INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®

A

CHARACTERIZATION OF THE ROLE FOR MYELIN-ASSOCIATED GLYCOPROTEIN AS AN INHIBITOR OF AXONAL REGENERATION

by

YINGJING SHEN

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

2000

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

6/12/00
Date

Chair of Examining Committee
Dr. Marie T. Filbin, Hunter College

6/27/00
Date

Executive Officer
Dr. Richard L. Chappell

Dr. Laurel Eckhardt, Hunter College

Dr. Jesus Angulo, Hunter College

Dr. Moses V. Chao, New York University School of Medicine

Dr. Lloyd A. Greene, Columbia University

Supervising Committee

The City University of New York

ABSTRACT

CHARACTERIZATION OF THE ROLE FOR MYELIN-ASSOCIATED GLYCOPROTEIN AS AN INHIBITOR OF AXONAL REGENERATION

By

YINGJING SHEN

Advisor: Professor Marie T. Filbin

Following injury, axons in the adult mammalian CNS do not normally regenerate. It is suggested that the presence of inhibitory molecules in CNS myelin contributes substantially to this effect. However, the precise nature of these inhibitory molecules is not known. Myelin-associated glycoprotein (MAG), a well-characterized transmembrane protein of both CNS and PNS myelin, was previously reported to promote neurite outgrowth from newborn dorsal root ganglion (DRG) neurons. Here, we demonstrate that MAG can also function as a potent inhibitor for neurite regeneration. When expressed by CHO cells, MAG strongly inhibits neurite outgrowth from cerebellar neurons of all postnatal ages and DRG neurons older than postnatal day 3. The inhibitory effect of MAG on neurite outgrowth is further confirmed when MAG is expressed by immortalized nonmyelinating Schwann cells, which are normally an excellent substrate for neurite outgrowth. The expression of MAG by these Schwann cells significantly changes their permissiveness as a neurite growth substrate. These results indicate that MAG has strong inhibitory effect on neurite growth and may contribute to the lack of regeneration of the CNS.

We also show that expression of MAG by Schwann cells significantly reduces neurite branching from DRG and superior cervical ganglion (SCG) neurons. Therefore, in addition

to its neurite extension inhibitory function, MAG also inhibits neurite branching. It has been suggested that myelin and its associated neurite growth inhibitors restrict the collateral sprouting of nerve terminals. Our data agree with these previous observations and suggest that MAG may also contribute to limited collateral sprouting in myelinated regions.

It was previously shown that MAG binds to neurons in a sialic acid-dependent manner. Here we map the sialic acid binding site on MAG by site-directed mutagenesis of arginine 118 (R118). Arginine 118 is conserved among all members of the Siglec family, a subgroup of sialic acid binding proteins in the Ig superfamily, to which MAG belongs. Mutation of R118 into either alanine (R118A) or aspatic acid (R118D) completely abolishes the binding of MAG-Fc to neurons. Therefore, we suggest that R118 is important for sialic acid binding of MAG to neurons. However, when expressed by Schwann cells, R118-mutated MAG retains the ability to inhibit neurite outgrowth. We suggest that MAG has two recognition sites for neurons, the sialic acid binding site at R118 and a distinct neurite growth inhibition site. A two-site model for the inhibition function of MAG is proposed.

The mechanism by which MAG inhibits neurite outgrowth was investigated and we suggest that the inhibition effect of MAG is modulated by a neuronal cAMP-dependent activity. Elevation of cAMP levels in neurons completely abolishes the inhibition by MAG. However, the direct involvement of cAMP-dependent activity in MAG induced inhibition is unlikely, because blocking cAMP-dependent activity by an antagonist of cAMP, Rp-cAMP, or a PKA inhibitor, KT5270 does not affect neurite outgrowth. Therefore, MAG can be added to the growing number of molecules whose effect on neurite growth is regulated by endogenous level of neuronal cAMP.

ACKNOWLEDGMENTS

I am deeply grateful to my thesis advisor, Dr. Marie T. Filbin, for all the guidance, supervision and scientific training she has provided. Through all these years, she has always been there, supporting me and helping me. I thank her for all she has done for me.

I thank the members of our laboratory, Kejia, Manhar, Gitali, Maria Elena, Song, Wenhui, Dongming, Ying, Elena, Adijat, Ilia, Willy, Susanna, Jane, Yamina, Chhaya, Oliver, Steven and others, for their friendship and support. We have enjoyed a lot of happy time over all these years. I especially thank Gitali, who gave me important help and advice when I was a new student in Dr. Filbin's lab. We worked together on the same project and results presented in Chapter III of this thesis were shared by both of us. Gitali did all the neurite measurements. I also thank Song, who worked together with Gitali and me on the project of mapping the sialic acid binding site of MAG by mutagenesis, and had contributed to the results presented in Chapter V. I also have a nice time working together with Dongming on the effect of cAMP and neurotrophins on the inhibition of neurite outgrowth by MAG. The effect of PKA inhibitor on neurite outgrowth in Chapter VI was quantitated by her.

I thank the members of my thesis committee and all professors who have given me invaluable advice and help: Dr. Jesus Angulo, Dr. Moses Chao, Dr. Laura Eckardt, Dr. Lloyd Greene, Dr. James Salzer, Dr. Patrick Doherty. I especially thank Dr. Thomas Schmidt-Glenewinkel for the technical training in molecular biology he provided to me and for his unlimited advice on experimental problems I encountered.

I thank my parents and my sister for their understanding and unquestioning support. I thank my husband for his consideration, support and patience. This dissertation is a gift to them.

TABLE OF CONTENTS

List of Figures

List of Table

Chapter I

Introduction

1.1 General Understanding to Nerve Regeneration

Injured axons in the adult mammalian CNS normally fail to regenerate. Although the immature mammalian CNS does have a certain capacity to repair itself after injury, this capacity is lost during the early stages of development (Nicholls and Saunder, 1996). In contrast, adult mammalian PNS nerves regenerate following injury and a significant degree of neuronal function is restored (Cajal, 1928; Johnson, 1993). Since the begining of this century, great efforts have been made to understand the mechanism underlying axonal regeneration and to manipulate it to promote functional recovery in the injured CNS.

Early this century, the lack of regeneration in the mature mammalian CNS was thought to be the result of a permanent loss of regenerative abilities in adult CNS neurons (Clark Le Gros, 1942, 1943; Johnson, 1993). CNS axons have a low regenerative potential. Although some damaged CNS neurons can send out sprouts, they fail to grow through the lesions and fail to make connections (Cajal et al., 1928). This pessimistic view changed in the early 1980s when Aguayo and his colleagues made the striking findings that showed that pieces of peripheral nerve grafted into the damaged region of adult brain, supported axonal regeneration of up to several hundred fibers across the transplanted bridge for a length of upto 30 mm (David and Aguayo, 1981). Using similar transplants of peripheral nerves, Aguayo's group tested the regeneration ability of spinal cord, brain, and optic nerve fibers and found that a majority of CNS neuron types were able to regrow fibers into a nearby peripheral nerve graft (Aguayo et al., 1981).

These results suggested that the lack of axonal regeneration in the mature CNS is not completely due to an intrinsic inability of these neurons to regrow their axons, instead, influences arising from the environment also play an important role in the success or failure of regeneration (Aguayo et al., 1981). The environment in the injured CNS is inhibitory for axonal regeneration. In contrast, the distal stump of injured peripheral nerve provides a favorable environment that fosters axonal regeneration. When the inhibitory CNS environment is substituted by a growth-promoting environment of peripheral nerve, the regeneration of injured CNS axons is significantly enhanced (Bahr and Bonhoeffer, 1994). As Aguayo pointed out, the regenerative potential of central neurons may be expressed only when CNS neuroglial environment is changed to resemble that in the PNS (Aguayo et al., 1981).

Axonal regeneration is a cooperative process. The speed and extent of axon growth are influenced both by the properties of the surrounding environment and the properties of the neuron itself (Fawcett, 1992). Evidence suggests that neurons have certain tightly regulated developmental programs that control their intrinsic ability to extend axons (Fawcett et al., 1992; Carpenter et al., 1986). On the other hand, environmental factors interacting with neurons also influence the neuronal growth state. It is likely that they provide the signals that trigger specific cellular and molecular programs in the injured neurons and therefore affect the regenerative capacity of the neurons (Broude et al., 1997; Bregman et al., 1998a; Bregman et al., 1998b). In the mature mammalian CNS, the low regenerative potential of

neurons and the growth-inhibitory environment combine and lead to the failure of nerve regeneration.

1.2 Developmental changes in the capacity of neurons to regenerate

Both PNS and CNS neurons decline in their potential for axonal growth during maturation (Nicholls and Saunders, 1996; Fawcett, 1992). This is commonly seen in the tissue culture dish, where axon growth from embryonic tissues tends to be much faster and more profuse than that from postnatal tissues, although older neurons can grow long axons given optimal conditions, and after an initial lag period (Collins and Lee, 1982; Argiro et al., 1982, 1984). Embryonic dorsal root ganglion (DRG) neurons extend long neurites on neonatal optic nerve cryosections, but the neurite length declines sharply with age. Rat retinal ganglion cells (RGC) extend neurites on neonatal optic nerve cryosections only when they are younger than E14-15 (Shewan et al., 1995). A precipitous decline was seen at postnatal day 2 in the ability of hamster retinal axons to reinnervate tectal target, even when the target is embryonic (Chen et al., 1995). *In vivo,* when embryonic rat CNS tissues were grafted into spinal cord lesion sites in newborn and adult rats, the amount of axonal growth of host neurons was substantially greater in newborn rats than in adult rats (Bregman et al., 1993; Iwashita et al., 1994). Regrowing fibers of the neonatal rats can grow into, through and beyond the embryonic grafts, while in adults, similar grafts can induce sprouting but not lead to comparable regeneration (Xu et al., 1995).

Moreover, embryonic neurons have the ability to grow long axons in mature CNS environment, which is inhospitable for both adult CNS and PNS neurons (Shewan et al., 1993). Mature CNS tissue, which does not support regrowth of its own damaged axons, does support axonal growth of embryonic CNS neurons implanted into the adult animal (Nicholls and Saunders, 1996; Fawcett, 1992). For example, embryonic substantia nigra reextend their axons and make extensive local dopaminergic connections in the host striatum (Bjorklund et al., 1992) and grafts of embryoic spinal cord regenerate and form connections with the host spinal cord (Bregman et al., 1993). Human forebrain neuroblasts implanted into lesioned striatum of adult rats extended axons along major myelinated fiber tracts for distances of up to 20 mm (Wictorin et al., 1990, 1992), in contrast to the fact that adult CNS neurons fail to penetrate mature CNS tissue by more than 1 mm (Cajal, 1928; Vidal-Sanz et al., 1987). *In vitro*, embryonic DRG neurons extend axons on adult optic nerve cryosection, while their neonatal and adult counterparts cannot (Shewan et al., 1993, 1995).

It has been suggested that certain developmental programs control the changes in the ability of neurons to extend axons, presumably involving sequential switching of genes which will determine the speed and extent of axonal growth and its response to the environmental cues (Fawcett, 1992; Shewan et al., 1995; Chen, et al., 1997). First, as neurons mature, the decrease in growth ability is companied by down-regulation of cell adhesion molecules (Daniloff et al., 1986) and receptors of growth promoting ligands (Cohen et al., 1989). For example, laminin-binding $\alpha 6\beta 1$-integrin receptors are lost from RGC neurons when they innervate the tectum, which leads to the loss of response to the growth-promoting effect of

laminin (Cohen et al., 1986, 1987, 1989; deCurtis et al., 1991). In addition, some molecules switch to different isoforms with maturation. N-CAM in adult nervous system contains an exon, VASE, which negates much of the growth-promoting response to N-CAM (Martini and Schachner, 1988; Bates and Meyer, 1997; Saffell et al., 1994). One exception is in adult mammalian olfactory neurons that continue to produce the embryonic form of N-CAM. Interestingly, these neurons retain the ability to grow in the adult CNS (Miragall et al., 1988; Fawcett et al., 1992).

Second, the expression of receptors for putative inhibitory molecules is likely to be turned on at particular time points during development, probably after axons contact their normal targets in vivo (Shewan et al., 1995). In support of this suggestion is the study of the co-culture of embryonic RGCs with mature oligodendrocytes (Ard et al., 1991), showing that the axonal growth from embryonic RGCs are not affected by the oligodendrocytes expressing myelin-associated inhibitors that can block the growth of adult CNS axons. In another study, it was shown that embryonic RGCs and DRG neurons produce axons penetrating astrocytic tissue which correlates with glial scar *in vivo*, while the same type of neurons from the postnatal animals fail to grow (Fawcett et al., 1989).

Third, in addition to the changes in expression of cell surface receptors, expression of molecules involved in signaling pathways also changes, as in the case for GAP-43, suggesting a change in integrating extracellular signals (Gordon-Weeks, 1989). GAP-43 is a general early marker of postmitotic neurons, where it is expressed at high levels during

axonal growth and synaptic formation. It accumulates in axonal growth cones and presynaptic nerve terminals where it associates with the cortical cytoskeleton (Skene, 1989; Meiri and Gordon-Weeks, 1990). Down regulation of GAP-43 to essentially undetectable levels coincides with the onset of synapse elimination (Moya et al., 1988; Caroni and Becker, 1992).

Finally, this developmental change also involves the changes in expression of cytoskeleton proteins, such as tubulin, neurofilament and microtubule-associated proteins (MAPs). For example, a certain microtubule-associated protein isoform is only expressed during embryonic stages (Fawcett, 1992). Such changes might directly influence the rate of regrowth.

Together, all these changes may work in concert and contribute to the decline of regenerative potential and in the CNS, may contribute to the lack of regeneration in adulthood.

1.3 Adult CNS neurons differ in their ability to regenerate

Even with the adult CNS, the ability of neurons to regenerate differs greatly. Given a permissive environment, such as Schwann cells or embryonic CNS tissues, many CNS neurons are able to regenerate their axons. However, some axons regenerate better than others. When peripheral nerve grafts are inserted into the thalamus, over 80% of the axons

growing into the grafts come from the GABAergic neurons of the reticular nucleus of the thalamus, whereas other neurons show little regeneration (Morrow et al., 1993). Similarly, in the cerebellum, axons of purkinje cells will not regenerate, whereas olivary axons regenerate readily (Rossi et al., 1995; Fawcett, 1997). After spinal cord lesion in adult rat, combined treatment with neurotrophin-3 (NT-3) and IN-1 antibody, which neutralizes some myelin-associated growth inhibitors, significantly increases regeneration of CST axons in some animals, however, only 5-10% of the CST fibers show long distance growth (Schnell et al., 1994).

At the molecular level, the differences in the regenerative responses of neurons correlates with the expression of a number of growth-associated genes in neurons, including immediate early genes, cytoskeletal proteins, and GAP-43 (For reviews, see Schwab and Bartholdi, 1996). These genes are typically expressed in developing neurons and are seen to be reactivated in response to a lesion. Two types of neuron with high regenerative potential, for example, are those of the thalamic reticular nucleus and the substantia nigra; these neurons express particularly high levels of GAP-43 when their axons are cut (Vaudano et al., 1995). In the retina and in the rubrospinal pathway, only those neurons that regenerate their axons into peripheral nerve grafts up-regulate GAP-43 (Fawcett, 1997). Similarly, c-Jun protein is up-regulated substantially in DRG cells regenerating their injured central branches of axons into peripheral nerve grafts (Broude et al., 1997). Expression of actin and tubulin is increased in retinal ganglion cells that regenerated axons into a peripheral nerve graft (McKerraacher et al., 1993b). Although the expression of these genes is closely associated

with successful regeneration, it is not sufficient to induce regeneration. In transgenic mice overexpressing GAP-43, injured CNS axons showed some increase in sprouting, however, their regeneration was not improved (Benowitz and Routtenberg, 1997).

1.4 Effect of neurotrophic factors on survival and regeneration of injured neurons

Neurotrophic factors play important roles in normal development of the nervous system. Upon contact with the target areas, most neurons acquire trophic dependence on the survival factors released from their targets, and continuously retrogradely transport them to the cell bodies (Davies et al., 1991, 1994; Mettlin et al., 1995; Robinson et al., 1996). Numerous members of the neurotrophins (NGF, BDNF, NT-3, NT-4/5), ciliary neurotrophic factor (CNTF), fibroblast growth factor (FGF), and transforming growth factor β families have been found to influence survival of different population of neurons (Davies, 1997; Jelsma and Aguayo, 1994).

