belong to three categories: extracellular matrix, myelin, and cell adhesion molecules. First, of the ECM molecules we examined, laminin and tenascin, only tenascin showed a change; its levels of expression after injury in the p75-MAG were twice as much compared to control mice. The amount of laminin remained the same after the injury in both control and transgenic nerves. It was apparent that the basal lamina tubes, which was rich in laminin, remained intact in both the transgenic and the control mice (Griffin, 1993). Second, when crushed nerves were immunostained for Po and MBP, it was obvious that there were more

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myelin ovoids in the transgenic nerves compared to the control nerves. However, when we examined these proteins by western blotting, we did not observe any major difference for either in permanently transected nerves. This discrepancy in the results between both detection methods for MBP and Po amounts, most likely is because the slower clearance of myelin in the p75-MAG nerves, is compensated by the much faster regeneration and myelination of axons in the control animals. Third, we found that transgenic Schwann cells express more NCAM and tenascin than control Schwann cells. It has been shown that de-differentiated Schwann cells will increase their L1, NCAM and tenascin levels (Martini and Schachner, 1988; Seilheimer and Schachner, 1987). However, it is unusual that the transgenic de-differentiated Schwann cells will have almost double amount of these proteins compared with their control counterparts. Seilheimer showed that Schwann cells will upregulate the expression of L1 but not NCAM when cultured in the presence of NGF (Seilheimer and Schachner, 1987). Nevertheless, transgenic Schwann cells have even higher levels of NCAM but not L1 after an injury or NGF treatment. One possible explanation for this increased NCAM and tenascin expression is that the transgenic Schwann cells remain longer and more persistently in a de-differentiated phenotype than the control cells. We are currently testing this hypothesis by looking at sympathetic unmyelinated axons and at the early stages of myelination, for levels of tenascin, CAMs and ensheathment of axons. However, there is another explanation for the slower rate of myelination in the p75-MAG mice, that because regeneration is so much slower in these animals, myelination is equally slowed down from lack of regenerated mature axons in the distal stump.

These results show that the altered expression of some of these molecules could not account for the lack of regeneration in the p75-MAG mice, except for the presence of MAG in Schwann cells during regeneration. There are several reasons indicating that MAG is the cause of lack of axonal regeneration. First, we have shown that MAG, which is a potent inhibitor of axonal regeneration for a variety of neurons is upregulated in the Schwann cells during their closest interaction with growing PNS axons (De Bellard et al., 1996; Mukhopadhyay et al., 1994). Second, that the most neurite-promoting molecules in the PNS, laminin, L1 and NCAM, remained unaffected or up-regulated in the transgenic mice. Third, although the levels of tenascin doubled the ones from control animals, tenascin is a

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non-permissive molecule for CNS regeneration (Faissner, 1990), but in the PNS it has been shown that its upregulation is associated with successful regeneration (Fischer et al., 1997; Fruttiger et al., 1995; Langenfeld-Oster et al., 1994). In other words, lack of regeneration in the p75-MAG nerves cannot be a consequence of increased amounts of tenascin and/or NCAM at the distal end of the transgenic nerve. The reason is that the effects of these molecules after an injury is to promote regeneration of axons, not to cause inhibition. That is to say, the only molecule present after an injury in the transgenic mice that can be accounted for the delay in axonal regeneration is MAG. Our finding that the two promoting CAM molecules examined, L1 and NCAM, (Doherty et al., 1991; Doherty and Walsh, 1992; Martini, 1994), could not overcome *in vivo* the inhibition by MAG in our transgenic mice further confirmed our previous *in vitro* results (Tang et al., 1997). In those experiments, neurite outgrowth from cerebellar neurons growing on cells expressing NCAM or on L1-Fc coated slides, was inhibited by a soluble form of MAG, MAG-Fc. Here we found that even in the presence of abundant amounts of L1 or NCAM, regeneration in the sciatic nerve was still inhibited by MAG-expressing Schwann cells. This finding further implies that the addition of these molecules to an injured spinal cord probably will not help regeneration if MAG is present at the injury site.

In our regeneration analyses, we observed that the extent of regeneration was different if counting for neurofilament-positive from counting GAP43-positive fibers in the distal stump. By counting the number of GAP43-positive fibers, we obtained a greater difference in numbers between transgenic and control mice than when counting fibers positive for neurofilament. This difference in the numbers of fibers is because actively growing axons express high levels of GAP43, while stable axons do not have GAP43 but neurofilament. In other words, immunostaining for GAP43 indicates the amount of regeneration taking place at the time the animal was sacrificed, while, neurofilament immunostaining shows all the axons that have successfully regenerated up to that time point. Thus, GAP43 stain is a more accurate marker for axonal regeneration. Hence, our finding that in the p75-MAG mice there is a marked decrease in GAP43-positive fibers compared to neurofilament-positive fibers indicates that axonal regeneration is being dramatically delayed in the transgenic nerves. The second and very telling difference we observed from using GAP43 to stain growing axons was that the number of GAP43-positive fibers after

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14dpi was still very low in the p75-MAG nerves, especially at 100mm from the crush site. The reason for this difference is either that by 14dpi most of the axons have already grown beyond this point or because the axons have not been able to regenerate at all up to that distance and time. If the axons have successfully regenerated past these two points in the transgenic nerves at 14dpi, we should have seen an increase in the number of neurofilament positive fibers, however we did not, on the contrary, the percentage of reduced neurofilament-positive fibers remained the same and much lower than in control nerves. Together these finding indicate that the axons had not grown much farther in the transgenic animals even after 14dpi.

Although these animals show a higher level of axonal debris after the injury, for several reasons this cannot account for their lack of regeneration, as is the case in the C57BL/Wld^s mice with delayed Wallerian degeneration for several reasons. First, the delay we observed in our animals does not correlate with the reports for delayed Wallerian degeneration in the C57BL/Wld^s. For example, it has been found that in the C57BL/Wld^s mice, there is down-regulation of tenascin expression (Fruttiger et al., 1995). However, in the p75-MAG mice we found the opposite, there is an increase in the amounts of tenascin. The second reason is that morphologically the type debris that is still left in the nerve is not like that found in the C57BL/Wld^s mutant mice after several months post injury, which consists of intact, undegenerated myelinated axons (Brown, 1992; Lunn et al., 1989). The delay in Wallerian degeneration we observed in the transgenic mice is a consequence of the delay in axonal regeneration, not a cause for lack of regeneration. It has been observed that vascularization in the distal stump of a nerve depends on robust axonal regeneration. However in our mice, since axonal regeneration is definitely impaired, the normal angiogenic process in the PNS has been compromised. Third, when MAG-deficient mice were crossed with the C57BL/Wld^s mice, there was an improvement in PNS regeneration compared with C57BL/Wld^s mutant mice (Schafer et al., 1996). This data shows that; 1) even in the presence of delayed Wallerian degeneration, axonal growth can still take place provided MAG is not encountered by the regenerating axons, and 2) that lack of Wallerian degeneration is not sufficient to stop regeneration. To put it another way, the presence or absence of MAG at the distal stump is sufficient to make a difference in axonal regeneration as shown by those experiments and the ones presented here.

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The results presented here show unequivocally that MAG can inhibit axonal regeneration *in vivo*. However, the findings with the MAG-deficient mice after a spinal cord section, do not appear to be in agreement with our findings (Bartsch et al., 1995; Li et al., 1996). Those experiments should have shown some difference in the extent of axonal regeneration after the injury, however only the ones carried out by Li and co-workers showed an increase in CNS regeneration in the MAG-deficient mice compared with wildtype MAG mice. As already mentioned in the introduction of chapter 2, there are several explanations for these discrepancies; for example, the different mouse backgrounds used, the method for scoring regeneration, the likely presence of compensatory molecules or that the effect of myelin inhibitors is not additive. One factor, which is very important, is the background mouse line used. It is not the first time that a mice background makes a dramatic difference in the phenotypic outcome of a protein-deficient mouse (Deroche et al., 1997; Orioli et al., 1996; Sibilia and Wagner, 1995). Henkemeyer found out that when he backcrossed his C57BL/6J Eph3 and 5 receptor-deficient mutants with the CD1 background, their phenotype increased dramatically (Orioli et al., 1996). In the two MAG-deficient studies, the mouse background was different. The group of Roder used a C57BL/129/Sv/CD1 inbred line (Li et al., 1994), the group of Schachner used the C57BL/6J line (Montag et al., 1994). That the mouse background made a difference in the MAG-deficient mice experiments is noticed by pointing out that in the C57BL/6J wildtype mice the maximum length of regeneration was only 1mm, while in the CD1 after the same time post injury was about 8mm. In other words, in the C57BL/6J mice there is another factor(s) that is hindering regeneration, maybe a more persistent post-injury inflammation or more reactive astrocytes. In either case, the different mouse lines gave varied results perhaps due to inherent differences in each line.