After injury, when the retrograde transport of trophic factors from the target to the neuronal cell bodies is disrupted, the first challenge for the injured neurons is survival. In the PNS, the distal stump supplies an abundant source of survival factors for the injured neurons (Meyer et al., 1992; Funakoshi et al., 1993; Meyer et al., 1992; Curtis et al., 1993, 1994). In contrast, in the CNS, neurotrophic support is insufficient (Cajal, 1928; Rudge et al., 1995), and axonal injury often causes extensive nerve cell death or neuronal atrophy. Exogenous sources of trophic factors provided can significantly increase neuronal survival and prevent neurons

from atrophy. Intravitreal administration of BDNF greatly increased the survival of RGCs: virtually all RGCs survive by day 7, and more than 80% survive by day 10. Rapid loss of RGCs occurred when injections were stopped (Mansour-Robaey et al., 1994). Application of BDNF or NT-3 helped to prevent the axotomy induced atrophy in rubrospinal neurons (Bregman et al., 1998). These results suggest that neurotrophic factors greatly improve the survival of neurons. This effect will consequently increase the chance of subsequent regeneration.

Neurotrophic factors also exert an important role in promoting regeneration. Without neurotrophin treatment, brainstem axons do not enter Schwann cell grafts placed into thoracic level lesions. Administration of BDNF and NT-3 significantly supports the regrowth into the grafts (Ye and Houle, 1997; Xu et al., 1994; 1995). When used together with fetal spinal cord transplantation, exogenous application of BDNF, NT-3 and NT-4/5 increases both the distance of axonal growth and the density of axonal growth within the transplant (Bregman et al., 1997). In the rubrospinal pathway, the number of neurons regenerating into peripheral nerve grafts is greatly increased upon application of BDNF, which is companied by an increase in the expression of GAP-43 (Tetzlaff et al., 1994). These results suggest that neurotrophic factors, especially when acting synergistically with peripheral nerve grafts, greatly increase the regenerative potential of neurons.

Neurotrophic factors have also been shown to affect other neuronal responses after injury such as cytoskeletal synthesis and transport (Lasek and Katz, 1987). On the other hand, the

low abundance of appropriate neurotrophic factors restricts axon regeneration in the injured CNS environment (Johnson, 1993).

1.5 Permissive and inhibitory influence in the environment

In addition to the intrinsic neuronal growth capacity, another factor that appears to be critical in determining the extent of regeneration is the permissiveness of the environment. CNS neurons do not spontaneously regenerate after injury, however, they do regenerate into a peripheral nerve bridge when transplanted into the damaged region (Aguayo, 1981).

After injury to the peripheral nerve, loss of axonal contact initiates a sequence of significant changes in the distal stump, referred to as Wallerian degeneration. Here, the environment dramatically changes and becomes growth promoting for the regenerating axons (Fawcett and Keynes, 1990). This is normally associated with the recruitment of macrophages, which remove axonal and myelin debris (Beuche and Friede, 1984, 1986; Perry, 1987). Schwann cells (Salzer and Bunge, 1980) down-regulate myelin-related proteins, up-regulate growth-promoting cell adhesion molecules (NCAM, L1, Martini and Schachner, 1988) on their cell surfaces, secrete neurotrophic factors (NGF, Brown et al., 1991a) and extracellular matrix components (laminin, merosin, Sanes et al., 1990), and proliferate within their basal lamina to form the bands of Bungner. These changes combine to create a favorable environment for axon regeneration. Wallerian degeneration is a necessary prerequisite for the adult peripheral nerve regeneration (Brown et al., 1991a). In the mutant mouse strain, the C57BL/Wld^{s},

Wallerian degeneration is markedly delayed (Brown et al., 1991b) and axons and myelin sheaths persist for weeks after axotomy, rather than a few days in the wildtype mouse. Regeneration fails in nerves such as the phrenic, which have very few unmyelinated axons; in nerves with more unmyelinated axons, some regeneration does occur, but only in association with the non-myelinating Schwann cells in these fibers (Brown et al., 1994). Consistent with this, DRG neurons dissociated from adult rats regenerate their neurites only when cultured on cryosections of the distal segment of previously lesioned sciatic nerves, but not when grown on sections of normal nerves (Bedi et al., 1992). The up-regulation of growth-promoting molecules in the degeneration process contributes to the axonal regeneration. In addition, the observation that regenerating neurites will not grow along undegenerated nerve tract or nerve section which contain myelin also suggests that peripheral nervous system myelin components is inhibitory for neurite outgrowth and removal of these components is an important reason for axonal regrowth (Bedi et al., 1992). In support of this, it was reported that growth inhibitory properties could be detected in extracts of PNS myelin devoid of laminin (David et al., 1994). Thus, it is both the up-regulation of growth-promoting molecules and also the removal of growth-inhibitory activities during the process of Wallerian degeneration that changes the PNS environment to support extensive regeneration.

In contrast to the PNS, Wallerian degeneration is rare in the CNS (Cajal, 1928; Stoll et al., 1989). After injury, CNS neurons encounter a non-permissive environment that is very different from that of the PNS. There are few growth-promoting molecules in the adult CNS.

On the other hand, presence of an abundance of inhibitory factors actively blocks regeneration.

Growth-inhibitory activities in the adult CNS are mainly associated with reactive astrocytes and myelin debris (Schwab, 1993). Following injury, mature astrocytes undergo distinct metabolic changes. They proliferate and invade the site of lesion where they form a tightly compact glial scar with interdigitated processes, connected by extended gap junctions, leaving little space for the passage of growth cones (Johnson, 1993). These scars were suggested to form a physical barrier for axonal regeneration (Johnson, 1993). In some cases, loose scars with few gap junctions were also observed, however, these scars are associated with axons in their scar matrix and appear to be permissive for axon regeneration (Alonso and Privat, 1993). Growth-inhibitory molecules are also suggested to be present on the non-permissive scars and mediate contact inhibition of axonal growth. This is supported by the results from *in vitro* experiments in which these reactive astrocytes provide poor or even non-permissive substrates for regenerating axons (McMillan et al., 1994). Chondroitin sulfate proteoglycan (CSPG) is one of the growth-inhibitory molecules expressed on the surface of reactive astrocytes (Dow et al., 1993, 1994). Up-regulated in adult astrocytes, CSPG has been shown to mask the growth-promoting activity of heparin sulfate proteoglycan (Dow et al., 1993, 1994). Other molecules such as tenascin have also been suggested to contribute to the inhibitory activity of reactive astrocytes (Schachner, 1994).

Growth-inhibitory activities have been shown to be highly enriched in CNS myelin and oligodendrocyte membranes (Schwab, 1993). When dissociated, these membrane preparations provide an inhibitory substrate for neurite outgrowth even in the presence of optimal levels of neurotrophic factors like NGF, BDNF or NT-3 (Caroni and Schwab, 1988a; Savio and Schwab, 1989). *In vivo*, a close relationship is observed between the failure of regeneration of CNS axons and the appearance of myelin in their tracts (Schwab, 1993). Rats and hamsters lose their ability to regenerate injured spinal cord fibers at age 4-6 days, corresponding to the time when myelin appears in a large part of the white matter (Schwab and Schnell, 1989). On the other hand, when oligodendrocyte development and myelin formation were prevented by x-irradiation, adult corticospinal tract fibers regenerated for 4.5-6 mm distal to the lesion, compared to about 1 mm in normal rats (Savio and Schwab, 1990). Similar effects were also observed in chick (Keirstead et al., 1992, 1995). Another important effect observed in these myelin-disrupted nerve tracts was the increased number of sprouts from these fibers and the extension of side branches to neighboring areas (Collelo and Schwab, 1994; Schwegler et al., 1995). This lead to the suggestion that not only does myelin inhibit axonal extension, it also inhibits neurite branching from the regrowing fibers. The latter function is thought to be related to its role in the normal, adult nervous system where they might participate in restricting the collateral sprouting of axons and stabilizing the established nerve connections (Colello et al., 1994).

The growth inhibitory activity of CNS myelin has been demonstrated to be associated with membrane-bound myelin proteins. Schwab and his colleagues designed an *in vitro* spreading

assay with 3T3 cells to assess the substrate nonpermisiveness of CNS myelin membrane fractions and of isolated membrane proteins reconstituted in liposomes, because they found that the unfavorable substrate properties of CNS myelin not only affected neurite outgrowth but also affected the spreading and migration of 3T3 cells (Caroni and Schwab, 1988b). In a series of experiments, Schwab and his colleagues showed that CNS nonpermissiveness was abolished by treatment with proteases and was not associated with myelin lipid (Caroni and Schwab, 1988b). Myelin proteins were size fractionated by SDS-PAGE and the separated proteins were recovered from the gel and their substrate nonpermissiveness were tested in fibroblast spreading assays and in neurite extention assays. Two inhibitory protein fractions corresponding to molecular weight 35 kD and 250 kD were found to account for most of the nonpermissive substrate activity of gel-extracted CNS myelin proteins. Removal of these two protein fractions yielded a CNS myelin fraction with permissive substrate properties (Caroni and Schwab, 1988b). When supplemented to liver protein or sciatic nerve protein they converted these permissive substrates into nonpermissive ones. These molecules, referred to as NI-35 and NI-250, are enriched in all CNS white matter and mature oligodendrocytes, but are absent from peripheral nerves (Caroni and Schwab, 1988b). Monoclonal antibodies were raised against these proteins. IN-1and IN-2 antibodies which were originally developed against NI-250, turned out to recognize not only NI-250 but also NI-35, suggesting a substantial degree of structural similarity between these two inhibitors (Caroni and Schwab, 1988c). When included in culture media, IN-1 reversed the inhibitory activity of mature oligodendrocytes and optic nerve explants (Caroni and Schwab, 1988c; Bandtlow et al., 1990). *In vivo*, neutralization of these inhibitors with IN-1 antibody

significantly enhanced regeneration. Corticospinal tract (CST) fibers re-extended to a maximum distance of 6.5 mm in the presence of IN-1 antibody and this length was further increased to 20.3 mm when NT-3 was applied at the same time (Schnell et al., 1994). Application of NT-3 alone did not have a significant effect on the length of the regenerative fibers, although it stimulated sprouting of the transected CST (Schnell et al., 1994). Moreover, when IN-1 antibody was continuously supplied by secretion from transplanted hybridoma cells, partial recovery of reflex and locomotion function was observed (Bregman et al., 1995). Despite their significant contribution to the inhibitory activities of CNS myelin, however, the identity of these two molecules is still not known.

It is of note that when IN-1 antigens were neutralized in the CST, although a significant improvement in regeneration was seen, only 5-10% of the injured neurons grew long axons across the lesion site (Schnell et al., 1994). This, as pointed out by Schwab, suggests that NI-35 and NI-250 are not the only inhibitors in CNS myelin, and that other molecules are also present (Barinaga et al., 1994). Searching for the other molecules led to the finding that two protein fractions purified from the bovine CNS myelin by DEAE-chromatography exhibit neurite growth inhibitory activity, and in one of them, the inhibitory activity can be immunodepleted by anti-MAG (myelin-associated glycoprotein) antibody (McKerracher et al., 1994). Independently, our lab showed that MAG, when expressed on the surface of CHO cells, potently inhibits neurite outgrowth from postnatal cerebellar neurons (Mukhopadhyay et al., 1994). These results suggested that MAG, a well-characterized myelin protein, may be another important inhibitor in CNS myelin.

1.6 Myelin-associated glycoprotein: structure

Myelin-associated glycoprotein (MAG) was first detected by the incorporation of radioactive sugar precusors into glycoproteins that were present in purified CNS myelin fractions (Quarles et al., 1973). When isolated from rat brain, MAG has an apparent molecular weight of 100 kD, of which 30% is carbohydrate (Frail and Braun, 1984). The independent sequencing of cDNAs for MAG from rat brain in three different laboratories (Arquint et al., 1987; Lai et al., 1987; Salzer et al., 1987) revealed that it is an integral membrane protein with a large extracellular disposed amino-terminal segment, a single transmembrane segment, and a cytoplasmic carboxy-terminal segment.

The extracellular segment of MAG contains five immunoglobulin (Ig) homology units. Therefore, MAG is a member of the immunoglobulin gene superfamily (IgSF, Arquint et al., 1987; Lai et al., 1987; Salzer et al., 1987). Among the five Ig-like domains, domain 2 (D2) to domain 5 (D5) were characterized as C2-like (constant type 2) Ig domains, because they have intermediate features between variable (V) and constant (C) Ig domains: they contain seven β-strands and cysteine spacing typical of C domains, but their amino acid sequence have more homology to V domains (Salzer et al., 1990).

In contrast, the first Ig-like domain (D1) belongs to V-like domain. It has nine β-strands, designated A-G, making up two β-sheets, the GFCC'C" sheet, and the ABED sheet (Wiliams and Barclay, 1988; Salzer et al., 1990). In this domain, the second and third cysteines, in β-

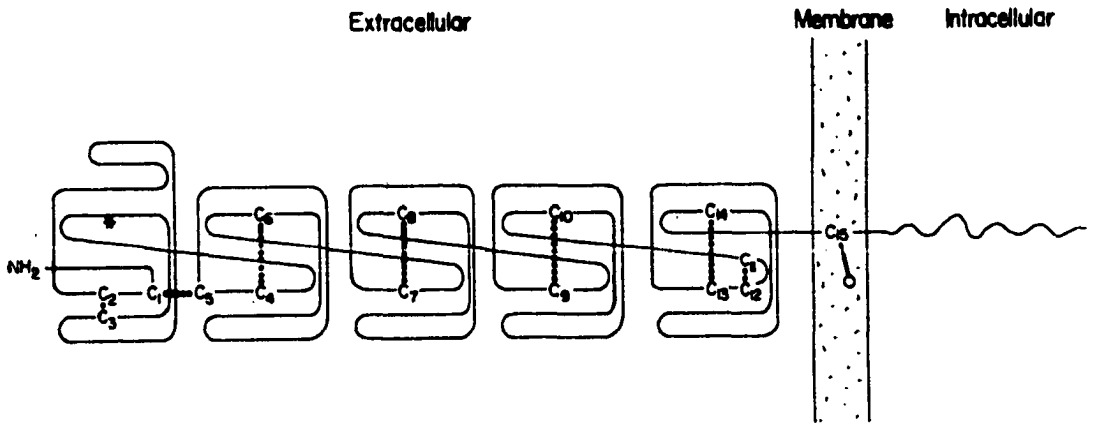


Figure 1.1 Model of the conformation of MAG. MAG has five immunoglobulin domains in the extracellular segment. The first domain folds as a variable domain and other domains fold constant (C2) domains. The predicted position of the cysteins and their disulfide linkages are indicated including an interdomain disulfide linkage of the first and fifth cystein, and the intrasheet linkage of the second and third cystein. The predicted position of the RGD sequence (*) of MAG within the first domain is also shown (Pedraza et al., 1990).

strands B and E respectively, form an unuausal intrasheet disulfide linkage, and the first cysteine in D1 is therefore placed at a position to form a interdomain disulfide linkage with cysteine 5 in D2 (Salzer et al., 1990; Figure 1.1). The consequence of this folding is that the tripeptide sequence Arg-Gly-Asp (RGD), located between D1 and D2 of MAG, falls into the interior of D1 and thus has limited accessibility (Pedraza et al., 1990). The RGD sequences are frequently an important determinant of binding sites for integrins (Ruoslahti and Pierschbacher, 1987), however, due to the steric inaccessibility, the functional importance of this sequence in MAG in mediating binding with integrins is limited (Quarles et al., 1992). Supporting this is the inability of the anti-RGD antibody to recognize MAG in its native

conformation, although it reacts well with SDS denatured MAG (Salzer et al., 1990). Also consistently, a synthetic peptide containing the RGD sequence failed to block the adhesion of MAG, incorporated into liposomes, to axons (Sadoul et al., 1990).

The structural characteristics of the V-like domain of MAG is not commomly found within the Ig-superfamily, and it is unique for a subgroup of IgSF members termed siglecs (Kelm et al., 1994; 1998). Siglecs are a homologous group of cell surface Ig-like glycoproteins that recognize distinct sialylated glycoconjugates (Kelm et al., 1994, 1998). Other members of the siglec family include the murine macrophage-restricted cell surface molecule sialoadhesin, the lymphoid differentiation antigen CD33, the B cell adhesion molecule CD22, and SMP (Schwann cell myelin protein, a protein similar to MAG but found only in avian species). Members of this family share considerable sequence similarity in the first four Ig-like domains, however, each member has different binding specificity (Kelm et al., 1994; 1998). MAG prefers α 2,3-linked sialic acid attached to O-linked glycoconjugates, CD22 binds to α 2,6 linked sialic acid attached to N-linked glycoconjugates, while sialoadhesin and CD33 recognize α 2,3-linked sialic acid attached to either N- or O-linked glycoconjugates (Kelm et al., 1994; Freeman et al., 1995).