When we scored regeneration by the number of GAP43-positive fibers in the nerve sections, there was a more dramatic difference in numbers between transgenic and control animals than when counting neurofilament-positive fibers. This difference did not diminish but increase with distance and time post-injury. This observation is somewhat indicative of a delay in the growth of axons after encountering MAG. In light of these findings with the

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p75-MAG mouse, we would like to suggest that MAG may cause its effect on axonal regeneration by stopping completely the growth of axons or neurites. What we are suggesting is that MAG might not have much of a direct effect in the total length that an axon can grow, but its effect is mostly in the process of neurite outgrowth initiation. Several findings support this hypothesis on the mechanism for inhibiting axonal regeneration by MAG. One finding is provided by the studies on CNS regeneration with the MAG-deficient mice. If this hypothesis is correct, there should be a higher number of fibers regenerating past the wound in the MAG-/- mice, though these may not be necessarily dramatically longer in comparison with the MAG+/+ mice. In accordance with this hypothetical role for MAG, the total number of axons past the injury in the MAG-/- mice is larger than in MAG+/+ mice, however they grew only twice as long as control axons (Li et al., 1996). Similar results were reached when scoring regeneration in the $^{MAG-/-}$C57BL/Wld^{s}; compared with the $^{MAG+/+}$C57BL/Wld^{s}, the MAG-deficient mice have twice as many axons regenerating along the myelin sheaths, than the wildtype mice also mutant for Wallerian degeneration (Schafer et al., 1996). A second finding came by scoring for neurofilament positive fibers. We observed that in general, there was about a 50% reduction in their numbers, and this did not vary much with distance or time after the injury. These observations indicate that axonal regeneration did not improve much with time, as in control animals, but remained dramatically delayed. Third, that consequently with an effect on initiation not on extension by MAG, the total absolute length that was achieved in the MAG-deficient mice of Li, was not much longer than the total length achieved in IN-1 treated wildtype mice (Bartsch et al., 1995; Schnell and Schwab, 1990). Fourth, recent findings in our lab have shown that inhibition by MAG and myelin can be reversed provided the levels of cAMP remain high (Cai et al., 98), but if they are not, MAG can still inhibit neurite outgrowth. This observation may explain the reason why when neurons are grown on MAG-expressing cells or on MAG-deficient myelin for very long periods of time, there is little or no inhibition of neurite outgrowth compared with controls (unpublished results and (Bartsch et al., 1995). What we are suggesting is that, after long time in culture neurons can increase their internal cAMP levels and hence overcome the inhibition of neurite outgrowth by MAG. This hypothesis on the cAMP levels in the neurons helps explain also why in our neurite outgrowth assays on MAG-expressing CHO cells, we find a large

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number of neurons without any neurite at all or at most, only a very short one, but at the same time, there are always a few neurons with neurites (De Bellard et al., 1996; Mukhopadhyay et al., 1994). Similar results have been observed for other growth cone collapsin molecules; that if neurons are left in the coculture for long time, the neurites are able to grow back again (Luo, 1993). Hence, the differences in neurite outgrowth on MAG-expressing CHO cells by individual neurons and after long periods of culture, may be a consequence of their internal cAMP levels. Fifth, it has been suggested that MAG/myelin has a function in keeping axonal stability and preventing uncontrolled sprouting from the nodes of Ranvier in myelinated axons (Filbin, 1996; Schwegler et al., 1995). This putative function could easily take place if MAG has such a halting effect on axonal growth. In support of this role for MAG on sprouting, recent findings in our lab have shown that when neurons are grown on MAG-expressing Schwann cells, their neurite branching is also significantly reduced (Shen et al., 1998). That is to say, MAG can potently inhibit sprouting in neurons. Altogether, the results discussed above support a role for MAG as a molecule whose function is to prevent any growth from a neuron/axon after it has established contact with it.

Regeneration is not the only process that can be affected by encountering MAG. Since these p75-MAG animals now express MAG in neurons from areas responsible for motor coordination (cortex, cerebellum and caudate) and learning (hippocampus) they may have some subtle abnormalities in their CNS. In addition, myelination in these animals may be affected because of the p75 receptor-induced, early expression of MAG in Schwann cells (Bartsch et al., 1997; Owens and Bunge, 1991). We are currently in the process of monitoring more precisely the learning and motor abilities in these mice, as well their onset of myelination in the PNS and CNS. Finally, because the p75 receptor promoter we used is turned on in the brain as early as embryonic day 11.5 (Huber and Chao, 1995), we want to study the possible consequence in axonal pathfinding and targeting for the above neurons.

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CHAPTER 4

The Receptor

Introduction

The data we have accumulated to date, indicates that MAG is a potent inhibitor of axonal regeneration from a variety of neurons *in vitro* and *in vivo*, and brings up an important question: what is the MAG receptor? For many years, MAG was hypothesized to have a role in the initiation of myelination, behaving as a receptor on glial cells, for a ligand on the surface of the axon (Salzer et al., 1990). After the findings by Filbin and co-workers, MAG has also been shown to bind and inhibit neurite outgrowth. Here, MAG is behaving as a ligand for a putative receptor on the axon (Filbin, 1995). However, the nature of this receptor remains to be determined. In addition, it is not known if the receptor is a single protein or several, as are the CNTF and neurturin receptor complexes (Baloh et al., 1997; Davis et al., 1993; Stahl and Yancopoulos, 1994).

There have been a number of indications as to the nature of the receptor for MAG. Studies on the binding properties of MAG to extracellular matrix components lead to the suggestion that MAG had a role in the process of fibrillogenesis of collagen, because it was able to change this process (Bachmann et al., 1995; Fahrig et al., 1987; Probstmeier et al., 1992). However, MAG did not bind to collagen once it had formed. In another study, fibroblasts expressing MAG were shown to support heterotypic binding, demonstrating that MAG could bind/interact with a different molecule on the cell surface, and not with itself as had been proposed (Afar et al., 1991; Johnson et al., 1989). Moreover, neurons were capable of binding to MAG and the growth of their neurites was promoted when exposed to MAG, either incorporated into liposomes or expressed on the surface of fibroblasts (Johnson et al., 1989; Poltorak et al., 1987). It has also been shown that MAG-expressing oligodendrocytes respond to MAG by increasing the complexity of their processes as well as increasing their survival when grown on surfaces coated with MAG (Gard et al., 1996; Kreider et al., 1996). Taken together, these results suggest that MAG has a receptor on the surface of neurons and oligodendrocytes, rather than being a molecule whose function is to alter the extracellular matrix of neurons and glia.

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The first clue as to the nature of the MAG binding component was from a sequence alignment of MAG with CD22 and sialoadhesin, two sialoadhesin family members (Fig.1). These molecules bind to their respective receptors on cells in a sialic acid-dependent manner (Kelm et al., 1996). Binding assays carried out with MAG-Fc showed that MAG could also bind to neurons in a sialic acid-dependent manner (De Bellard et al., 1996; Kelm et al., 1994). Furthermore, the complete removal of sialic acid and re-linking sugars in a specific manner enzymatically demonstrated that MAG preferentially binds to α2,3-O linked sialic acids. In summary, MAG is a member of the sialoadhesins, a growing sub-family of molecules from the immunoglobulin superfamily that engage in sialic acid-dependent binding to their receptors on the cell surface (Kelm et al., 1994; Kelm et al., 1996).

From these studies came the suggestion that the putative receptor for MAG was a ganglioside. These glycolipids are abundant on myelinated axons, and some carry the correct linkage required for MAG binding. Moreover, it has been shown that MAG can bind to GQ1b and GT1b gangliosides in a sialic acid-dependent manner (Collins et al., 1997; Yang et al., 1996). A caveat to these results is that, like those mentioned above with collagen, the observed interactions were not performed in a way that mimics the one taking place at the axon-glia interface. For example, they used purified gangliosides to measure their binding to fibroblasts expressing MAG, or they measured binding of purified gangliosides to MAG-Fc alone. However, it has not been shown that an antibody to any of these gangliosides can block either binding to MAG or of MAG to the neurons. More importantly, we and others have shown that MAG binds to any molecule that has a sialic acid in α2,3-O linkage, irrespective of the nature of the interaction (Afar et al., 1991; Kelm et al., 1994). For example, we have been able to detect strong binding of MAG-Fc to red blood cells and this binding can be abolished by the 513 antibody or by sialidase treatment (Kelm et al., 1994; Tang et al., 1997). Finally, as shown in Chapter 1, there was a 50% reduction in binding to MAG-Fc when neurons are trypsinized before the binding assay, indicating that MAG binds to a sialoglycoprotein. Taken together the data shows that the putative MAG receptor on neurons is unlikely to be a ganglioside.

We decided to pursue the identification and characterization of the neuronal receptor. For this purpose, we use the chimeric form of MAG, MAG-Fc as a ligand. With

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this strategy, we would be able to identify any component on the neuron that can bind to MAG. Once peptide sequences were obtained, we would screen a neuronal cDNA library with degenerate oligonucleotides to identify and clone the binding proteins (Bergemann et al., 1998).

Results

MAG binds to two proteins of ~190 and ~250kD

In order to study the nature of the neuronal components that binds MAG we designed a modified "immunopurification" approach (Fig.30). This approach consisted of surface labeling the neurons with biotin, incubating the cell lysate with the soluble, chimeric MAG-Fc, precipitating the protein complexes with protein A sepharose, and running the dissociated proteins on a PAGE. Results from these experiments using different types of neurons, showed that MAG-Fc specifically precipitated a number of proteins (Fig.31). The specificity in the precipitation was demonstrated using a control chimera, MUC18-Fc, which does not bind to neurons, and by including the monoclonal MAG antibody, 513 in the precipitation mix. The advantage of this method was that it provided a reliable way to distinguish the surface proteins from cytoplasmic proteins, as the biotin used here does not enter the cells. Therefore, the bands detected with streptavidin HRP-conjugated reflected extra-cellular membrane associated proteins, not cytoplasmic proteins nor glycolipids.

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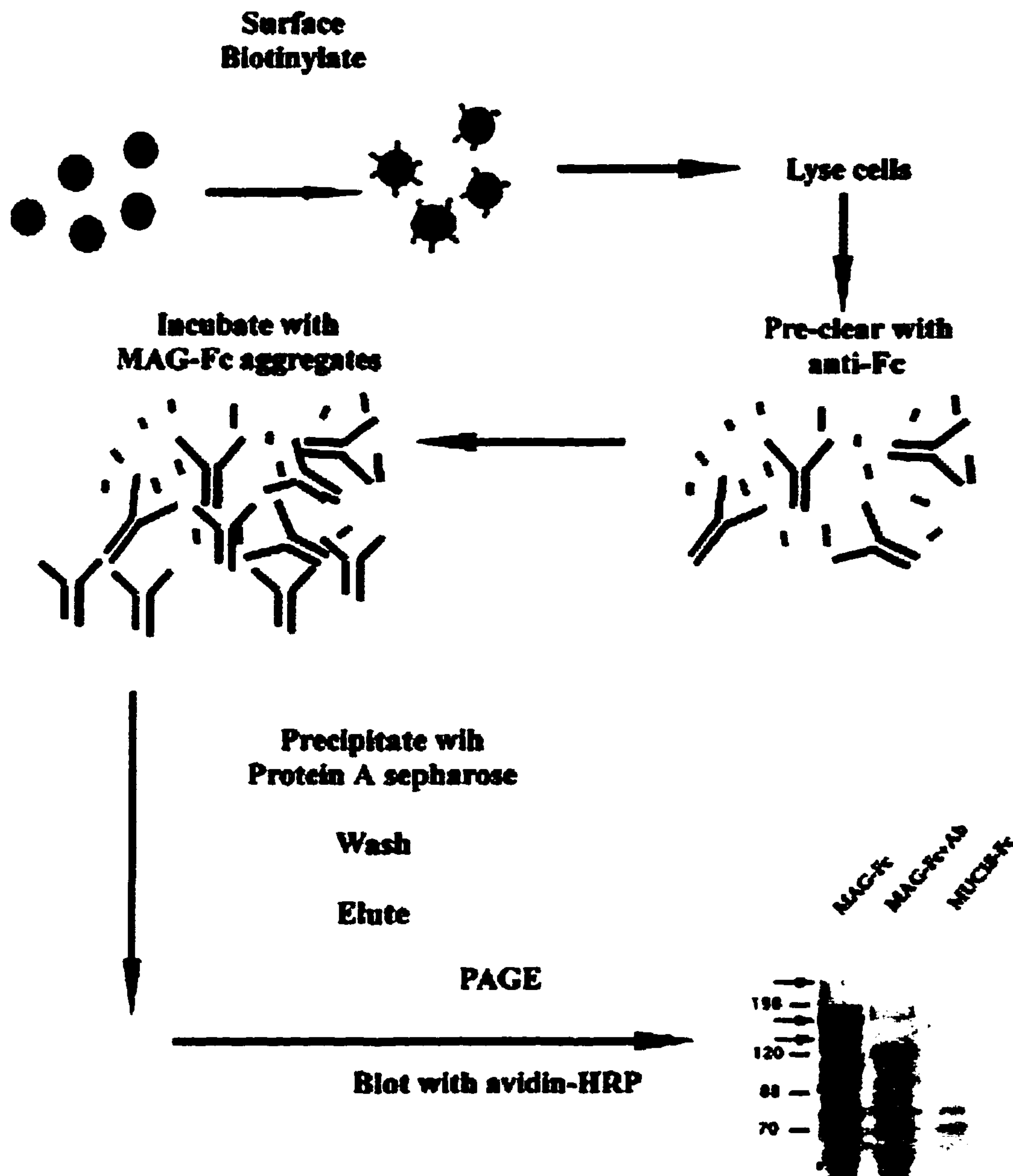

Fig. 30. Diagram of the precipitation protocol with Fc chimeras.

We used three different types of neonatal neurons for several reasons. First, in order to assess if there were any common proteins among those that are specifically precipitated by MAG-Fc. This criterion is very important because as it is already known, MAG can bind

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to any sialylated glycoprotein on a cell, which would be largely nonspecifically, without any physiological relevance. In other words, MAG-Fc can precipitate proteins from the surface of neurons other than the receptor, but by using different neurons that are known to be inhibited by MAG, we can more accurately distinguish and pick the most likely candidates to be the putative receptor. Second, by this approach we can also determine which type of neuron will provide the optimum yield of the putative receptor. Third, we decided to use neonatal neurons because these are easy to isolate in populations that are more homogeneous and their tissue dissociation gives the highest yield of cells per area dissected.

When DRG and cerebellar neurons were used to identify MAG binding components, several biotinylated proteins were apparent. Most of the precipitated proteins were nonspecific, as determined by their presence in the lysates precipitated in the presence of a MAG antibody or when MUC18-Fc was used. However, two proteins of molecular weights of approximately 190 and 250kD were specifically precipitated (Fig.31A) from both types of neuronal lysates. Similar results were obtained when the PC12 neuronal cell line was used (Fig.31B). In addition, two other proteins were also observed: a 90kD present in DRG neurons precipitates, that was not observed in the cerebellar or PC12 cell precipitates, and a 130kD present in both cerebellar and DRG neurons precipitates. We also tested retinal neurons in a similar way, and found that MAG-Fc precipitated specifically only one detectable protein of about 190kD from these cells. In summary, MAG-Fc bound specifically to at least two proteins of molecular weights of about 190 and 250kD. A summary of the results from these experiments is shown in Table VIII.