MAG has two developmentally regulated isoforms resulting from alternative splicing of the primary MAG transcript (Lai et al., 1987). The molecular weight of L-MAG and S-MAG are 72 kD and 67 kD, respectively. However, since MAG is highly glycosylated *in vivo*, both isoforms co-migrate on SDS-PAGE as a single, diffuse band of about 100 kD. L-MAG and

S-MAG share the same sequence in their extracellular domains and transmembrane domains, but differ in length and sequence of their cytoplasmic domains. Two consensus recognition sites for protein kinase C and one site for tyrosine kinase are present in L-MAG but not in S-MAG (Trapp, 1990). These sites are known to be phosphorylated (Edwards et al., 1989).

1.7 Myelin-associated glycoprotein: localization

MAG is found in both CNS and PNS myelin where it represents approximately 1% and 0.1% of the total myelin protein, respectively (Trapp, 1990). MAG is expressed by oligodendrocytes in the CNS prior to the onset of myelination and by Schwann cells in the PNS at the time of axonal contact (Trapp, 1990). In the CNS, L-MAG is the principal form during early and active myelination stages, whereas S-MAG predominates in the mature CNS (Bartsch et al., 1989). In the PNS, S-MAG represents more than 95% of the total MAG at all stages of myelination (Martini and Schachner, 1986). MAG is located in the periaxonal membrane of both CNS and PNS myelin, as well as in other non-compact regions in the PNS such as the paranodal loops, the Schmit-Lanterman incisures, and the outer mesaxon membranes (Trapp, 1989).

1.8 Myelin-associated glycoprotein: function in myelination

Because of its early expression during myelination and its subcellular location close to the axon, MAG has long been suggested to play an important role in the initiation of myelination

(Quarles, 1984; Martini and Schachner, 1986; Owens and Bunge, 1989). Evidence from *in vitro* studies strongly supports this suggestion. When co-cultured with DRG neurons, Schwann cells prematurely expressing L-MAG initiate myelination at an earlier stage (Owens et al., 1990). On the other hand, when the expression of MAG is prevented by anti-sense MAG RNA, Schwann cells fail to initiate the myelin spiral (Owens and Bunge, 1991). However, observations in the MAG-deficient mouse are in apparent contradiction to these results. The initiation and periodity of myelin are normal in the PNS (Li et al., 1994). Subtle morphological abnormalities were observed in CNS myelin (Li et al., 1994; Montag et al., 1994; Bartsch et al., 1995), where the periaxonal cytoplasmic collar was reduced or absent, and some axons were surrounded by 2 or more myelin sheaths. Significant disruption of myelin structure is not shown until the age of 8 months, when the maintenance of the axon-myelin unit is disrupted in the PNS, resulting in both axon and myelin degeneration (Fruttiger et al., 1995). Companying this, neurofilament phosphorylation is greatly reduced in MAG-/- axons and a marked shrinkage in axonal diameter starts from the age of 3 months (Yin et al., 1998). Taken together, these results suggest that MAG has several crucial functions in myelination: MAG is involved in the recognition between oligodendrocytes and axons, MAG participates in the formation of the periaxonal cytoplasmic collar, MAG is also important in maintaining the integrity of both axons and myelin (Meyer-Franke and Barres, 1994). Its role in the initiation of myelination, however, needs to be examined further. Instead of a knockout mouse, a transgenic mouse line that prematurely expresses MAG in Schwann cells may provide a suitable experimental model.

1.9 Myelin-associated glycoprotein: function in axonal regeneration

As an Ig-superfamily member, MAG was initially found to be a neurite-outgrowth promoting molecule. Neurite outgrowth from neonatal DRG neurons was promoted by recombinant MAG expressed by transfected fibroblast cells (Johnson et al., 1989). Consistent with this observation, down-regulation of MAG by interferon-gamma and tumor necrosis factor-alpha on immortalized Schwann cells reduced the neurite outgrowth of neonatal DRG neurons (Schneider-Schaulies et al., 1991). However, it was recently reported that MAG has a bifunctional effect on neurite outgrowth: it promotes neurite outgrowth from neonatal DRG neurons, but inhibits growth from adult DRG neurons and all postnatal cerebellar, retinal, spinal, hippocampal, and superior cervical ganglion neurons (Mukhopadhyay et al., 1994; DeBellard et al., 1996). Grown on a monolayer of MAG-expressing, transfected CHO cells, neurite lengths extended from all these postnatal neurons are 70-90% shorter than those grown on control transfected cells (DeBellard et al., 1996), an effect which can be reversed by a polyclonal anti-MAG antibody (Mukhopadhyay et al., 1994). This suggests that MAG has a potent inhibitory effect on neurite outgrowth and this inhibitory effect is widespread for postnatal neurons from a variety of regions of the nervous system.

In support of our observation, another strong indication of the inhibitory effect of MAG comes from the growth cone collapse assay. About 60% of the growth cones of PND 1 hippocampal neurons collapsed when they encountered polystyrene beads coated with

recombinant MAG (Li et al., 1996). Such collapse was not observed with denatured MAG. However, when immunopurified soluble MAG was used instead of MAG-coated beads, another group found that no morphological changes were evoked in PND1 and adult DRG neurons (Bartsch et al., 1995). Considering that MAG coated on beads can work in a multimeric manner while soluble MAG is monomeric, the differences in these results suggest that MAG, like other cell adhesion molecules, needs to form clusters at the cell surface to function correctly.

McKerracher et al. (1994) have shown that MAG contributes significantly to the inhibitory properties of CNS myelin. Immunodepletion of MAG from myelin is accompanied by a 60% loss in inhibition by this membrane. They also showed that neurons grown on the purified, extracellular domain of MAG were inhibited in the formation of neurites (McKerracher et al., 1994). Moreover, we have recently found that soluble forms of MAG, either chimeric MAG-Fc (consisting of the extracellular domain of MAG fused to the Fc portion of human IgG), or dMAG (which is a proteolytic fragment of its extracellular domain released from myelin *in vivo*), potently inhibits neurite outgrowth (Tang et al., 1997b). The importance of these results is that they demonstrate that MAG can exert its effects either as a substrate molecule in myelin or as a soluble factor released from myelin, and that these effects do not require the cooperation of another molecule.

As evidence accumulated suggesting a strong inhibitory effect of MAG on neurite outgrowth, the question of its functions *in vivo* as an inhibitor of axonal regeneration has

recently been addressed using the MAG-deficient mice. There are discrepancies in the results reported. McKerracher's group examined *in vivo* axonal regeneration after spinal cord lesion and showed a significant increase in the number of axons that grew across the lesion in MAG-/- mice (49 axons in 15 mice) compared to that in wild type mice (9 axons in 8 mice). The regenerating axons reached a maximum distance of 13.2 mm distal to the injury in MAG-/- mice, which is twice longer than that in wild type mice (Li et al., 1996). Consistently, when neurons were grown on purified CNS myelin from MAG-/- mice, the average neurite length from NG108 cells was about 30% longer compared to that on myelin from normal mice. However, while the inhibitory activity in CNS myelin can be fractionated into several peaks by DEAE-chromatography (McKerracher et al., 1994), a more significant reduction (49%-55%) of inhibitory activity was noted in the first major peak corresponding to MAG than that observed in the intact MAG-/- myelin (Li et al., 1996). These results agree with the observations from previous *in vitro* experiments and suggest that MAG contributes significantly to axonal growth inhibition associated with CNS myelin. On the other hand, these results also suggest that MAG is not the only inhibitor in CNS myelin, and that the lack of MAG in MAG-/- mice is partially masked due to the presence of a variety of other inhibitors (Li et al., 1996).

However, different results were reported by another research group. Using a different line of MAG-/- mice, Barstch et al (1995) reported that no significant improvement in regeneration of injured spinal cord and optic nerve in the MAG-/- mice compared to the wild type mice. Nor is the neurite outgrowth extended on cryosections of optic nerves of MAG-/-

mice. Improved outgrowth was observed from NG108 cells but not from PC12 cells and adult DRG neurons when grown on purified MAG-/- myelin (Bartsch et al., 1995). On the other hand, the IN-1 antibody can partially reverse the inhibition on both types of myelin extracts and enhanced in vivo regeneration. Despite the discrepancies in the two reports, one point is confirmed by both: the existence of numerous inhibitors other than MAG in CNS myelin which includes the IN-1 antigens and other as yet unidentified factors. While differences could exist between the two independently developed mouse lines, it should be noted that the possibility of the presence or up-regulation of other compensatory molecules was not addressed in these studies. Although an unequivocal contribution of MAG to the inhibition of CNS myelin cannot yet be concluded, considering that MAG is only one member of a large variety of inhibitors in CNS myelin, it is perhaps not surprising, especially if some of the other inhibitors are up-regulated in the knockout animals, that positive effects on regeneration of the CNS are difficult to be fully revealed in mice lacking MAG.

In contrast to the CNS, a distinct *in vivo* demonstration of the inhibitory effects of MAG has been provided by regeneration experiments in the PNS (Schafer et al., 1996). As discussed above, after injury to the PNS, regeneration will only occur when myelin debris is removed. In a mutant mouse with abnormally slow Wallerian degeneration (C57BL/Ola), there is unusually poor axonal regeneration. However, in the MAG-/-: C57BL/Ola mice (generated by cross-breeding C57BL/Ola mice with MAG-/- mice), the number of regenerating axons in association with persistent, apparently intact myelin sheaths were

increased compared to the C57BL/Ola mice expressing MAG. These results suggest that MAG when present, is a potent inhibitor for axonal regeneration *in vivo* in the PNS.

1.10 Mechanisms by which environmental cues affect neuronal growth states

Whether an axon will regenerate after it is cut depends on the balance between the intrinsic ability of the axon to regrow and the permissiveness of its environment. Regenerative capability of neurons differs significantly when different environments are provided (David and Aguayo, 1981), suggesting that the neuronal growth state can be largely affected by environmental cues the regenerating axon encounters. However, signal transduction mechanisms underlying many of these processes, which lead from the interaction with either extrinsic growth-promoting or inhibiting molecules to the change in axonal growth, are not fully understood.

Environmental factors were shown to affect the regenerative state of neurons by affecting the expression of a number of growth-associated genes (Broude et al., 1997; McKerraacher et al., 1993a; 1993b; Schwab and Bartholdi; 1996). For instance, c-Jun expression was rapidly up-regulated in successfully regenerating PNS neurons (Aldskogius et al., 1992), but is only transiently reactivated in axotomized CNS neurons (Lieberman, 1971), whose regenerative attempts were aborted in the inhibitory CNS glial environment. However, if the injured CNS neurons encounter a peripheral nerve graft (McKerraacher et al., 1993a; 1993b) or a fetal spinal cord transplant (Broude et al., 1997), this transient reactivation was prolonged and

regeneration is significantly improved. The expression of GAP-43 also seems to be influenced by local environment factors. Injury to either the peripheral nerve branch or the dorsal root axon branch of a DRG neuron will lead to rapid upregulation of GAP-43 expression. Interestingly, peripheral nerve lesions elicited much higher GAP-43 levels in DRG than dorsal root lesions (Schreyer and Skene, 1993; Chong et al., 1992; 1994). It is possible that the presence of CNS myelin has a suppressive effect on GAP-43 expression.

Receptor-mediated signaling cascades are likely to be involved and to lead to regulation of regeneration-associated gene expression in neurons (as discussed above), and/or in cytoskeletal reorganization and function (Tanaka and Sabry, 1995; Goodman, 1996; Schwab and Bartholdi, 1996). One example comes from studies on a group of cell adhesion molecules (CAMs), NCAM, L1 and N-cadherin, which were shown to promote axonal outgrowth (for reviews, see Walsh and Doherty, 1996; 1997; Green et al., 1997). These molecules are normally found on both the advancing growth cone and also on cellular substrates. In some circumstances, homophilic binding of the CAM in the substrate to the CAM in the neuron activates neuronal fibroblast growth factor (FGF) receptors, possibly via a direct interaction in cis between the CAM and FGF receptor (Williams et al., 1994; Saffell et al., 1997). This leads to the sequential stimulation of phospholipase C-γ (PLC γ) and diacylglycerol (DAG) lipase to generate arachidonic acid (for review, see Doherty and Walsh, 1994), which in turn increase Ca^{2+} influx via N- and L-type channels and activate protein kinase C (PKC). Recent results show that GAP-43, a major growth cone component associated with axonal growth, guidance and synaptic plasticity, is a downstream effector in

this cascade and is phosphorylated on serine-41 in response to CAMs stimulation (Meiri et al., 1998). Previous experiments show that GAP-43 is enriched in growth cones at the interface between membrane receptors and cytoskeleton (Meiri and Gordon-Weeks, 1990; Moss et al., 1990), and that its phosphorylation state influences cytoskeleton dynamics and affect growth cone motility (Dent and Meiri, 1992). Phosphorylated GAP-43 stabilizes long actin filaments, whereas unphosphorylated GAP-43 prevents actin polymerization (He and Meiri, 1997). These results provided a framework for a model whereby CAMs influence axonal growth by modulating the ratio of phosphorylated to unphosphorylated GAP-43 so as to regulate actin cytoskeleton dynamics in the advancing growth cones.

It is now realized that one environmental factor could simultaneously activate more than one signaling cascade in neuronal cells. Therefore, an interactive signaling network, which integrates several parallel signaling pathways, is more likely to be the underlying neuronal response, rather than a simple linear pathway. Studies on lysophosphatidic acid (LPA)-induced neurite retraction are a good example in point. LPA is a serum-derived neurite growth inhibitor (Tigyi and Miledi, 1992). Binding of LPA to its neuronal receptor activates both Gi and Gq heterotrimeric G protein-linked signaling pathways (Tigyi et al., 1996a). Interestingly, a similar (PIP)-Ca^{2+} second messenger system, as seen in the CAMs pathway, is induced downstream of Gq activation, but plays only a permissive role in the neurite retraction response. Blockers of PLC and PKC both diminish retraction by interfering with LPA-induced Ca^{2+} mobilization, which is required for the withdrawal of neurites. However, activation of this (PIP)-Ca^{2+} cascade alone is not sufficient to cause neurite retraction. The

Ras-like small GTP-binding protein Rho, which has been suggested to play a role in actin cytoskeleton reorganization, is shown to play a crucial role in mediating LPA-induced neurite retraction. Inactivation of Rho by the ADP-ribosylation with the *C. botulinum* exoenzyme C-3 not only abolishes LPA induced neurite retraction, but also induces neuronal differentiation (Tigyi et al., 1996b). Evidence from a separate experiment shows that activation of Gq is sufficient to activate Rho (Katoh H et al., 1998). Therefore, it is likely that an as yet unidentified pathway, parallel to the (PIP)-Ca^{2+} cascade, might also be induced downstream of Gq and lead to the activation of Rho (Tigyi et al., 1996b). In contrast, activation of Gi does not appear to be involved in the neurite retraction process itself (Tigyi et al., 1996b). However, elevation of cyclic AMP levels triggers a PKA-dependent mechanism that prevents LPA-induced retraction. It is likely that the coupling of LPA-receptor to Gi might lower cyclic AMP levels so as to protect and promote the functioning of the morphoregulatory signaling system. Therefore, the stimulation by LPA activates multiple signaling pathways, each conducting required, permissive and protective functions, and orchestrating the neuronal response through complex regulation.

It must be noted that the surrounding environment will present a combination of different factors that might affect the growth of a regenerating axon. Complicated coordination of different signaling systems will be expected to contribute to the neuronal state of regrowth.

1.11 The goal of studies in this thesis

MAG was previously shown to promote neurite outgrowth from newborn DRG neurons. My thesis work started when our lab found that MAG has a bifunctional effect on neurite outgrowth, it promotes growth from newborn DRG neurons, but inhibits neurite outgrowth from late postnatal DRG neurons and all postnatal cerebellar neurons. However, in these studies, MAG was expressed on surface of Chinese hamster ovary (CHO) cells that do not normally express MAG. Investigation of the inhibitory effects of MAG on neurite outgrowth when it is expressed in Schwann cells, the cells that normally express MAG, may have more physiological relevance. In addition, the mechanism for this inhibition is not known. How does MAG interact with its neuronal receptors? What signal transduction pathway does it induce in the neurons? To address these questions, the following points were addressed:

1. The effect of MAG on neurite outgrowth of neurons at different ages was investigated using MAG-expressing CHO cells (Chapter III).

2. The inhibition effect of MAG on neurite outgrowth was examined further when MAG was expressed in Schwann cells (Chapter IV).

3. The binding of MAG to neurons was shown to be sialic acid-dependent. By site-directed mutagenesis, the sialic acid binding site on MAG was identified and the effect of mutation at this site on the function of MAG was assessed (Chapter V).

4. Using chemicals that interfere with various signal transduction pathways, the signaling mechanism for MAG's inhibition was investigated (Chapter VI).

Chapter II

Methods and Materials

2.1 Cell culture

Transfected CHO cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), proline (40 mg/liter), thymidine (0.73 mg/liter), and glycine (7.5 mg/liter) at 37°C in 5%CO_2.

A spontaneously transformed Schwann cell line (Schwann cell 117, kindly provided by Dr. James Salzer (Owens et al., 1990)), and its MAG-expressing sublines were maintained in DMEM with 10% dialyzed FCS at 37°C in 5% CO_2. For transfected cells, 0.4 mg/ml of G418 was included into the media.

2.2 Isolation of Neurons

Neurons were isolated as previously described (Doherty et al., 1990). Briefly, for rat pups up to 10 days of age, cerebellar, DRG and SCG neurons were removed from two animals. Like tissue was combined and placed in 4 ml of 0.025% trypsin in PBS, triturated, and incubated for a further 10 min at 37°C. Trypsinization was stopped by addition of 4 ml of DMEM containing 10% FCS, and cells were centrifuged at 800 rpm for 5 min. The cells were resuspended to a single-cell suspension in 2 ml of SATO (Doherty et al., 1990). For late postnatal DRG neurons, ganglia were removed from two animals and incubated in 5 ml of L-15 containing 0.025% trypsin and 0.15% collagenase type I (Worthington) for 30 min at 37°C. The ganglia were triturated with a fire polished Pasteur pipette. Trypsinization was

stopped by adding 5 ml of DMEM containing 10% FCS, centrifuged at 800 rpm for 5 min and resuspended in SATO. Cells were counted with a Coulter counter.

2.3 Neurite outgrowth on control and MAG-expressing CHO and Schwann cell monolayers

Wells of an 8-Chamber tissue culture slide (Lab-Tek) were coated with 16.6 μg/ml of poly-L-lysine (Sigma) for 30 min at room temperature, washed 3 times with distilled water, and incubated with 10 μg/ml of fibronectin (Sigma) at 37°C for 2 hr. Confluent monolayers of control and MAG-expressing CHO or Schwann cells where indicated were established over a 24 hr period in individual chambers of the precoated slides. Cocultures were established as previously described (Doherty et al., 1990) by adding 10,000 cerebellar or DRG neurons to the CHO monolayers or 4000 cerebellar neurons to the Schwann cell monolayer. Culture medium was serum-free SATO. For studies for signal transduction pathways, various reagents were included in SATO media at the indicated concentration.

After periods of time, as indicated, cocultures were fixed for 30 min with 4% paraformaldehyde, and permeabilized with methanol for 2 min at -20°C. The cells were then blocked for 30 min with DMEM containing 10% FCS and incubated for 2 hr with a rabbit antibody against GAP-43 (1:4000; from R. Curtis and G. Wilkins). Cells were washed three times with PBS-BSA (0.05%) and then incubated for 30 min at room temperature with a biotinylated donkey anti-rabbit Ig (1:500; Amersham), washed three times, and incubated

with streptavidin-conjugated Texas Red (1:300; Amersham) for 45 min. After three more washes, the slides were mounted in Permfluor (Baxter) and viewed with a Zeiss fluorescence microscope. The length of the longest neurite for each GAP43-positive neuron was determined by computer-assisted digitalized image analysis (Doherty et al., 1990)

2.4 Neurite extension and branching on Schwann cell monolayers

4000-6000 DRG or SCG neurons from PND 7 rat pups were seeded on confluent monolayer of control or MAG-expressing Schwann cells. After incubation for 12 hr, cocultures were fixed and stained for GAP43, as described above. Neurite extension of each GAP43-positive SCG or DRG neuron was analyzed by manually counting the number of neurons with neurites longer than 3 times the diameter of cell body and calculating the percentage of this population of neurons. For analysis of neurite branching, the image of each selected neuron was superimposed with a pattern of concentric circles. The smallest circle, which has a radius of 7 μm, was centered over the neuronal soma. The subsequent circles increased in radius each by 14 μm. The number of intersections of the neurite branches with each circle was determined by computer assisted image analysis and was plotted against the distance from the soma. This analysis provided information on the extent of branching at different distance from the soma.

2.5 Neurite outgrowth on immobilized myelin membranes

Wells of an 8-chamber slide were first coated with 16.6 μg/ml poly-L-lysine at room temperature for 1 hr and washed with 0.1 M $NaHCO_3$. After washing the wells with DMEM, rat CNS myelin at 1.5 or 3 μg total protein/well were dried overnight onto the poly-L-lysine-coated wells and used as a substrate for neurite outgrowth. Cerebellar neurons from rat pups up to PND 7 were seeded at 50,000 neuron/well, incubated at 37°C for 20 hr, and fixed and immunostained for GAP43. The length of the longest neurite for each GAP43-positive neuron was measured as described above.

2.6 Mutation of R118 in MAG

The Chameleon double-stranded site-directed mutagenesis kit (Stratagen) was used to mutate the nucleotide sequence coding for R118 in L-MAG to either an alanine or an aspartate as described by the manufacturer. L-MAG in antisense orientation in pBluescript was used for the initial annealing step. The mutations were confirmed by dideoxy sequencing. The R118A- and R118D-mutated forms of MAG were then subcloned into pSJL plasmid for expression in Schwann cells. To obtain Fc-chimeras of the mutated MAG proteins, an XhoI restriction fragment (nucleotide 182-1672 in MAG cDNA) which contains the mutated nucleotides were cut and used to replace the corresponding region in the MAG-Fc sequence harbored in pIG1 vector.

2.7 Schwann cell transfection

The pSJL plasmids containing the cDNA for L-MAG, its R118A-, R118D-mutated forms, or its 3'-5' orientated antisense control was introduced into Schwann cell 117 by lipofectin (GibcoBRL) mediated transfection. The transfected cells were selected in 400 μg/ml G418 (GibcoBRL). Colonies were lifted from the plates and were combined and cells were surface-stained for MAG by incubating with 513 MAG monoclonal antibody (mouse) (Boehringer Mannheim) at 1 μg/ml for 1 hr at room temperature. After washing in PBS, the cells were incubated with phycoprobe-conjugated anti-mouse IgG (goat) (Biomeda) at the dilution of 1:100 for 30 min at 37°C, then washed and resuspended in DMEM. The cells expressing high level of MAG were selected by FACS, and further expanded.

2.8 Western blot analysis of expression of MAG in Schwann cells and CHO cells

Cells (80%-90% confluent) were lysed in 0.5 M Tris-HCl (pH7.5) containing 2% SDS, 4% β-mercaptoethanol, and the following anti-proteases: 1 μg/ml chymotrypsin, 1 μg/ml pepstatin, 1 μg/ml phenylmethylsulfonyl fluoride. The lysate was homogenized by passage through a 23 gauge syringe. Protein concentration was measured with a Bio-Rad kit before the addition of β-mercaptoethanol. The lysates were incubated at 95°C for 3 min, after which they were subjected to SDS-polyacrylamide gel (8%) electrophoresis. The proteins were transferred to PVDF membrane, immunostained with a monoclonal antibody against rat MAG (1:100; from Dr. Richard Quarle) for 1 hr at room temperature; second antibody was alkaline phosphatase-conjugated goat anti-mouse (1:10,000; Sigma). The substrate was 5-

bromo-4-chloro-3-indolylphosphate, and the chromogen was nitroblue tetrazolin (kerkegaard and Perry Laboratory) used according to the manufacturer's instructions.

2.9 Indirect immunofluorescence of intact Schwann cells

Cells were grown overnight in chambers of an 8-well tissue culture slide coated with poly-L-lysine (Sigma) and fibronectin (Sigma). Culture media were removed and cells were incubated with a monoclonal antibody against MAG (5μg/ml; Boehringer Mannheim) for 2 hr at 4°C, then fixed in 4% paraformaldehyde for 10 min at room temperature. The cells were then washed three times with DMEM, blocked for 30min with 10% FCS in DMEM, incubated with phycoprobe-conjugated goat anti-mouse IgG (1:50; Biomeda) for 1 hr at room temperature, and wash three times with DMEM and once with ddH2O before being mounted with Gel-mount (Biomeda) and viewed with a Zeiss fluorescence microscope.

2.10 Preparation of Fc-chimeras

The cDNA for Fc-chimeras were constructed by fusing the extracellular domain of MUC18, wild type MAG, or mutated MAG to the Fc region of human IgG (Kelm et al., 1994), and were harbored in pIG1 plasmid vectors. To prepare Fc-chimeric proteins, these plasmids were transiently transfected into COS-1 cells with DEAE-dextran (Kelm et al., 1994). COS-1 cells of 70-80% confluency were covered by transfection solution containing 100ng plasmid DNA per $1.0x10^5$ cells, 250 μg/ml DEAE-dextran, and 100 μM chloroquine

diphosphate in 10 mM HEPES-buffered DMEM, and were incubated at 37°C for 3-5 hr or until vacuolation started. Cells were then osmotically shocked by PBS/10% DMSO for 2 min, washed twice with HEPES-buffered DMEM, and incubated in DMEM with 1% IgG-depleted serum at 37°C to allow recovery and production of Fc-chimeric proteins.

After 8-10 days of incubation, culture media of transfected COS-1 cells were collected and the chimeric proteins were purified by affinity chromatography. The media were incubated with 5 ml protein A Sepharose slurry overnight at 4°C. Bound Fc proteins were eluted from protein A Sepharose by 5 ml 100 mM glycine solution at pH 3.0, then neutralized with 0.5 ml 1 M Tris buffer at pH 8.0. The eluted proteins were concentrated to about 1-2 mg/ml by Centricon-30 microcentractors (Amicon) via repeated centrifugation, sterilized by filtration with sterile Ultrafree-MC filter units (Milipore). Protein concentration was estimated using a Bio-Rad protein assay kit.

2.11 Solid-phase Binding Assay with Immobilized Fc-chimeras

In order to maximize the chances for correct orientation of Fc-chimera, 15 μg/ml anti-human IgG (Fc-portion) (Sigma) was first coated onto an Immulon-3 96-well ELISA plate (Dynatech) for 2 hr at 37°C in 0.1 M bicarbonate buffer. Wild type or mutated MAG-Fc and the control MUC18-Fc, at a concentration of 10 μg/ml, were absorbed for 2 hr at 37°C to the coated plate. $2x10^5$ isolated cerebellar neurons were added to each well and incubated for 1 hr at room temperature. The plate was washed 2-4 times with PBS/0.25%BSA applied to

each well under gravity by allowing free flow from an elevated reservoir at an appropriated rate. Bound neurons were lifted up in Isoton solutions and their numbers were counted with a Coulter counter.

2.12 An ELISA Assay for Mutated Fc-chimeras

A 96-well ELISA plate (Dynatech) was coated with anti-human Fc (IgG) (Sigma) at 15 μg/ml in 0.1 M bicarbonate buffer for 2 hr at 37°C. The plates were washed with PBS and blocked with 5% nonfat milk in PBS for 30 min at room temperature. The Fc-chimeras were added at a concentration of 1 μg/ml and incubated overnight at 4°C. The MAG 513 monoclonal antibody (Boehringer Mannheim) was added at various concentrations, from 0.1 to 333 ng/ml, and incubated for 2 hr at 37°C. The plate was then washed and blocked again with 5% nonfat milk in PBS for 30 min at room temperature. Peroxidase-conjugated sheep anti-mouse IgG (Sigma) diluted 1:500 was added and color was developed using 4% wt/vol O-phenylenediamine and 0.2% H_2O_2 in citrate buffer. Absorbance was measured in an ELISA reader at the wavelength of 490 and 630 nm.

Chapter III

Inhibition of Neurite Outgrowth by MAG Expressed on CHO Cell Surface

3.1 Introduction

It is generally believed that the presence of inhibitory molecules in the mammalian central nerve system plays a major role in the lack of axonal regeneration after injury to the brain or spinal cord (Johnson, 1993; Schwab et al., 1993). Therefore, the identification of inhibitory molecules would help to determine the conditions that would permit nerve regeneration after injury. Axonal growth inhibitory molecules are particularly enriched in CNS myelin (Johnson, 1993; Schwab et al., 1993; Schwab abd Bartholdi, 1996). IN-1 monoclonal antibody recognizes at least two proteins that are likely to play a role in this inhibition (Caroni and Schwab, 1988a, b; Schnell and Schwab, 1990; Bregman et al., 1995). However, the identification of these two proteins has been very difficult and their molecular nature remains elusive.

In contrast, MAG is a well-characterized membrane protein found in both CNS and PNS myelin. It is a member of the Ig gene superfamily (Arquint et al., 1987: Lai et al., 1987; Salzer et al., 1987). Numerous members of this family of molecules have been shown to mediate cell-cell adhesion and to promote neurite outgrowth (Salzer and Colman, 1989; Walsh and Doherty, 1996, 1997). Indeed, in addition to its putative function in myelination, MAG has been shown to enhance neurite outgrowth from DRG neurons from 1-day-old rats (Johnson et al., 1989). The length of neurites from newborn DRG neurons grown on MAG-transfected fibroblast cells was about twice as long as those grown on control fibroblasts. Subsequent to this study, we examined the effect of MAG on neurite outgrowth of DRG

neurons, and another type of neurons, cerebellar neurons. In addition, to assess if the effect of MAG on neurite outgrowth changes during development, DRG and cerebellar neurons of different ages were studied. The system used in these studies was similar to that used by Johnson et al, co-cultures of neurons on confluent monolayers of Chinese hamster ovary (CHO) cells expressing MAG on their surface. Similar co-culture systems have been successfully used to study the effect on neurite outgrowth of a vatriety of cell adhesion molecules (Doherty et al., 1990). L-MAG cDNA was introduced into CHO cells by transfection and the L-MAG protein was shown to be expressed at the physiological level, and distributed evenly on the cell surface (Mukhopadhyay et al., 1994). MAG protein expressed in CHO cells has a molecular weight of approximately 100 kDa when glycosylated, the same as MAG found in sciatic nerve or brain, indicating that the proteins are glycosylated to the same extent as they are in vivo (Quarles et al., 1983). The effect of MAG on neurite outgrowth on CHO cell surface was examined. We showed not only that MAG enhanced neurite outgrowth from newborn DRG neurons but also that it has a pronounced inhibitory effect on outgrowth either from older DRG neurons or from developing cerebellar neurons. A sharp transition from promotion to inhibition in the response of DRG neurons to MAG was seen at postnatal day 2-3. This inhibitory effect of MAG may account for at lease part of the general inhibitory effect of CNS myelin.

3.2 Results

3.2.1 Neurite outgrowth of DRG neurons of different age on MAG-expressing and control CHO cells

It has been shown previously that MAG promotes neurite outgrowth from DRG neurons of PND1 rats. To confirm this result, PND1 DRG neurons were isolated and cultured on confluent monolayers of control and MAG-expressing cells. The co-cultures were subsequently fixed and stained for GAP-43 and the longest neurite of each GAP-43 positive neuron was measured. Consistant with the previous finding, the average neurite length from PND1 DRG neurons on MAG-expressing cells was about twice as long as that on control cells (Figure 3.1).

In contrast to PND1 DRG neurons, a different effect of MAG was found for older postnatal DRG neurons. When cultured on control CHO cells, adult DRG neurons extended substantial neurite outgrowth (Figure 3.2). However, when the same neurons were co-cultured with MAG-expressing cells, neurite outgrowth was reduced by about 40% (Figure 3.2c). This result suggested that during maturation, DRG neurons changed their response to MAG. While neurite outgrowth from newborn DRG neurons was promoted by MAG, neurite outgrowth from adult postnatal neurons was inhibited.