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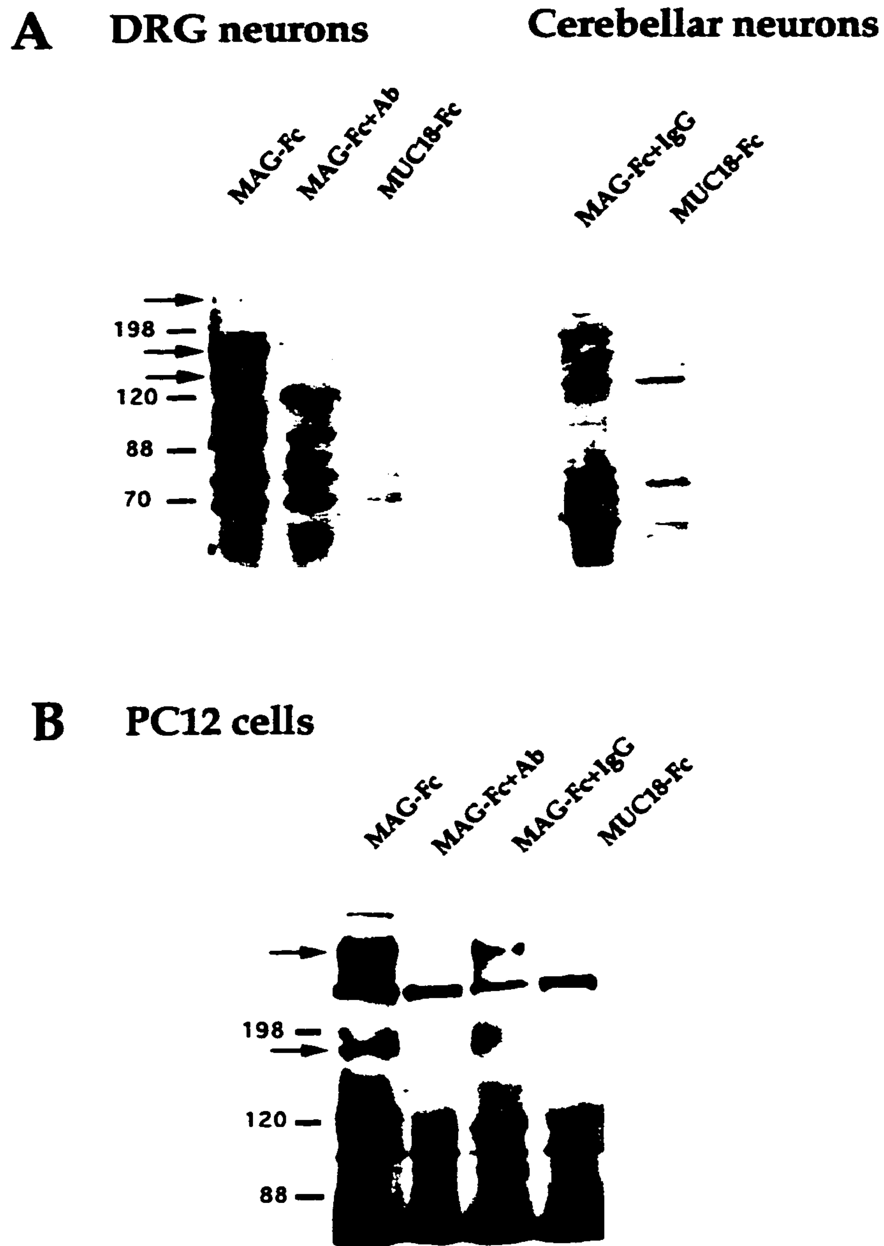

Fig. 31. Western blot of proteins precipitated with MAG-Fc. After surface biotinylation of proteins from PC12, DRG and cerebellar neurons, cells were lysed, incubated with Fc-chimeras (MAG-Fc or MUC18-Fc as control) and the complexes precipitated with protein A sepharose. Eluted proteins were ran on a PAGE, blotted onto PVDF membranes and visualized with streptavidin-HRP. Arrows indicate proteins that are specifically precipitated by MAG-Fc. Where indicated Ab refer to monoclonal 513 anti-MAG or mouse IgG as control.

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TABLE VIII.

Surface Proteins Co-precipitating with MAG-Fc	
Type of Neuron	Approximate Molecular Weight (kD)
DRG	250
	190
	130
	90
Cerebellar	250
	190
	130
Retinal	190
PC12	250
	190

When all the types of tissue used were compared for precipitated proteins, the 190kD band was always present and was the most abundant for all the neurons tested (DRG, cerebellar, retinal and PC12). This protein was consistently precipitated, regardless of the age and the type of the neurons. This suggests that this 190kD protein (p190) may represent the receptor for MAG, or one of the receptor components. In addition, p190 is not precipitated when the 513 anti-MAG antibody is included in the precipitation mix, or by MUC18-Fc.

It has been shown that MAG binds to neurons in a sialic acid-dependent manner and that this interaction is involved in the inhibition of neurite outgrowth (De Bellard et al., 1996). Hence, if either of the two proteins, p190 or p250, is the receptor or part of its complex, it also is likely to be a sialic acid-bearing glycoprotein. In order to assess if this is the case, postnatal DRG neurons were desialylated before incubation with the Fc chimeras. The results in Fig.32 show that this treatment indeed abolished the binding of MAG-Fc to the 190kD protein. The 250kD could only be observed after over-exposure of the blot, so is not visible in Fig.32, but binding of this protein to MAG-Fc was also abolished after desialylation. However, the p190, being the most conspicuous and abundant of the

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sialylated proteins on neurons that are precipitated by MAG is the most likely candidate to be either the putative receptor or an important part of a receptor complex.

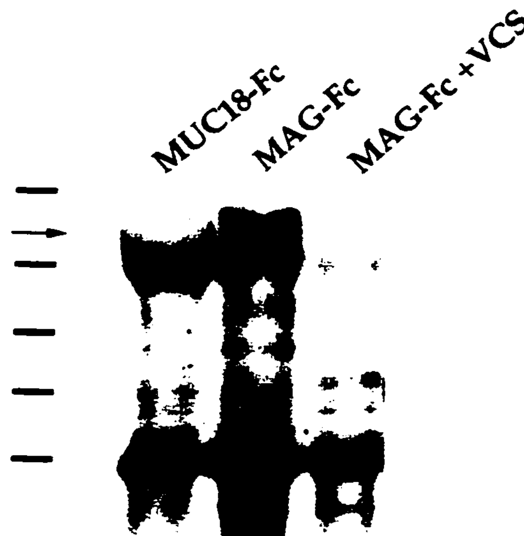

Fig. 32. Western blot of proteins precipitated with MAG-Fc after sialidase treatment. After surface biotinylation of proteins from DRG neurons, cells were lysed, incubated with Fc-chimeras (MAG-Fc or MUC18-Fc as control) and the complexes precipitated with protein A sepharose. Eluted proteins were ran on a PAGE, blotted onto PVDF membranes and visualized with streptavidin-HRP. Arrow indicates the p190 protein that is specifically precipitated by MAG-Fc. Where indicated +VCS refer to neurons treated with sialidase (50mU) before surface labeling. We used two forms of MAG-Fc, full length (1-5) and truncated (1-3).

Recently, several receptors involved in axonal guidance and neurite formation have been found to be glycosyl phosphatidylinositol-anchored (GPI-linked) (Baloh et al., 1997; Struyk, 1995; Yoshihara et al., 1995). In order to determine if the p190 we consistently precipitated with MAG-Fc is a GPI-linked molecule, DRG neurons were treated with phosphoinosityl-phospholipase C (PI-PLC) after surface labeling. The neuronal lysates were then incubated with MAG-Fc and precipitated with protein A sepharose. After this treatment the p190 protein was still present, albeit it was significantly reduced by about 50% (Fig.33). It is unlikely that this decrease in the amount of the p190 protein is because of the

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presence of contaminating proteases in the PI-PLC enzyme, because we repeated this experiment with three different commercial PI-PLC enzymes, currently used by other laboratories and found the same result. It is possible, from these results that the p190 protein is not one protein but two. One is GPI-linked, hence the decrease in total p190 after PI-PLC treatment. The other is not GPI-linked and is PI-PLC resistant, remaining on the cells after the enzymatic treatment.