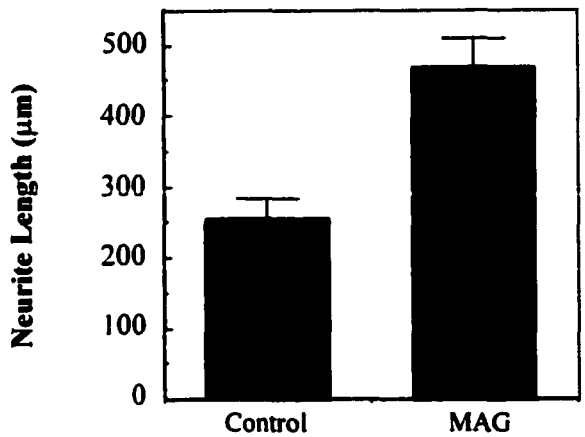


Figure 3.1 The effect of MAG on neurite outgrowth from PND1 DRG neurons. Dissociated DRG neurons from PND1 were cultured for 12hr on confluent monolayers of control CHO cells or MAG-expressing cells. The co-cultures were then fixed and stained for GAP-43, and the longest neurite of each GAP-43 positive neuron was measured. Results show the mean length of longest neurites per neuron (± SEM) for at least 100 individual neurons.

3.2.2 DRG neurons show a sharp transition in their response to MAG

To determine when the switch from promotion to inhibition of neurite outgrowth takes place for DRG neurons, a detailed time course of the effect of MAG on neurite outgrowth from postnatal DRG neurons was carried out. DRG neurons from rats of different postnatal ages, from PND1 to adult, were isolated and used in the neurite outgrowth assay described above.

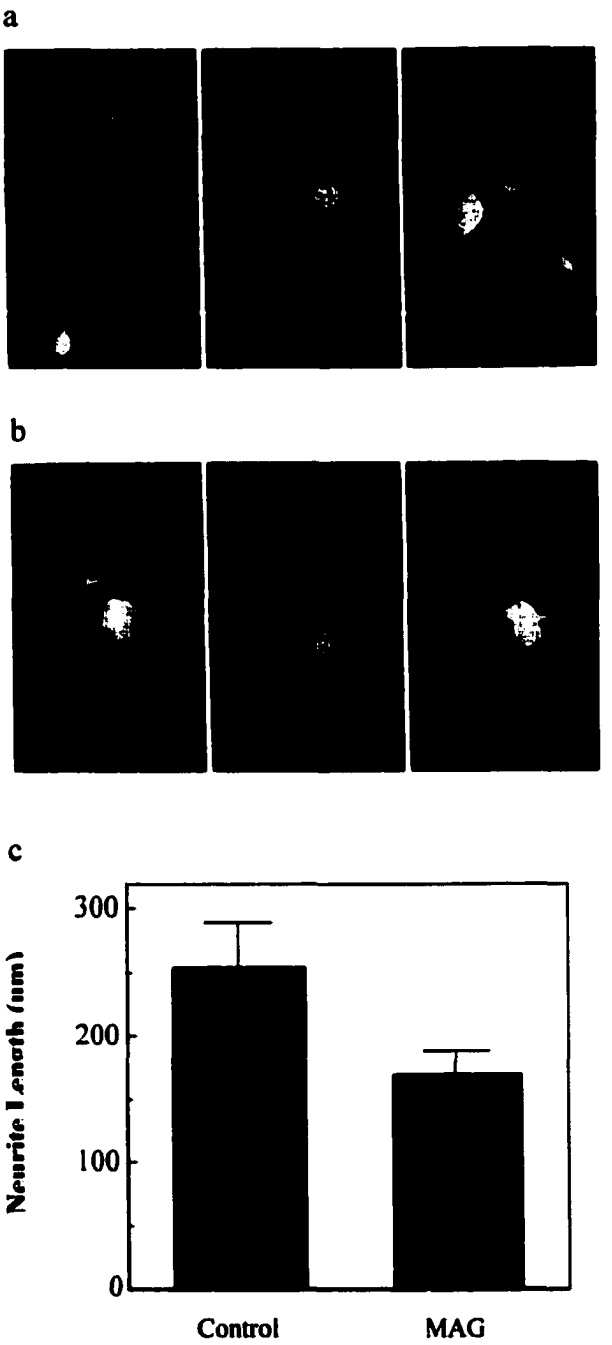


Figure 3.2 The effect of MAG on neurite outgrowth from adult DRG neurons. (a and b) Dissociated neurons from adult rats were cultured for 17 hr on confluent monolayers of MAG-expressing (a) and control (b) CHO cells before being fixed and immunostained for GAP-43. (c) The longest neurite of each GAP-43 positive neuron was measured. Results show the mean length of longest neurites per neuron (± SEM) for at least 100 individual neurons.

The results show that MAG promotes neurite outgrowth from PND1 DRG neurons by about 100%. At PND2, MAG also promotes outgrowth but by only 80% (Figure 3.3). In contrast, at PND3 MAG inhibited neurite outgrowth by about 40%. A similar inhibition was observed for all DRG neurons from rats of PND3 or older. The transition from MAG promoting to inhibition was sharp, occurring between PND2 and PND3. It is of note that at no stage in postnatal development did DRG neurons fail to respond to MAG; neurite outgrowth was always either inhibited or promoted.

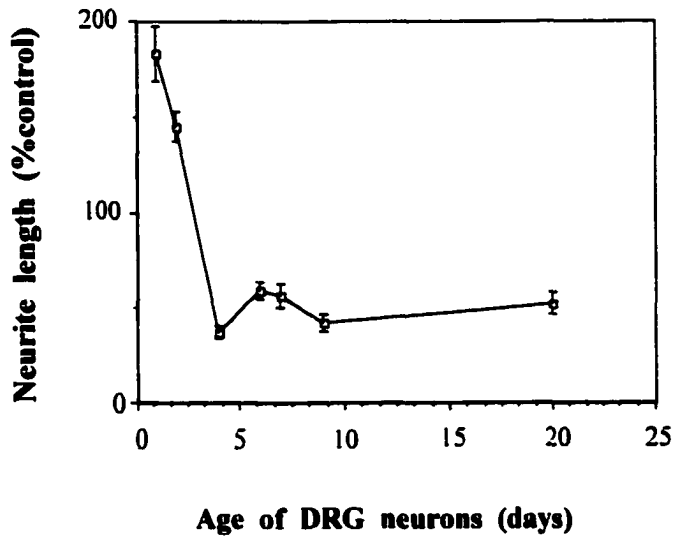


Figure 3.3 The effect of MAG on neurite outgrowth from DRG neurons of different ages. Dissociated PND 1, 2, 3, 4, 5, 7 and adult neurons were cultured for 12 hr on confluent monolayers of control and MAG-expressing CHO cells before fixing and immunostaining for GAP43 and neurite length was measured. Results show neurite length on MAG-expressing cells as a percentage of neurite length on control cells (±SEM). For each point 120-180 individual neurons were measured.

3.2.3 Neurite outgrowth of cerebellar neurons of different age on MAG-expressing and control CHO cells

To assess the effect of MAG on neurite regrowth from CNS neurons, rat cerebellar neurons isolated at postnatal day (PND) 1 were cultured on confluent monolayers of MAG-expressing CHO cells and neurite outgrowth was compared to that on control CHO cells not expressing MAG. The neurons and neurites were fixed and stained for GAP43 and the longest neurite from each GAP43 stained neuron was measured for at least 100 neurons.

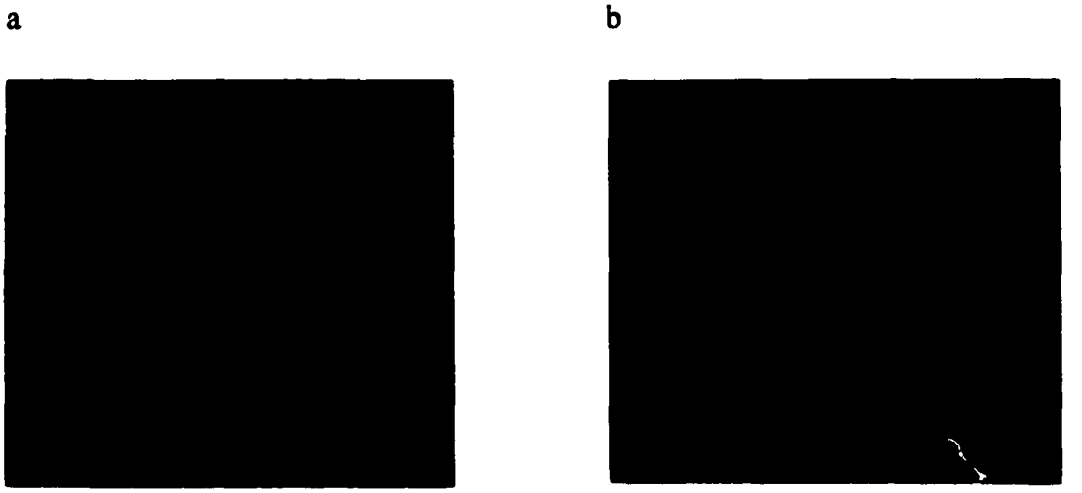


Figure 3.4 The effect of MAG expressed by CHO cells on neurite outgrowth from cerebellar neurons. Dissociated cerebellar neurons from PND1 rats were cultured for 16.5 hr on confluent monolayers of control (a) and MAG-expressing (b) CHO cells before being fixed and immunostained for GAP43.

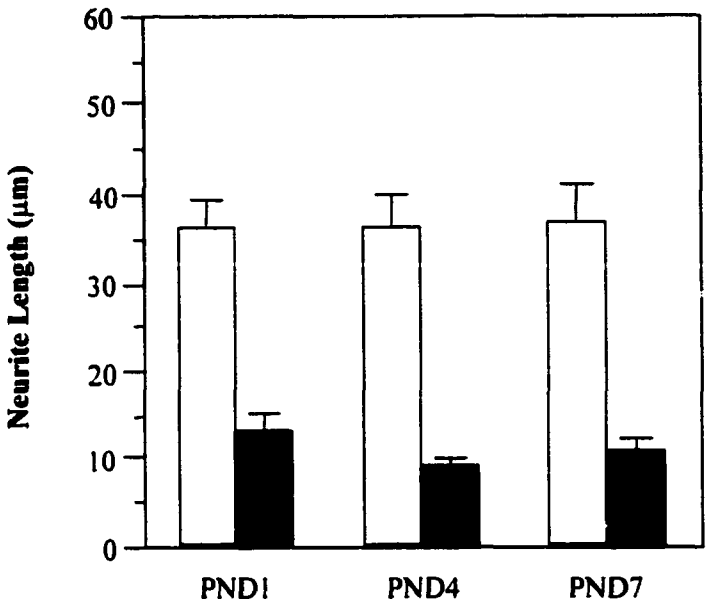


Figure 3.5 The effect of MAG expressed by CHO cells on neurite outgrowth from cerebellar neurons of different ages. Dissociated PND 1, 4 and 7 cerebellar neurons were cultured for 16.5 hr on confluent monolayers of control (open bars) and MAG-expressing (closed bars) CHO cell before being fixed and immunostained for GAP-43. Results show the mean length of the longest neurite per neuron (± SEM) for 120-180 individual neurons.

The result shows that PND1 cerebellar neurons extended long neurites when grown on control CHO cells, but they extended much shorter and fewer neurites when grown on MAG-expressing cells (Figure 3.4). Neurite outgrowth on MAG cells was dramatically reduced, by 70%, when compared to that on control cells (Figure 3.5). This result suggests that, in contrast to its promoting effect on the neurite outgrowth from PND1 DRG neurons, MAG

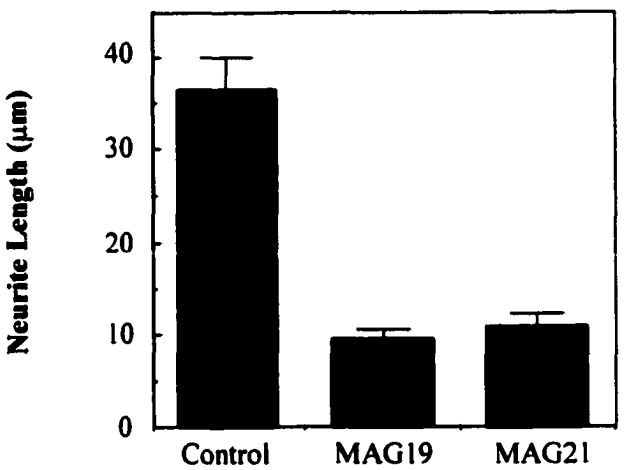


Figure 3.6 The effect of different MAG-expressing CHO cell lines on neurite outgrowth from cerebellar neurons. Dissociated PND4 cerebellar neurons were cultured on confluent monolayer of control and MAG-expressing CHO cell lines, MAG19 (open bar) and MAG21 (closed bar), for 17-18 hr. The co-cultures were then fixed and stained for GAP-43, and the longest neurite of each GAP-43 positive neuron was measured. Results show the mean length of longest neurites per neuron (± SEM) for at least 100 individual neurons.

shows a potent inhibitory effect on neurite growth from PND1 cerebellar neurons (Mukhopadhyay et al., 1994).

Since it has been shown that the response of DRG neurons to MAG in their ability to extend neurites changes during maturation, and since it is known that the ability of cerebellar neurons to extend neurites in response to other adhesion molecules changes during development (Doherty et al., 1990), we further investigated whether the ability of MAG to

inhibite neurite outgrowth from cerebellar neurons also changes with age. Neurite outgrowth assays were carried out with cerebellar neurons from PND1, 4, and 7 rats. Results shows a similar inhibition by MAG of around 70% for neurons of all ages tested (Figure 3.5).

To ensure that the inhibitory effect of MAG on neurite outgrowth from cerebellar neurons is not an artifact of one particular transfected CHO cell line, two different MAG-expressing CHO cell lines, MAG19 and MAG21, were tested. It was found that the ability of PND7 cerebellar neurons to extend neurites was reduced to a similar degree of 60-70%, when grown on a monolayer of either cell line compared to that on control CHO cells (Figure 3.6). Therefore, the inhibitory effect of MAG on neurite outgrowth was not likely to be an artifact of a particular cell line.

3.3 Conclusion and discussion

In conclusion, MAG, a member of the Ig superfamily, has a potent inhibitory effect on the growth of neurites from both PNS and CNS neurons. MAG inhibits neurite outgrowth from all postnatal cerebellar neurons by 60-70%. MAG inhibits neurite outgrowth from adult DRG neurons by about 40%. On the other hand, MAG promotes neurite outgrowth from newborn DRG neurons. The developmental switch of the effect of MAG on neurite outgrowth from DRG from promotion to inhibition occurs sharply at postnatal day 3.

The inhibitory effect of MAG on neurite outgrowth are dramatic, more so for cerebellar neurons than for adult DRG neurons, which may be relevant to the difference in the regenerative capacity of the two neuron types. The specificity of this effect was demonstrated in a number ways. First, because a number of different MAG-expressing CHO cell lines exert the same inhibitory effect on neurite outgrowth, the effect is unlikely to be an artifact of any one cell line. We carried out neurite outgrowth assays using four different MAG-expressing CHO cell lines, and the data of two of these cell lines were presented in this study (Figure 3.2c). Second, the fact that CHO cells expressing MAG promote outgrowth from newborn DRG neurons but inhibit outgrowth from older postnatal DRG neurons and all postnatal cerebellar neurons, eliminates the possibility that the inhibitory effects of MAG are just simply a result of expression a foreign protein by CHO cells, which would affect the inherent neurite-promoting properties of CHO cells in a nonspecific manner. In keeping with this suggestion is the observation that expression of comparable levels of Po, another myelin-

specific protein, by CHO cells had no effect on their ability to permit neurite outgrowth from cerebellar neurons (Mukhopadhyay and Filbin, unpublished observation). Finally, studies by other investigators in our lab have shown that a polyclonal antibody to MAG reverses inhibition by about 40% (Mukhopadhyay et al., 1994), whereas it has no effect on neurite extension on control CHO cells. Complete reversal was not achieved, most likely because the affinity of the antibody for MAG is not as strong as that of MAG for its putative receptor, and the two are in direct competition.

Inhibitory factors have been suggested to function by decreasing adhesion of neurons for their substrates (Caroni and Schwab, 1988a, 1988b). This is unlikely to account for the inhibitory effect of MAG, because it was observed that on MAG-expressing cells and on control cells, the plating efficiency is the same for all neurons tested, regardless of age or type (Mukhopadhyay and Filbin, unpublished observation). Furthermore, it has been shown by other investigators in our lab that radiolabled MAG binds equally well to newborn DRG neurons as to cerebellar neurons (Tang et al., 1997b), although the effect of MAG on the growth of these two types of neurons are the opposite. This indicates that MAG, when present on CHO cells, is unlikely to decrease the adhesion of neurons to these cells and that the inhibitory effect of MAG does not result from an inability of neurons to adhere to MAG-expressing cells. To date, the mechanism by which MAG exerts an effect on neurite outgrowth, either promotion or inhibition, is still poorly understood. The neuronal component with which MAG interacts to bring about the effects is also unknown.

Subsequent to this study, the effects of MAG on neurite outgrowth of a wide variety of neurons were tested (Debellard et al., 1996; Turnley and Bartlett, 1998). It has been shown that the inhibitory effects of MAG on neurite outgrowth is widespread for postnatal neurons from a variety of regions of both the CNS and the PNS, including retinal, spinal, hippocampal, and superior cervical ganglion neurons (DeBellard et al., 1996). A developmental switch of response to MAG from being promoted to being inhibited is also observed in a number of different neurons (Salzer et al., 1990; Debellard et al., 1996; Turnley and Bartlett, 1998). Except for DRG neurons, retinal ganglion neurons and spinal neurons switch their response to MAG by birth; embryonic retinal ganglion neurons and spinal neurons are promoted by MAG (Salzer et al., 1990; Turnley and Bartlett, 1998), while postnatal neurite growth from these neurons is inhibited (DeBellard et al., 1996). Recently, it was suggested that this developmental switch is likely to be mediated by a developmentally regulated decrease in endogenous neuronal cyclic AMP levels (Cai et al., submitted). The endogenous level of cAMP in retinal ganglion neurons and spinal neurons was shown to drop dramatically during development, and this spontaneous decrease in cAMP coincides with the switch from promotion to inhibition of neurite growth by MAG. It was also shown that inhibiting a signal downstream of cAMP activation (inhibition of PKA) can block early developmental promotion of neurite growth by MAG, and that inhibition in older neurons can be prevented by elevating cAMP levels (Cai et al., 1999; Cai et al., submitted).

In myelinating axons of both the CNS and the PNS, MAG is localized to the innermost myelin membrane, i.e., the membrane in contact with the axon (Trapp and Quarles, 1984;

Martini and Schachner, 1986). Because of this location and its early expression in development, MAG has long been suggested a role in the formation of myelin and in the maintenance of the stable interaction between axon and myelinating cell (Trapp et al., 1989). Indeed, the spacing between the axon and myelinating cell is abnormal in MAG-deficient mouse (Li et al., 1994; Montag et al., 1994; Bartsch et al., 1995). In addition, significant disruption of the axon-myelin unit occurs by the age of 8 months, resulting in both axon and myelin degeneration (Fruttiger et al., 1995). The neurite inhibitory properties of MAG are suggested as an additional function of this molecule in maintaining stable myelin by actively preventing growing axons from entering and disrupting sites of stable axon-myelin interaction. Such an action would be required during development of the CNS, when myelination of some axons is complete before the last axon has reached its target (Chiquet, 1989). More specifically, the processes of the majority of cerebellar neurons never enter myelinated areas (Jacobson, 1991), and MAG could contribute to this exclusion.

In addition, after injury, MAG may contribute to the inhibitory effect of CNS myelin. Consistent with our findings, McKerracher et al., (1994) has demonstrated by *in vitro* assays that MAG can account for part of the inhibitory effects of CNS myelin. Their research group has also shown that in MAG-/- mice there was a significant increase in the number of axons that grew across the lesion site compared to that in wild type mice. Moreover, the maximal length the regenerating axons extended was also longer in MAG-/- mice compared to that in wild type mice (Li et al., 1996). However, different results were reported by another research group. Using a different line of MAG-/- mice, Barstch et al (1995) reported that no

significant improvement in regeneration of injured spinal cord and optic nerve in the MAG-/- mice compared to the wild type mice. It should be noted that differences could exist between the two independently developed mouse lines, and that the possibility of the presence or up-regulation of other compensatory molecules was not addressed in these studies. An unequivocal contribution of MAG to the inhibition of CNS myelin cannot yet be concluded. However, considering that a combination of several growth-inhibitory components is likely to be present in CNS myelin including the IN-1 antigens, it is perhaps not surprising, especially if some of the other inhibitors are up-regulated in the knockout animals, that positive effects on regeneration of the CNS are difficult to be fully revealed by disruption of only one of these inhibitors. Studies on the IN-1 antigens showed that neutralization of IN-1 antigens and application of exogenous neurotrophins resulted in long-distance regeneration of approximately 5-10% of injured corticospinal fibers, however, the majority of spinal cord axons fail to regenerate (Schnell et al., 1994). These results strongly support the notion that there are redundant inhibitory factors in CNS myelin and that the absence of any one is insufficient to affect regeneration significantly.

In contrast to the CNS, regeneration does take place in the PNS after injury. However, the regenerating axons of adult peripheral nerves enter the distal stump only after myelin-axon degeneration has taken place so that myelin debris, and hence MAG, has been removed. In the mutant mouse C57BL/Ola in which myelin is removed very slowly or not at all, there is very limited regeneration. Some regeneration does occur, but only in association with the nonmyelinating Schwann cells where there is no MAG (Brown et al., 1994). On the other

hand, in mice with a double mutation for C57BL/Ola and MAG-/-, regeneration is faster after injury, even though myelin is not cleared in these mice (Schafer et al., 1996). In agreement with this, it was reported that adult DRG neurons only extended neurites on tissue sections of predegenerated peripheral nerve but not on the ones with intact myelin and an abundance of MAG (Bedi et al., 1992). This strongly points to a role for MAG in determining when and where regeneration in the PNS takes place.

In summary, in this study we have also shown for the first time that MAG is a bifunctional molecule for neurite regeneration. MAG promotes neurite regeneration from newborn DRG neurons, but it potently inhibits neurite outgrowth from cerebellar neurons and adult DRG neurons. It is also suggested for the first time that MAG could contribute to the poor regeneration of the mammalian CNS.

Chapter IV

Inhibition of Neurite Elongation and Branching by MAG Expressed on Schwann Cell Surface

4.1 Introduction

We have shown that MAG expressed by CHO cells inhibits neurite regeneration from a wide variety of neurons of the CNS and the PNS (Debellard et al., 1996). However, CHO cells, or other fibroblasts, do not normally express MAG. *In vivo,* MAG is expressed by the myelin-forming oligodendrocytes in the CNS and Schwann cells in the PNS.

Schwann cells, the glial cells of the PNS, differentiate during development from a common set of precursors into two distinct phenotypes--myelin-forming and non-myelin-forming Schwann cells. Myelin-forming Schwann cells form a single myelin sheath around a single axon, whereas non-myelin-forming Schwann cells ensheathe multiple small axons without forming any myelin sheath (Voyvodic, 1989). The two kinds of Schwann cells express distinct sets of molecules (for review see Jessen and Mirsky, 1991). Myelin-forming Schwann cells express high levels of the myelin-related proteins, including MAG, myelin basic protein (MBP), peripheral myelin protein 22 kDa (PMP22), and protein zero (Po), but express little or no neural cell adhesion molecule (NCAM) and L1. Conversely, non-myelin-forming Schwann cells express high levels of NCAM and L1, but do not express myelin-related proteins.

The two types of Schwann cells differ significantly in their properties as a substrate for neurite growth. PNS myelin formed by myelin-forming Schwann cells exhibits inhibitory properties for axonal regeneration, whereas non-myelin-forming Schwann cells provide a

permissive substrate for axonal growth (Brown et al., 1991b; Bedi et al., 1992; David et al., 1994). In a mutant mouse strain, the C57BL/Wld^s, where clearance of myelin sheath is markedly delayed (Brown et al., 1991b), regeneration is significantly impeded. Some regeneration does occur, only in association with non-myelin-forming Schwann cells, but rarely along the myelinated nerve tracts (Brown et al., 1994).

The differentiation into myelin-forming and non-myelin-forming phenotypes is reversible after peripheral nerve injury. During Wallerian degeneration, myelin sheaths in the distal stump break up and previously myelin-forming Schwann cells de-differentiate into phenotypes of non-myelin-forming characteristics. They dramatically decrease their synthesis of myelin-related proteins (including MAG) and glycolipids and up-regulate the expression of cell adhesion molecules such as NCAM and L1 (Scherer and Salzer, 1996). These Schwann cells were also shown to increase their secretion of a variety of neurotrophic molecules including NGF, BDNF, NT-3, NT-4/5, TGF-βs, CNTF and LIF, and to produce extracellular matrix components, such laminin, which promote axonal regeneration (Scherer and Salzer, 1996).

De-differentiated Schwann cells in the distal stumps play an important role in facilitating axonal regeneration. Studies have demonstrated that if Schwann cells are eliminated by freeze-thaw, PNS axons will still regenerate in the acellular nerve grafts, but not as well as in nerve grafts that contain live Schwann cells (Ide et al., 1990; Sketelj et al., 1989). Similarly, when segments of peripheral nerve are utilized to promote regeneration within the CNS, CNS

axons will only regenerate into peripheral nerve grafts that contain Schwann cells, but not into acellular peripheral nerve grafts (Berry et al., 1988). These studies suggest that Schwann cells in the degenerated distal stump are essential for axonal regeneration.

In the current study, we investigated the neurite growth-promoting properties of Schwann cells in culture. We showed that Schwann cells in culture were an excellent substrate for neurite growth of different types of CNS and PNS neurons. They supported extensive neurite outgrowth from isolated cerebellar, dorsal root ganglion (DRG) and superior cervical ganglion (SCG) neurons as well as to support tremendous neurite branching from DRG and SCG neurons. Our next aim was to investigate the inhibitory effect of MAG when it is expressed on the surface of Schwann cells. These studies would have more physiological importance than those with MAG expressed by CHO cells and may provide evidence for the possible contribution of MAG to the axonal-growth inhibitory property of PNS myelin. For this purpose, MAG was induced to express on the surface of the cultured Schwann cells and the ability of MAG-expressing Schwann cells to support neurite outgrowth was compared to that of the control Schwann cells. The results were consistent with that from CHO cell studies and confirmed that MAG has a strong inhibitory effect on neurite regeneration. In addition, MAG was shown to strongly inhibit neurite branching from DRG and SCG neurons. These results may help to generate a more complete understanding of the inhibitory function of MAG.

4.2 Results

4.2.1 Expression of MAG by Schwann cells

The Schwann cell line used in this study was originally derived from neonatal rat sciatic nerves. After repetitive passaging in the presence of glia growth factor (GGF) and foskolin, these cells lost normal growth control and were able to proliferate without added mitogens. Upon contact with axons, these cells can associate with axons in culture, deposit a basal lamina, and ensheathe axons, but they do not to myelinate axons (Porter et al., 1987). Full-length LMAG cDNA, under the control of the CMV promoter, was introduced into these cells by adenoviral infection (Owen et al., 1990). The MAG-expressing Schwann cell line and the control Schwann cell line were kindly provided by Dr. James Salzer.

Figure 4.1 Western blot analysis of MAG expression in Schwann cells. Immunodetection of MAG in cell lysates of control uninfected Schwann cells (lane 1), MAG-expressing Schwann cells (lane 2), and MAG-expressing CHO cells (lane 3). Each lane was loaded with 32 μg of total protein. Proteins were separated by polyacrylamide gel electrophoresis (8%), transferred to PVDF membrane, and immunostained for MAG with a monoclonal rat anti-MAG antibody (1:100). Bars refer to molecular weight standards. Arrow points to the bands of L-MAG.

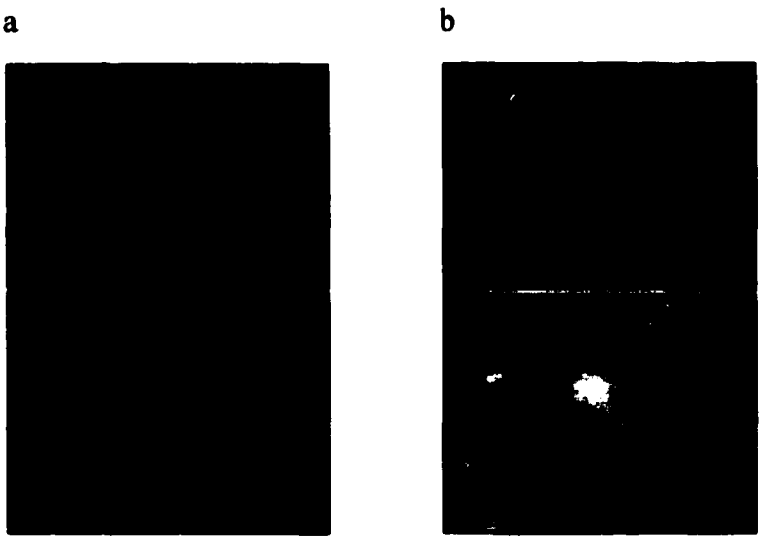


Figure 4.2 Surface detection of MAG on Schwann cells by immunofluorescence staining. Live Schwann cells, either control uninfected (a) or MAG-expressing (b) cells were incubated with 513 monoclonal MAG antibody (5 μg/ml), then fixed and incubated with phycoprobe-conjugated goat anti-mouse IgG (1:50).

The expression of MAG in these Schwann cells was characterized (1) by immunostaining for MAG with a MAG monoclonal antibody (B11F7, O'Shannessy et al., 1985) on Western blots of cell lysates, and (2) by immunofluorescence of live cells with 513 MAG monoclonal antibody which recognizes native MAG (mAb513, Poltorak et al., 1987).

Immunostaining of a Western blot shows that LMAG-expressing Schwann cells express an abundance of MAG (Figure 4.1). The amount of MAG expressed in these Schwann cells is comparable to that expressed by CHO cells, which we have already shown to be inhibitory for axonal growth (Mukhopadhyay et al., 1994). MAG expressed by these Schwann cells has

an apparent molecular weight of about 100 kD, similar to that of MAG expressed by CHO cells, indicating that MAG is glycosylated to the same extent in the two cell lines (Mukhopadhyay et al., 1994). No detectable MAG expression is seen on Western blots by control Schwann cells.

To determine whether MAG reaches the surface of these Schwann cells, live cells were immunostained with mAb513. As shown in Figure 4.2, LMAG-expressing Schwann cells were immnofluorescently stained with mAb513 and the staining was evenly distributed on the cell surface. Consistent with that observed by Western bolt, no MAG staining was detected on the surface of the control Schwann cells. Hence, these results indicate that MAG expressed by Schwann cells reaches the cell surface and has an even distribution.

4.2.2 MAG expressed by Schwann cells inhibits neurite outgrowth from cerebellar neurons

To investigate the effect of MAG on neurite outgrowth when it is expressed in Schwann cells, we carried out neurite outgrowth assays in the same manner as for the studies with MAG-expressing CHO cells. Cerebellar neurons from PND 7 rat pups were dissociated and plated on confluent monolayers of MAG-expressing Schwann cells and control Schwann cells which do not express MAG. After overnight incubation, the cocultures were fixed, stained, and the length of the longest neurite from each neuron was measured.