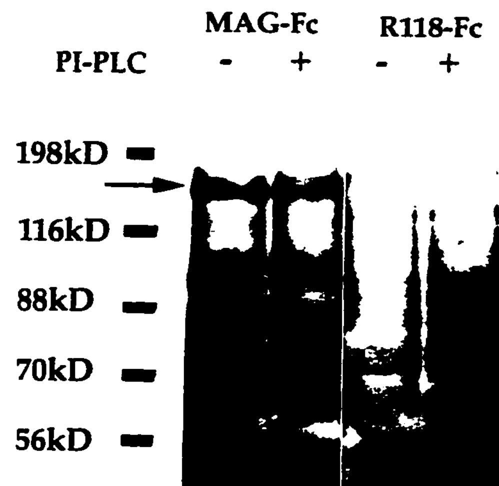

Fig. 33. Western blot of proteins precipitated with MAG-Fc after PI-PLC treatment. After surface biotinylation of proteins from DRG neurons, cells were lysed, incubated with Fc-chimeras (MAG-Fc or a mutated MAG, R118-Fc as control) and the complexes precipitated with protein A sepharose. Eluted proteins were ran on a PAGE, blotted onto PVDF membranes and visualized with streptavidin-HRP. Arrow indicates the p190 protein that is specifically precipitated by MAG-Fc. Where indicated +PI-PLC refers to neurons treated with the enzyme before surface labeling with biotin.

Binding of MAG-Fc to Live Neurons

Previous studies have shown that soluble MAG-Fc inhibits axonal regeneration after binding to growing neurons (Tang et al., 1997). However, we do not know where on the neuronal surface MAG-Fc is binding, or if it prefers to bind to a particular part of the neuronal morphology, e.g. neurites versus cell body. To address this question, DRG neurons were cultured for different times, which allowed them to extend neurites, and were then incubated with the Fc chimeras. Bound MAG-Fc was visualized using an anti-Fc

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antibody conjugated to a fluorescent dye. As seen in the Fig.34A and B, MAG-Fc bound strongly to neurites as well as to cell bodies of DRG neurons. This binding was independent of the culture time for these cells, since there was no detectable difference in the staining of the neurons and neurites that had been in culture for up to one week. We also observed that the binding of MAG-Fc to neurons was punctuate, indicative perhaps of clustering by MAG-Fc. In contrast, smooth staining was seen when using another Fc chimera, L1-Fc (Fig.34D). As with the other binding assays used, there was no detectable binding to MUC18-Fc or neurons that had been desialylated (Fig.34C and E) (Kelm et al., 1994). The results from this experiment show that MAG binds specifically to neurons and that this binding is distributed both on the neuronal cell body and neurites in a sialic acid-dependent manner. This demonstrates that a sialylated MAG-binding component is widely distributed on the neuronal cell body and their neurites.

In addition to these findings, we observed that when neurons were treated with PI-PLC to remove all the GPI-linked molecules on the surface, the binding of MAG-Fc to the DRG neurons was reduced by about 70% or 50% as before (Fig.34F). This strengthens the previous observation of a reduced amount of the p190 protein that MAG-Fc precipitates after PI-PLC treatment of neurons. However, when we carried out a solid binding assay with isolated cerebellar neurons, we could not detect a decrease in the binding of these cells to MAG-Fc after PI-PLC treatment (results not shown). In other words, there seems to be two components on the neuron, one that is GPI-linked and another that is not, however, only one of them is enough to confer binding of neurons to MAG-Fc.

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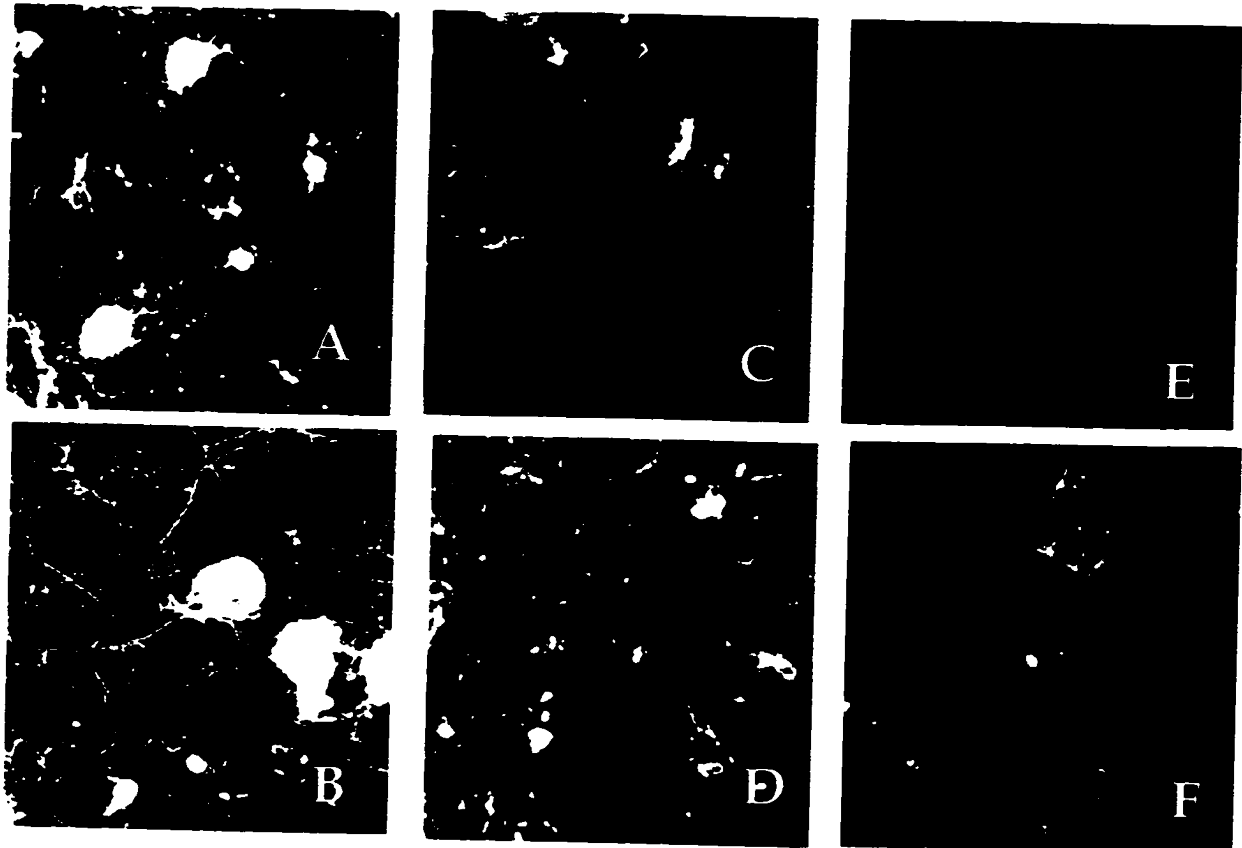

Fig. 34. DRG neurons labeling with MAG-Fc. DRG neurons were cultured on laminin for 2 days, after they had put out abundant neurites, Fc-chimeras were added: (**A,E**) MAG-Fc full-length (1-5) or (**B** and **F**) truncated (1-3) domains, **C**) MUC18-Fc as control, or **D**) L1-Fc were added for 1hr. The cultures were washed and a secondary anti-human Fc specific-Cy3 conjugated added for another 30min. Neurons were visualized under indirect immunofluorescence. DRG neurons were (**E**) desialylated (+VCS), or (**F**) treated with PI-PLC before adding the Fc-chimeras.

Screening with Different Antibodies to Identify the Receptor

It is possible that the putative receptor for MAG will be, or co-precipitates an already known and cloned protein (Apel et al., 1997; Kunz et al., 1996). In order to investigate this possibility, a screen by western blot of the MAG-Fc precipitates was performed using antibodies to different proteins, known to have an effect on axonal growth. The results are shown in Table IX.

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TABLE IX.