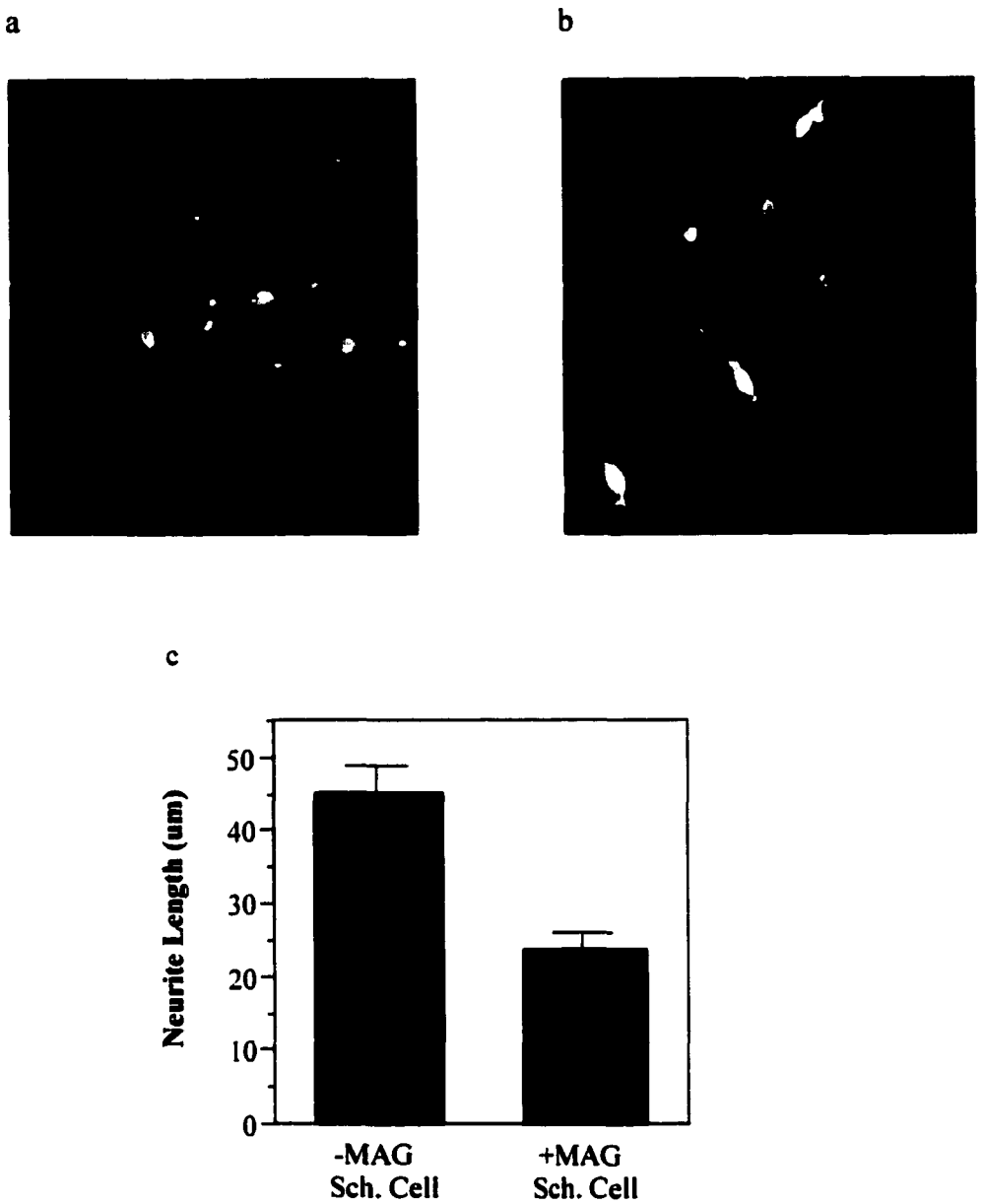


Figure 4.3 The effect of MAG expressed by Schwann cells on neurite outgrowth from cerebellar neurons. (a and b) Dissociated cerebellar neurons from PND 7 rats were cultured for 18 hr on confluent monolayers of control (a) or MAG-expressing (b) Schwann cells before fixed and immunostained for GAP43. (c) The longest neurite of each GAP-43 positive neuron was measured. Results show the mean length of longest neurites per neuron (± SEM) for at least 200 individual neurons.

As shown in Figure 4.3, control Schwann cells support neurite outgrowth from cerebellar neurons. However, neurites extended on MAG-expressing Schwann cells are shorter than those extended on control Schwann cells. Quantitative analysis of more than 200 neurons indicates that average neurite length from neurons grown on a monolayer of MAG-expressing cells was reduced by 47% compared to neurons grown on a monolayer of control Schwann cells. Similar results were obtained with a second, independently generated LMAG-expressing Schwann cell line (data shown in the next chapter). These results suggest that MAG can overcome the growth-promoting/ permissive properties of Schwann cells to inhibit neurite outgrowth from cerebellar neurons.

4.2.3 MAG expressed by Schwann cells inhibits neurite outgrowth from DRG neurons

We next tested the growth-inhibitory effects of Schwann cell-expessed MAG on DRG neurons---the sensory neurons of the PNS. As described in the previous chapter, MAG expressed by CHO cells inhibits neurite outgrowth from late postnatal DRG neurons but to a lesser extent than that for cerebellar neurons, perhaps due to a stronger regenerative capacity of DRG neurons. Now we tested if the robust growth of DRG neurites, which was supported by Schwann cells can be inhibited by MAG. DRG neurons from PND 7 rats were isolated and neurite outgrowth on a monolayer of MAG-expressing Schwann cells was tested as described above. It was found in this assay that control Schwann cells support not only extensive neurite outgrowth but also tremendous neurite branching from DRG neurons, as

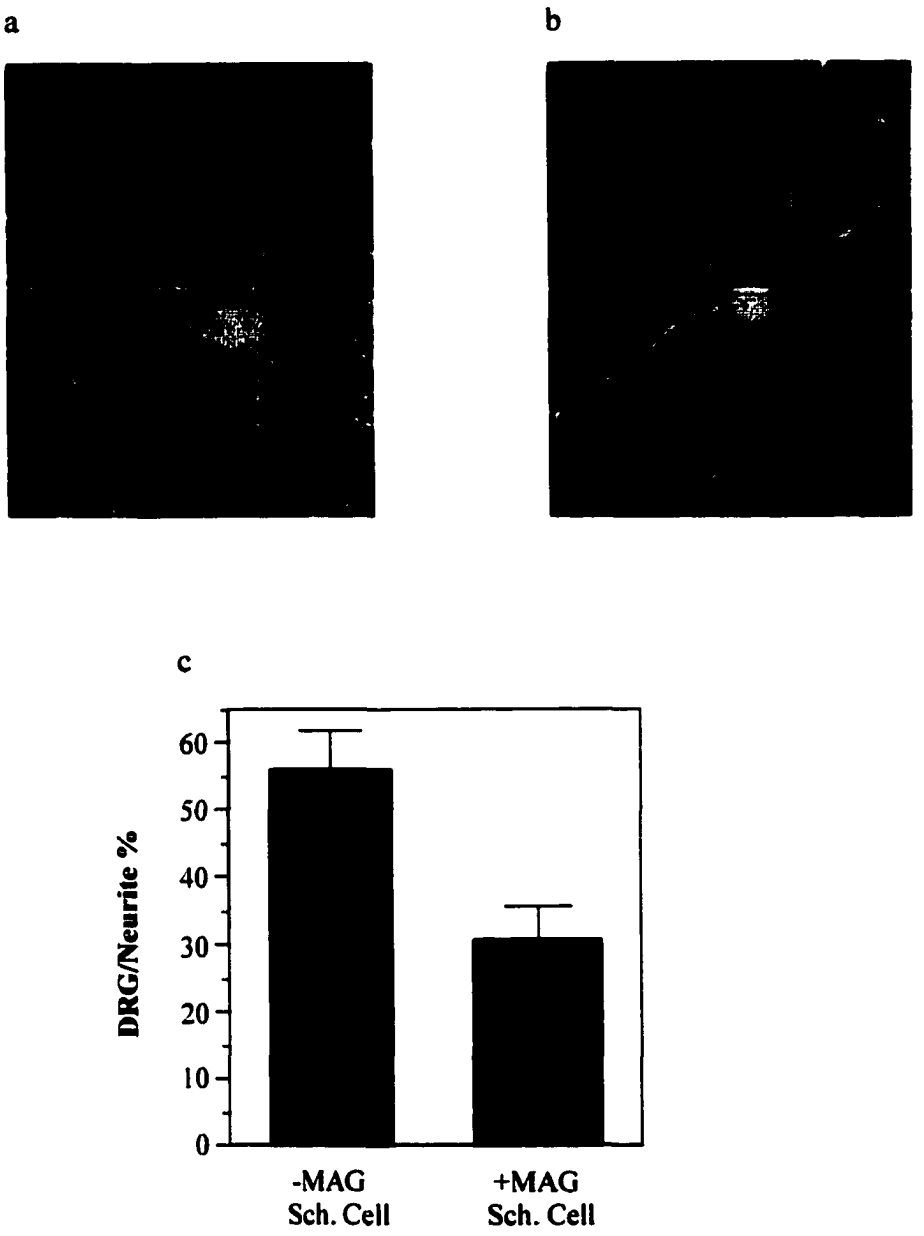


Figure 4.4 The effect of MAG expressed by Schwann cells on neurite outgrowth from DRG neurons. (a and b) Dissociated DRG neurons from PND 7 rats were cultured for 12 hr on confluent monolayers of control (a) or MAG-expressing (b) Schwann cells before fixed and immunostained for GAP43. (c) The number of neurons with neurites longer than three times their cell body was counted for about 200 neurons (± SEM). Results show the percentage of neurons with neurites of that length.

can be seen in Figure 4.4b. Because of this extensive branching, it is not possible to measure the length of an individual neurite, as we do for cerebellar neurons or for DRG neurons on CHO cell surfaces. Therefore, instead of measuring individual neurite length, the percentage of neurons with neurites longer than 3-times the diameter of their cell bodies was counted. As shown in Figure 4.4c, the percentage of DRG neurons extending neurites longer than 3-times the diameter of their cell bodies was reduced by 43% when grown on MAG-expressing Schwann cells compared to that on control Schwann cells that do not express MAG. These results suggest that MAG has a strong inhibitory effect on neurite outgrowth. It can inhibit neurite extension from DRG neurons when expressed by Schwann cells.

4.2.4 MAG expressed by Schwann cells inhibits neurite branching from DRG neurons

As stated above, DRG neurites extended on Schwann cells are highly branched and form a complex network. However, the extent of neurite branching and the complexity of neurite networks was dramatically reduced when these cells were grown on MAG-expressing Schwann cells (Figure 4.4). To assess the effect of MAG on neurite branching, we quantitated the extent of neurite branching by counting the number of times that neurites crossed a series of concentric circles that were superimposed onto the image of the neuron, centered on the cell body (Figure 4.5); the greater the number of crosses at a given distance from the cell body, the greater the branching. The average number of crossings was plotted against the radius of the concentric circles, which represented the distance from the center of

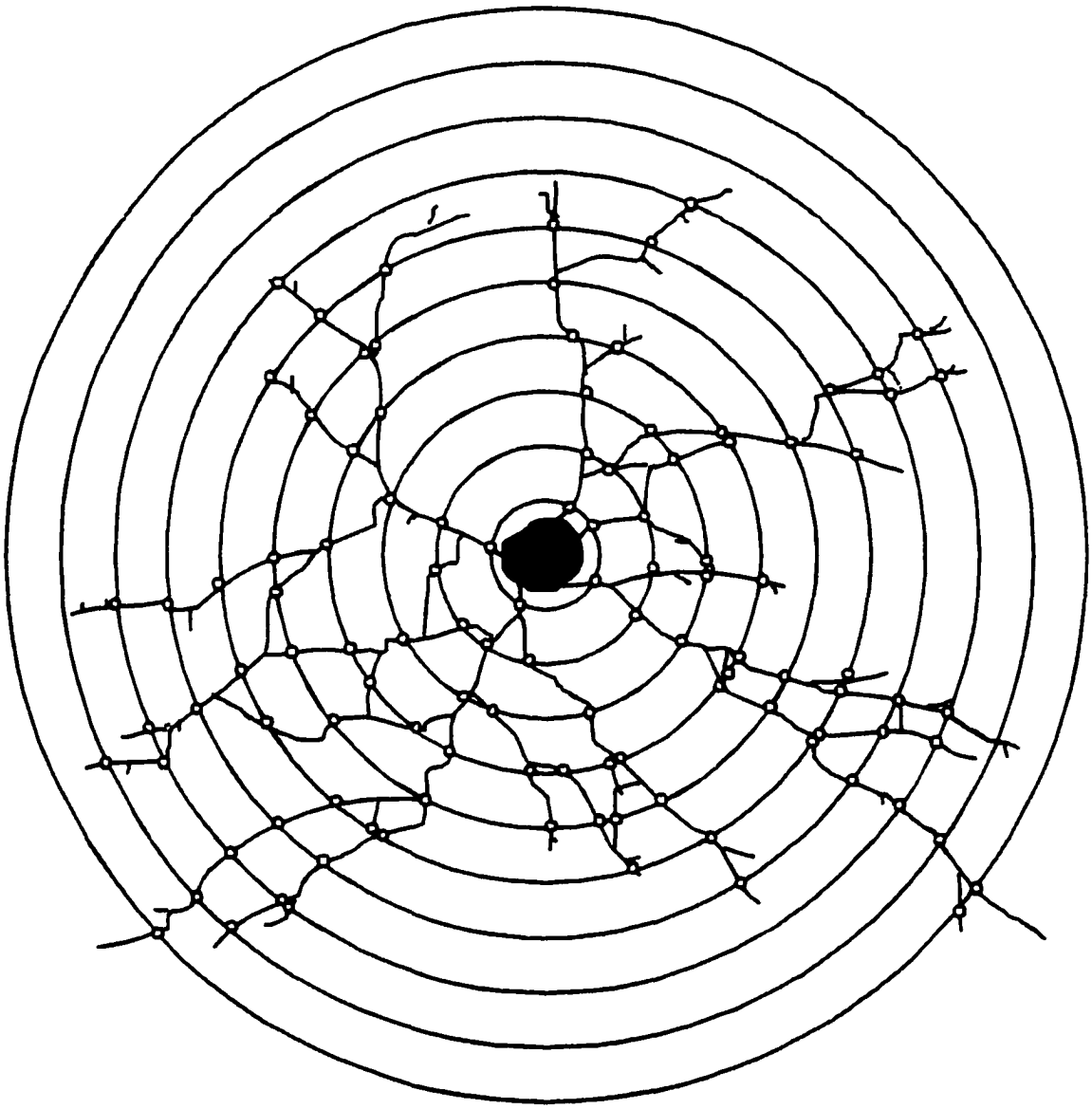
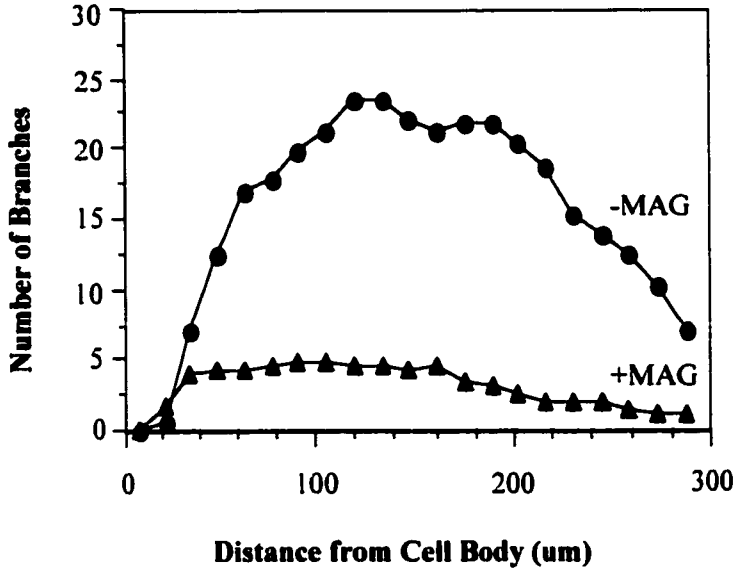


Figure 4.5 A method to quantitate the effect of MAG on neurite branching of DRG neurons. Each selected neuron was superimposed with a series of concentric circles. The smallest circle with the radius of 7 μm was centered over the neuronal soma. The subsequent circles increased in radius by 14 μm. The number of intersections of the neurite branches with each circle was determined. The average number of crossings was plotted against the radius of the concentric circles, which represented the distance from the center of the cell body.

a



b

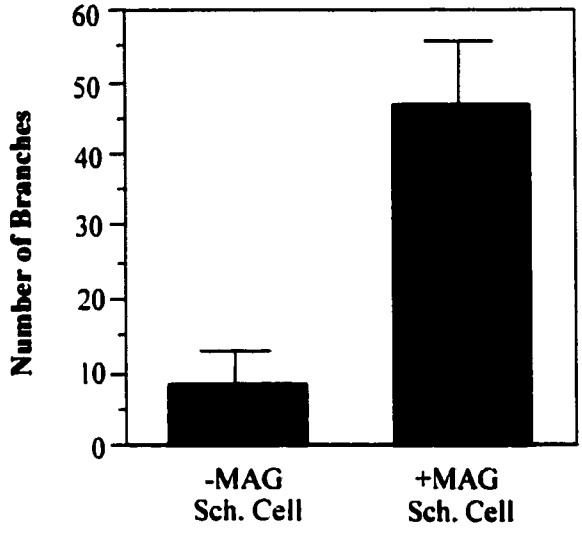


Figure 4.6 Quantitation of neurite branching from DRG neurons on MAG-expressing and control Schwann cells. Dissociated DRG neurons from PND 7 rats were cultured for 12 hr on confluent monolayers of control or MAG-expressing Schwann cells before fixed and immunostained for GAP43. A series of concentric circles was centered over the neuronal cell body and neurite branching was quantitated as described in Figure 4.5. (a) An overall profile of neurite branching from DRG neurons, either on MAG-expressing or on control Schwann cells, was generated by plotting the average number of branches crossed with each circle against the distance of this circle from the cell body. (b) The number of branches crossed with a circle of 133 μm away from the cell body was calculated and compared for MAG-expressing (+MAG Sch. cell) and control (-MAG Sch. cell) Schwann cells.

the cell body. Figure 4.6a shows an overall profile of neurite branching from DRG neurons grown on MAG-expressing as well as on control Schwann cells. The number of branches from DRG neurons grown on control Schwann cells increased significantly as neurites extended away from the cell bodies and reaches a peak of 20-25 branches per neuron at a distance of 100-200 μm from the cell body. In contrast, the number of branches extended from DRG neurons grown on MAG-expressing Schwann cells was at a consistently low level of 4-5 branches per neuron regardless of the distance from the cell body. The greatest difference in the number of neurite branches was observed at a distance of 133 μm from the cell body (Figure 4.6b), where the average number of branches extended from each neuron grown on control Schwann cells was about five times more than that on MAG-expressing cells. These results indicate that MAG can strongly inhibit neurite branching from DRG neurons supported by Schwann cells.