Antibodies to known Surface Proteins used to screen for MAG Receptor

Protein	**Signal**	**Reference**
α-8 and β-1 Integrins	-	(Felsenfeld, 1994)
Ret	-	(Baloh et al., 1997)
Contactin	-	(Peles et al., 1995)
Cranin	-	(Smalheiser and Kim, 1995)
MAPs	110kD	(Tanner et al., 1998)
GPI-GP150	-	(Yoshihara et al., 1991)
BIG-1	-	(Yoshihara et al., 1995)
Pan-Trk	-	(Snider, 1994)
CSPG	-	(Lander et al., 1998)
Amyloid (APP)	-	(Qiu et al., 1995)
BIG-1	-	(Yoshihara et al., 1995)
CASPR	90, 250kD	(Peles et al., 1997)
Fish Neurexin	250kD	(Russell et al., 1997)
Neurofascin	250kD	(Volkmer et al., 1996)
N-CAM	-	(Williams et al., 1994)
Neuropilin	-	(Kolodkin et al., 1997)

Of all the antibodies tested, a few showed some reactivity with the transferred proteins on the western blots. However, none reacted with the p190 protein. One such group with some crossreaction was the set of antibodies that recognizes the neurexins or a similar molecule, CASPR. These antibodies recognized a 250kD protein that was present only in the MAG lanes. The neurexins are a very large family of molecules, of sizes that range from to kD, that recently have been classed as proteoglycans, due to the large size of their carbohydrate moieties (Missler and Sudhof, 1998; Petrenko et al., 1996). These large molecules (neurexins) are abundant in the nervous system, particularly at the synapse, and have a variety of sugar moieties and linkages, including the type 2,3-O linked sialic acid that MAG binds to on the cell surface (Russell and Carlson, 1997). CASPR is a protein of 190kD that is found at the axon-node interface (Peles et al., 1997; Peles et al., 1997). Probably these two antibodies (neurexin and CASPR) crossreact with the p250 because of some carbohydrate similarity between their epitopes and the p250, not because this p250

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MAG-binding protein is a neurexin or a CASPR dimer. The other band detected on blots with the CASPR antibody was a 90kD protein. This protein was only apparent in the MAG-Fc precipitates when full-length MAG-Fc, not the truncated 1-3 domains protein was used. This distinction raises the question of why MAG precipitates a different protein from the p190 we get in larger amounts, and of a different size from the full-length CASPR. It is known that CASPR undergoes rapid degradation, which would make its molecular weight range between 50 and 150kD (Dr. Peles personal communication). In other words, this p90 protein may be a partly degraded CASPR. In order to determine if this is the case, neutralizing functional antibodies that will recognize CASPR in *in vitro* assays are being raised and will be tested in both binding and neurite outgrowth assays.

The antibody for neurofascin also showed some cross-reactivity with a 250kD molecular weight band. Neurofascin is an L1-like, CAM family member, involved in axonal growth (Hortsch, 1996; Rathjen et al., 1992). However, the molecular weight of this glycoprotein is 150kD, not a 250kD we observed in westerns using this antibody. The 250kD protein we see with the neurofascin antibody most likely results from a non-specific reaction with the antibody as appears to be the with the neurexin and CASPR antibodies.

The p250 protein that MAG precipitates in a small amount may be a proteoglycan. Because most antibodies to proteoglycans react with the sugar moiety on the molecule, the availability of specific antibodies to individual proteoglycans is limited. There are few antibodies to the core protein. Because of this, it is very difficult to test by western if the p250 is a known proteoglycan. Hence, we decided to test this hypothesis by an enzymatic reaction. One of the most reliable ways of determining if a molecule is a proteoglycan is to digest it with an enzyme that will cleave all the proteoglycans from the surface of the cells. Chondroitinase ABC is an enzyme that cleaves all the proteoglycans from cell surfaces. When we treated DRG neurons with chondroitinase ABC in the live soluble binding assay, there was no change in the binding of MAG-Fc to those neurons (results not shown). This suggests that the receptor is not a chondroitin proteoglycan.

Recently, it has been reported that recombinant MAG binds to a transmembrane form of MAP1B (Tanner et al., 1998). However, when we used an antibody to MAP1B on our blots, we did not see such a 150kD band, but a 110kD. Moreover, to date no

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transmembrane MAP has been identified (Dr. Gordon-Weeks personal communication and (Nunez and Fischer, 1997).

Affinity Purification of the Receptor for MAG

A logical consequence of the finding that MAG-Fc can specifically precipitate a protein in a small scale purification scheme, is that a column with MAG-Fc could be prepared in order to purify enough receptor for microsequencing. However, when such preparations were made they did not yield enough receptor to obtain a successful microsequencing.

These experiments were carried out in two ways. One was by immobilizing MAG-Fc in a sepharose CL-4B column and passing solubilized, crude membranes from whole brain through the column. When this was carried out on a small scale (1-2 brains), detectable amounts of the p190 protein were observed after biotinylation of the membrane proteins and subsequent elution from the column. Nevertheless, when the experiment was repeated with larger amounts of equivalent solubilized membranes from more brains (>10), we were not able to elute the receptor from the column. Elution was tried with low and high pH buffers using standard protocols with no success. Boiling the beads with MAG-Fc and the receptor, under reducing conditions yielded some amounts of p190. However, there was not enough protein eluted from the column by this method for microsequencing.

The other strategy was to prepare crude membranes and incubate them with MAG-Fc aggregates without coupling to a column. Previous experiments showed that the amounts of precipitated p190 protein were greater when using an anti-human Fc-specific antibody to aggregate MAG-Fc than using just MAG-Fc, and that postnatal DRG neurons gave the highest yields of p190 and the cleanest gels from the biotinylated precipitation. In order to obtain enough material for microsequencing crude membranes from the DRG of 260 rat pups (PND12-15) were used. The p190 band that was cut out and sent for microsequencing yielded several tryptic peptides, the largest of which was the only one to yield

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microsequencing information. However, the results showed that it corresponded to goat IgG, from the goat anti-human Fc used to aggregate MAG-Fc.

Discussion

In order to identify the receptor for MAG we designed our strategy based on two pieces of information. First, soluble MAG-Fc binds to neurons and inhibits neurite outgrowth from cerebellar neurons (De Bellard et al., 1996; Tang et al., 1997). Second, from the results of the binding assays after trypsin treatment, we determined that the putative receptor for MAG is a sialoglycoprotein, not a sialoglycolipid (De Bellard et al., 1996).

We took advantage of the structure of soluble MAG-Fc and designed two modified immunopurification methods. The first consisted in surface labeling neuronal proteins with biotin, incubating their lysates with the chimeras (MAG-Fc or MUC18-Fc as control), and the proteins were then precipitated with protein A sepharose. From these experiments, we were able to determine that MAG binds to and specifically precipitates several neuronal surface proteins. In addition, the amount of proteins increased when MAG-Fc was aggregated before incubation with the lysates. These results indicate that we could affinity-purify the MAG binding components and that the avidity of MAG for its putative receptor was increased when aggregated, as we had found before with our binding assays (Chapter 1 and (Kelm et al., 1994). These results show for the first time that MAG specifically binds to and precipitates proteins from neurons. These findings are very important because, to date, there was no approach to isolate the receptor for MAG. However, now we know we could take advantage of this method and design a column to affinity purify the receptor, this was the second approach taken in the present studies.

With the first method, using different types of surface labeled neurons (cerebellar, DRG, retinal and PC12) we observed that MAG-Fc specifically and reproducibly precipitated two proteins. The approximate molecular weights of these proteins were 190kD and 250kD. Furthermore, when the neurons were desialylated before surface labeling and precipitation with MAG, the binding to the p190 and the p250 was abolished.

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Of these two proteins, p190 was the most abundant and distributed compared with the p250. These results suggest that the p190 molecule might be the sialic acid bearing glycoprotein that MAG binds on the neurons. The p250 either could be a minor component in a putative MAG-receptor complex or just happens to be a sialylated protein that MAG binds and precipitates. Because of its abundance, we decided to pursue the isolation and microsequencing of the p190.

We decided to try identifying the p190 protein as the most likely candidate to be the putative receptor for MAG or a major part of its complex, for several reasons. 1) This protein is more abundant and its precipitation more reproducible than the p250. 2) The p190 protein was found on the surface of CNS, PNS and transformed neurons. In other words it is present in significant amounts in neurons that are inhibited by MAG. 3) When we aggregated MAG-Fc the yield of p190 increased substantially, while the yield of p250 did not. This indicates that MAG-Fc binds to p250 irrespective of its avidity, in contrast to what we found from binding assays, that MAG-Fc will bind more efficiently and tightly to neurons if we pre-aggregated MAG before the assay. 4) The p190 protein was precipitated by full length MAG-Fc and truncated MAG-Fc (domains 1-3), not by a mutated MAG on R118, R118-Fc. 5) Finally, that the p190 protein carries a sialic acid, which is a requirement for binding to MAG. However it should be noted, that we also have established that sialic acid binding is not necessary for inhibition of axonal regeneration by MAG, because MAG has two domains: one for binding in a sialic acid-dependent manner and one for neurite outgrowth inhibition (Tang et al., 1997; Tang et al., 1997). In other words, p190 might not be the protein responsible for inhibition of axonal regeneration, unless it also carries the site for inhibition of neurite outgrowth, besides carrying a sialic acid in a α2,3-O-linkage. If this is not the case, then there should be another component on the neurons that interacts with the neurite outgrowth inhibition site on MAG. In support of the existence of a putative second protein that interacts with MAG, we found that: 1) when neurons were treated with PI-PLC to remove all the GPI-linked molecules on the surface of neurons, the amount of the p190 was reduced by 50%. 2) The binding of MAG-Fc to live neurons was also reduced by 50%. However, when we carried out assays of neurons binding to immobilized MAG-Fc, after PI-PLC treatment we did not see any reduction in binding. That is to say, PI-PLC treatment of neurons reduced binding of soluble MAG to neurons,

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but not of neurons to immobilized MAG. The results from the binding assay and MAG-Fc precipitates after PLC treatment suggest the existence of a p190-GPI-linked component that can interact, with MAG-Fc. The reason we still observed binding of neurons to MAG-Fc was because there is another molecule, not GPI-linked, that is enough to confer binding of neurons to an immobilized MAG-Fc. However, in the soluble binding assay and in the precipitates, the total binding of MAG-Fc to neurons/p190 protein is reduced in about half, because half of the MAG binding components (p190) has been removed. A proposed model for this is shown in Fig.35. 3) The observation that a CASPR antibody recognized a 90kD protein when using full-length MAG not with a truncated MAG (1-3 domains), suggest that there is another protein that can interact with a full-length MAG but not with the truncated form. In other words, this p90 may be the protein responsible for neurite outgrowth inhibition. However, we do not have the tools to currently test this hypothesis until the appropriate antibodies become available.

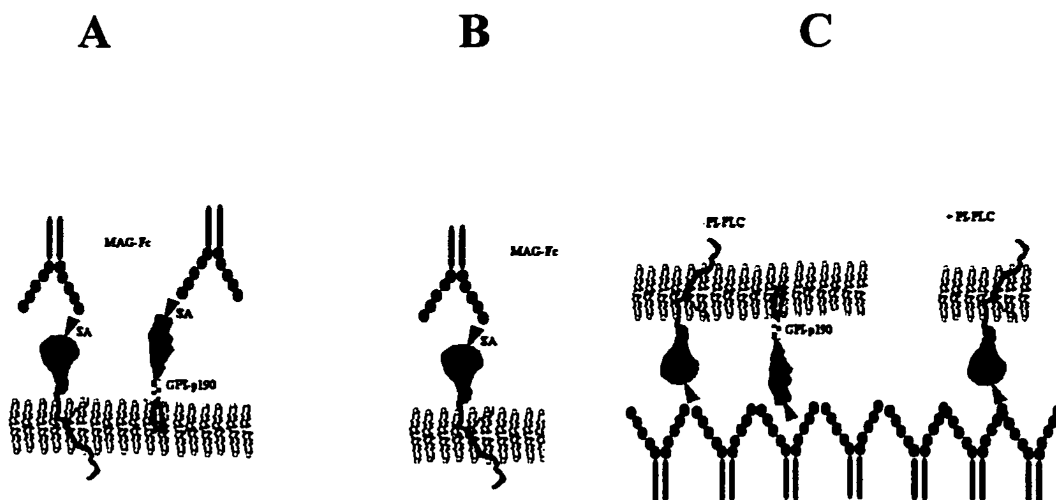

Fig. 35. Model for MAG bindings assays. A) Soluble binding assay of MAG-Fc to the two neuronal binding components before PI-PLC treatment. B) Soluble binding assay of MAG-Fc after PI-PLC treatment, binding to neurons is reduced by about 50%. C) Solid-phase binding assay of neurons to immobilized MAG-Fc, before and after PI-PLC treatment. Note that neurons can still bind to MAG-Fc after removing the GPI-linked p190.

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At the same time as preparing a large-scale preparation of p190 to microsequence, we tested different antibodies by western blots, to determine if the p190 protein is already known. We used antibodies to different molecules previously known to affect neurite outgrowth in these same westerns, yet we did not see any specific crossreaction that would have identified unequivocally the receptor or the p190. However, we observed that antibodies that have been raised against proteoglycans or similar molecules (neurexin and CSPG or CASPR), or neurofascin, gave some crossreaction with a p250 protein from our MAG precipitates. Nevertheless, this protein size (250kD) does not correspond to the original antigen that each of these antibodies was raised against. Therefore, these antibodies may crossreact with large proteoglycans in a non-specific manner, because of similarities in the carbohydrate composition of their respective epitopes to the p250 that is precipitated by MAG, they give a positive signal when we use them in our blots. Regardless of these discrepancies with the p250, this protein may well be a proteoglycan, considering its large size and weak crossreaction with a CSPG antibody. However, when we treated DRG neurons with chondroitinase ABC before the soluble binding assay, we did not see any change in the binding of MAG to the neurons, suggesting that the receptor for MAG is not a proteoglycan or at least not its major binding component. These observations do not exclude the possibility that MAG can actively bind to proteoglycans in a sialic acid-dependent manner, although non-specifically, especially in light of our findings that MAG binds strongly to red blood cells, provided they carry a α2,3-O-linked sialic acid (Kelm et al., 1994). However, this type of interaction will not be physiologically relevant, because sialic acid is necessary and sufficient for binding, but not for neurite outgrowth inhibition (see Chapter 1 discussion).

Another possibility is that the receptor for MAG is a ganglioside, as has been suggested by Schanaar and co-workers from their findings that MAG binds to GQ1b and GT1b gangliosides in a sialic acid-dependent manner (Collins et al., 1997; Yang et al., 1996). This suggestion could not explain the results presented above, that several proteins associate with MAG in a sialic acid-dependent manner. However, because binding of MAG to neurons was reduced after trypsin treatment, not abolished, it is possible that a ganglioside (protease resistant) is part of that MAG-receptor complex. We would like to suggest that the role of gangliosides in axonal regeneration takes place in a cis-manner.

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That is to say, on the glial cell itself, not in a trans-interaction, with the neuron responding to MAG. There are several observations that support more a hypothetical cis-interaction instead of a trans-interaction for gangliosides. For example, we know that: 1) MAG should only interact with one sialylated component on the opposing membrane at a time when inhibiting neurite outgrowth; and 2) we have shown that there is a sialoglycoprotein involved in MAG binding and neurite outgrowth inhibition. Therefore, MAG is either binding in a sialic acid-dependent manner either to a sialoglycoprotein or to a ganglioside, but not to both simultaneously. We suggest this, in light of, first, the steric hindrances that such an interaction will put on MAG. The reason for this is that the sialic acid binding site of MAG is the R118 located between domains 1 and 2. If MAG is binding to a large sialoglycoprotein, such as p190, sterically it is very difficult, when MAG has clustered, for MAG to also bind the relatively small sugar chain of a ganglioside, which is barely above the plasma membrane. Second, glial cells have gangliosides as well, and these could bind to MAG in a cis-interaction and thus modulate its effect on neurons and/or glial cells (Tropak and Roder, 1997). Furthermore, it has been shown that gangliosides can modulate the neurite outgrowth promoting abilities of NCAM and N-cadherin, not by activating the CAM specific pathway but by potentiating their neuritogenic effect (Doherty et al., 1992; Doherty et al., 1985). This is more likely for an interaction of a ganglioside with the CAM, helping them to cluster in a similar way as proteoglycans help dimerize FGF. Third, if a ganglioside is the sialylated component that MAG binds on the neurons, there still must be at least another component on neurons that carries the transduction of such an interaction into the cell and causes inhibition of axonal growth. This component may be the p190 we precipitate with MAG.

The second approach we took to isolate and purify the p190 was a large-scale preparation of solubilized membranes and through a column of immobilized MAG-Fc. This is a classical affinity purification method using the ligand to purify its receptor, and has proven very efficient in the isolation of many receptor proteins (Noda et al., 1982). However, when we used it, we never obtained enough material for microsequencing. The major reason was that we could not efficiently elute the receptor from the column without using drastic measures, and still this was not effective. This phenomenon is not unusual as other researchers have found the same problem when using Fc-chimeras in a column to

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identify binding partners of other sialoadhesin family members (P. Crocker and S. Kelm personal communication). When this approach was unsuccessful, we decided to use MAG-Fc aggregated with an anti-Fc, incubate it with solubilized crude membranes as before, and isolate the complexes with protein A sepharose. However, this strategy also was unsuccessful because when we sent what we thought was enriched p190 for microsequencing, it was the anti-Fc used to aggregate MAG. This shows that there were some small amounts of this antibody still running as a dimer despite the reducing conditions used. Nevertheless, these were high enough to be picked by the HPLC instead of the neuronal p190 we wanted to microsequence. Since both of these approaches were not successful, despite different conditions and tissue used, we decided that a functional screening of a neuronal cDNA library would prove more successful. The assay currently being developed in our lab consists in taking advantage of the feasibility that MAG activates a Gi protein (Cai et al, 1998). Knowing this, we are designing a screening method to choose clones that activate a Gi protein when we add MAG-Fc to COS cells expressing a neonatal cerebellar library (Kurkinen et al., 1997).

The effect of MAG on axonal regeneration also raises the questions of how and why MAG causes inhibition. Since MAG is not present at the time of axonal pathfinding and synapse formation, it is unlikely to have a guidance role during development of the nervous system. Nevertheless, it ought to have a real and relevant physiological function, which is related to axonal growth, because it has a powerful effect on a growing axon after an injury (see previous chapter as well as (Li et al., 1996; Mukhopadhyay et al., 1994).

There are several important pieces of information regarding the role of MAG at the axon-glia interface that are provided by the different MAG mutant mice. It has been observed that after a certain period, some of these mice develop a variety of abnormalities (Fruttiger et al., 1995; Li et al., 1998; Yin et al., 1998). The most obvious and relevant is that in these mutant mice the PNS undergoes axonal degeneration, as well demyelination after 8 months of age (Fruttiger et al., 1995; Li et al., 1998). That is to say, MAG appears to play a crucial role in the long-term maintenance of the integrity of both axons and myelin. The other piece of information is that in the PNS of these mice there is a reduction in the caliber of the axons, from lack of neurofilament phosphorylation at the internodes of the

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axon (Yin et al., 1998). Together all these observations suggest that MAG may have a functional role in modulating the maturation and stability of myelinated axons, and that this role is carried out by its physiological receptor in the neurons. In summary, the identification of the neuronal receptor for MAG will provide crucial answers for a lot of the questions that have been asked over the past years regarding the mechanism that governs axonal growth and maintains axon stability. The implications of such information are further understood when considering the devastating effects that spinal cord injury and some neuropathies have on humans. It has been the goal of these studies to provide some clues to how both of these phenomena take place in the nervous system and how to circumvent the problem of lack of axonal regeneration in the CNS.

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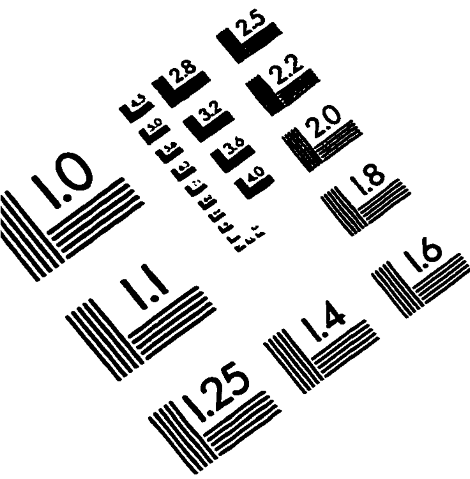
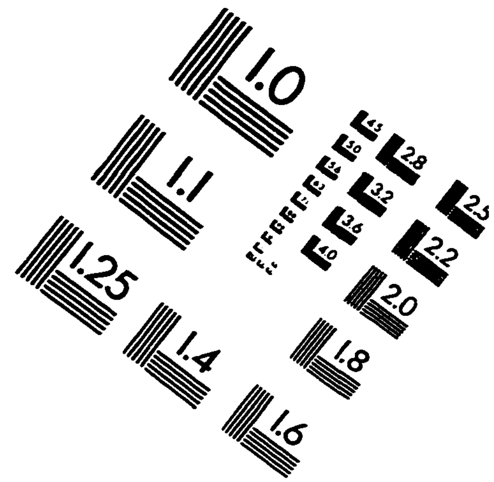
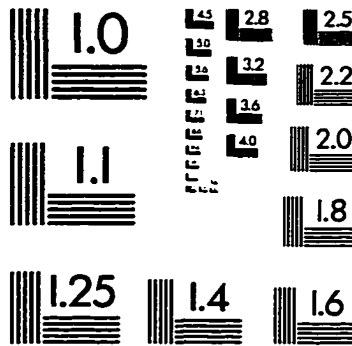
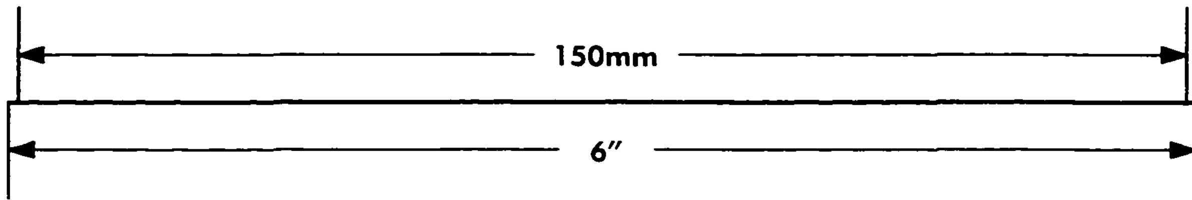
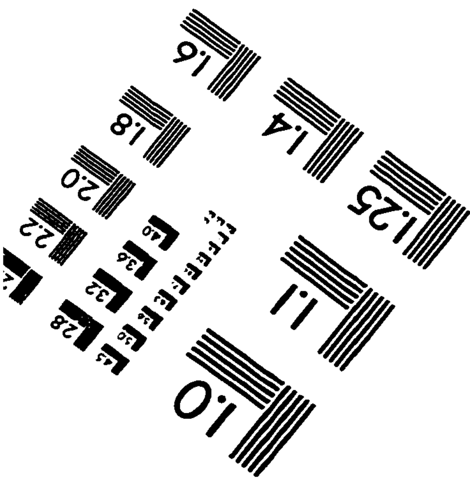
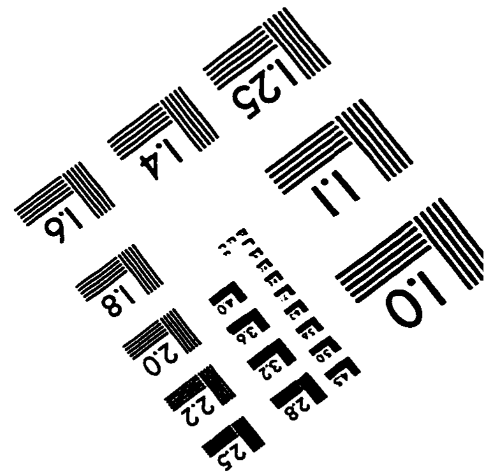
IMAGE EVALUATION TEST TARGET (QA-3)

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