4.2.5 MAG expressed by Schwann cells inhibits neurite extension and branching from superior cervical ganglion (SCG) neurons

As we have shown that MAG expressed by Schwann cells can inhibit both neurite extension and branching of DRG neurons, we would like to determine if these effects also apply to other neuronal types. Another peripheral ganglion neuron, superior cervical ganglion (SCG) neuron, was tested (Figure 4.7). Previous studies showed that, for postnatal ages, neurite outgrowth from these neurons was inhibited by MAG expressed by CHO cells.

SCG neurons from PND 2 rats were isolated and plated on Schwann cell monolayers as described in the previous experiments.

First, the number of neurons with neurites longer than 3x the diameter of the cell bodies was counted. From Figure 4.7c, it can be seen that the percentage of SCG neurons extending neurites was reduced by 38% when grown on MAG-expressing Schwann cells compared to those on control Schwann cells not expressing MAG.

Second, we analyzed the extent of neurite branching. As describe above, a series of concentric circles were superimposed onto the image of the neuron, centered on the cell body (Figure 4.5), and the number of neurites crossing each circle was counted. The average number of branches per neuron at different distance from the cell body was plotted against the distance from the cell body. Figure 4.8a showed an overall profile of neurite branching from SCG neurons on MAG-expressing Schwann cells as well as on control Schwann cells. The number of branches from SCG neurons grown on control Schwann cells increased significantly as neurites grew away from the cell body and at a distance of 133 um, an average number of 17 branches were extended from each neuron on control Schwann cells. In contrast, SCG neurons grown on MAG-expressing Schwann cells had only 3-4 branches per neuron, regardless of the distance from the cell body. Comparing the number of neurite branches at the distance of 133um from the cell body (Figure 4.8b), we showed that branching on MAG-expressing cells was reduced by more than 70% compared to that grown on control Schwann cells.

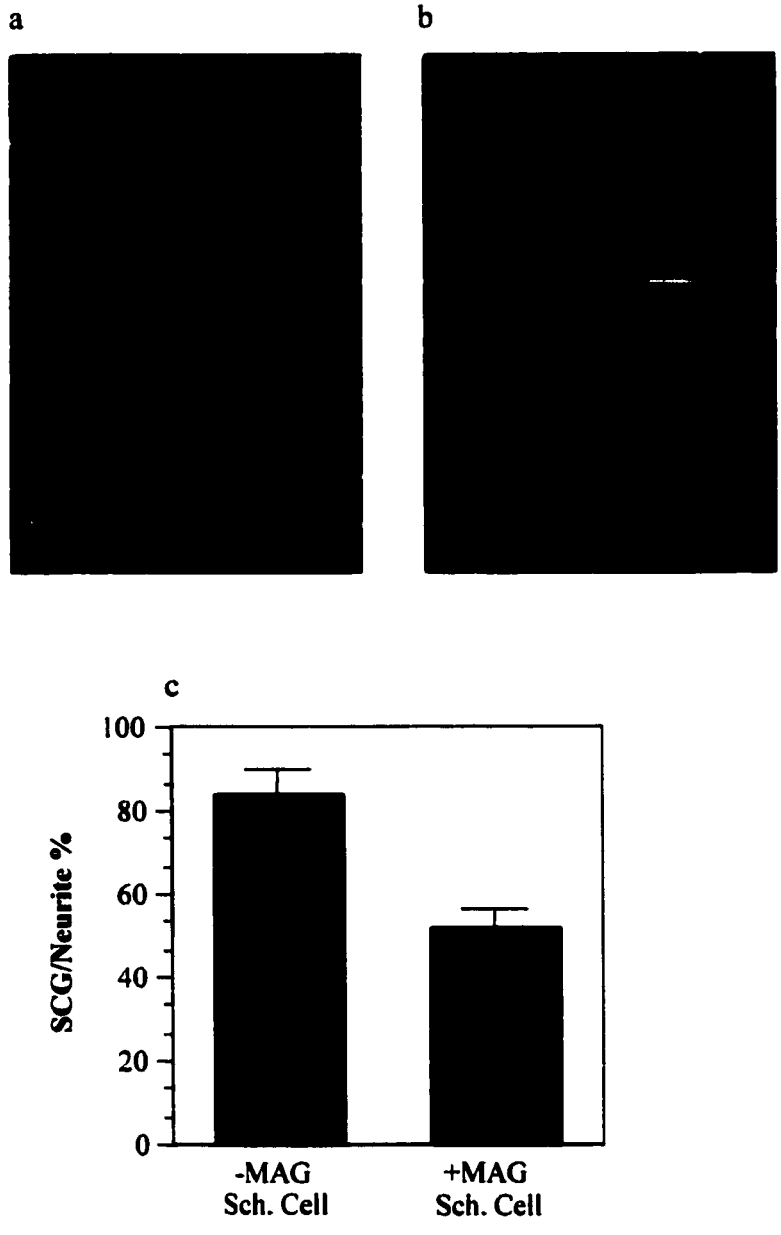
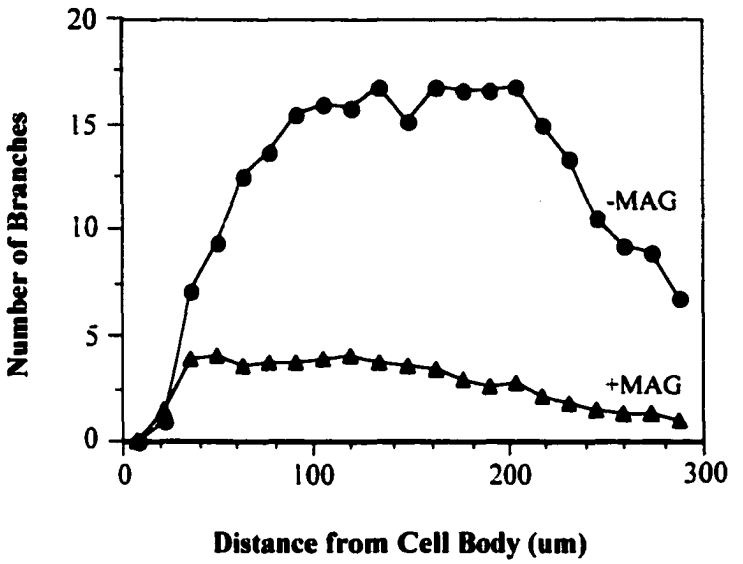


Figure 4.7 The effect of MAG expressed by Schwann cells on neurite outgrowth from SCG neurons. (a and b) Dissociated SCG neurons from PND 2 rats were cultured for 12 hr on confluent monolayers of control (a) or MAG-expressing (b) Schwann cells before fixed and immunostained for GAP43. (c) The number of neurons with neurites longer than three times their cell body was counted for about 200 neurons (± SEM). Results show the percentage of neurons with neurites of that length.

a



b

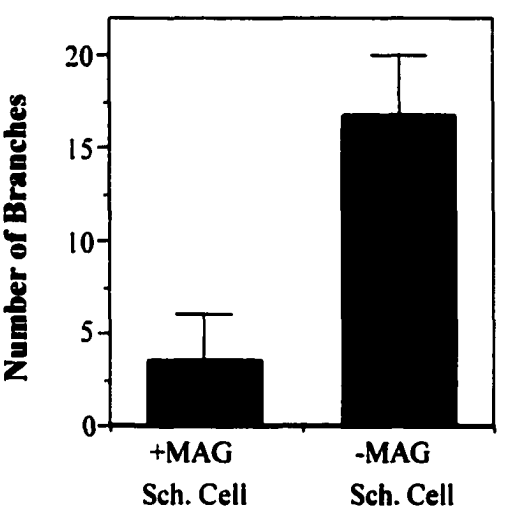


Figure 4.8 Quantitation of neurite branching from SCG neurons on MAG-expressing and control Schwann cells. Dissociated SCG neurons from PND 2 rats were cultured for 12 hr on confluent monolayers of control or MAG-expressing Schwann cells before fixed and immunostained for GAP43. Neurite branching was quantitated as described in Figure 4.5. (a) An overall profile of neurite branching from SCG neurons, either on MAG-expressing or on control Schwann cells, was generated by plotting the average number of branches crossed with each circle against the distance of this circle from the cell body. (b) The number of branches crossed with a circle of 133 μm away from the cell body was calculated and compared for MAG-expressing (+MAG Sch. cell) and control (-MAG Sch. cell) Schwann cells.

Taken together, these results show that MAG can inhibit both Schwann cell-supported neurite extension and branching from SCG neurons.

4.3 Conclusion and Discussion

MAG is normally expressed by oligodendrocytes of the CNS and Schwann cells of the PNS. Although we have shown in the previous chapter that MAG expressed by CHO cells strongly inhibits neurite outgrowth, it is necessary to examine this inhibitory effect when MAG is expressed by Schwann cells, one of the cells that express MAG in vivo. We show here that cultured Schwann cells are an excellent substrate for neurite outgrowth. The results that MAG expression by these Schwann cells significantly changes their permissiveness as a neurite outgrowth substrate confirms our previous results with CHO cells and suggests that MAG is a potent inhibitor of axonal outgrowth.

More than one individually cloned MAG-expressing Schwann cell line was used in these studies. Another LMAG-expressing Schwann cell line (cloned in our lab) was generated by transfection. In this cell line, LMAG cDNA was under the control of the mouse metallothionein promoter so that MAG is expressed at a lower level compared with that of the LMAG-expressing Schwann cell line generated by infection. Neurite outgrowth assays with cerebellar neurons were carried out with this cell line and similar results were obtained for these two cell lines (see the next chapter). In addition, an SMAG-expressing Schwann cell line (also provided by Dr. James L. Salzer) was used in parallel with LMAG-expressing Schwann cells for both neurite extension and neurite branching assays for cerebellar, DRG and SCG neurons. The same results were obtained from LMAG and SMAG-expressing Schwann cells. Hence, the inhibitory effects of MAG observed in these studies are unlikely to

be an artifact of any particular cell line. The fact that LMAG and SMAG exert the same inhibitory effect on neurite outgrowth and neurite branching is consistent with our previous observation that the two isoforms of MAG, when expressed on CHO cells, inhibit neurite outgrowth to a similar extent (Mukhopadhyay and Filbin, unpublished observation).

Schwann cells in the regenerating peripheral nerve are highly permissive for axonal growth (Fawcett and Keynes, 1990; Scherer and Salzer, 1996). During regeneration, Schwann cells de-differentiate and down-regulate the expression of myelin-related proteins, including MAG. At the same time, growth-promoting molecules such as L1 and N-CAM are up-regulated (Martini and Schachner, 1988). A key demonstration in this study is that the expression of MAG greatly reduces Schwann cell permissiveness, highlighting the requirement for MAG removal after injury, before PNS regeneraion can occur. On the other hand, these results also suggest that when expressed by the myelin-forming Schwann cells *in vivo*, MAG could contribute to inhibitory properties of PNS myelin. Supporting this suggestion is the report that the mutant mouse, C57BL/Wld^s, removes myelin only very slowly after injury and concomitantly exhibits slow PNS regeneration (Brown et al., 1991a, 1992). In contrast, in the double mutant C57BL/Wld^s and MAG-/- mice, in which MAG-free myelin persists after injury, there is much more robust axonal regeneration. The number of myelinated nerve tracts associated with regrowing axons is double in the MAG-deficient /C57BL/Wld^s mice compared to that in MAG-expressing C57BL/Wld^s mice (Schafer et al., 1996). Taken together, these results suggest that MAG can act as an inhibitory molecule in

PNS myelin that has to be removed from Schwann cells during Wallerian degeneration to facilitate axonal regrowth in lesioned peripheral nerves.

The observation that MAG dramatically reduces neurite branching in addition to inhibiting neurite length, may indicate a physiological role for MAG or other myelin-specific inhibitory molecules. It is reported that in the CNS of embryonic or neonatal animals, after partial lesion, uninjured fibers can sprout and expand their connections. However, in adult animals, collateral sprouting is scarce and is limited to lightly myelinated areas such as the olfactory system, the hippocampus, the septum, the molecular layer of the cerebellum, and substantia gelatinosa (Schwegler et al., 1995). It is also reported that in the CNS, if myelination is prevented or greatly reduced by killing dividing oligodendrocytes through spinal cord irradiation at birth, sprouting of uninjured fibers increase dramatically and the total fiber number increased by 34% (Colello and Schwab, 1994; Schwegler et al., 1995). An interesting coincidence is that the onset of myelination is well correlated with the end of long distance fiber growth (Caroni and Schwab, 1989) and the growth-permissive environment present during early developmental stages becomes an inhibitory one at the onset of myelination (Kapfhammer and Schwab, 1994). Therefore, it is suggested that myelin and its associated neurite growth inhibitors play an important role in preventing sprouting and stabilizing neuronal connections (Schwegler et al., 1995). MAG, expressed by both CNS and PNS myelin at periaxonal membrane in direct interaction with axons, is likely to contribute to this function. Here, based on our observations, we suggest that in normal animals, MAG may

also be responsible for restricting axonal sprouting to stabilize the fiber number and to protect the existing nerve circuits.

In summary, we have shown that MAG not only reverses the permissiveness of growth-promoting Schwann cells but also inhibits their ability to promote neurite branching. It is very likely that MAG in vivo carries out a similar function and contributes to the control of axonal sprouting. Since it is also suggested to play roles in maintaining axon:myelin stability, MAG is likely to be a multifunctional molecule in stabilizing nerve connections in normal animals.

Chapter V

Mapping the Site on MAG for Sialic Acic Binding to Neurons

5.1 Introduction

We have shown that MAG is a potent inhibitor of neurite regeneration and neurite branching and also, depending on the age and type of neuron, can promote neurite growth. In addition, it was recently reported that MAG influences axon stability in the intact, mature nervous system via neurofilament phosphorylation (Fruttiger et al., 1995; Yin et al., 1998). In MAG-/- mice, a marked shrinkage in axonal diameter companied by greatly reduced neurofilament phosohorylation was observed by the age of 3 months (Yin et al., 1998) and by the age of 8 months, axon-myelin unit was disrupted in the PNS, resulting in both axon and myelin degeneration (Fruttiger et al., 1995). These observations imply that MAG could interact with a neuronal component and by transducing signals to neurons, affects neurite growth and axon stability. To date, the identity of the putative neuronal receptor(s) for MAG in these events remains elusive and whether the molecule is the same for all these situations remains to be resolved.

The recent demonstration that MAG is a sialic acid-binding protein and consequently belongs to a subgroup of Ig superfamily, termed siglecs, may shed some light on the possible identity of the neuronal component with which MAG interacts (Kelm et al., 1994). Siglecs are a homologous group of cell surface glycoproteins that recognize distinct sialylated glycans (Kelm et al., 1994, 1998). Sialic acid is found on the cell surface, mostly as terminal components of glycoconjugates and is suggested to play important roles in cellular interaction (Schauer, 1985; Varki, 1997; Kelm and Schauer, 1997). We have shown that, regardless of

whether their neurite outgrowth is promoted of inhibited, MAG binds to neurons in a sialic acid-dependent manner (Kelm et al., 1994; DeBellard et al., 1996). If neurons are desialated, the binding of MAG is abolished (Kelm et al., 1994). In addition, the binding of MAG to neurons is trypsin-sensitive, therefore, it is concluded that a neuronal sialoglycoprotein, rather than a sialoglycolipid is involved in this binding (DeBellard et al., 1996). More importantly, if neurons are desialylated before the neurite outgrowth assay, inhibition by soluble MAG is completely lost. However, inhibition of neurite outgrowth by MAG expressed by CHO cells is only partially reversed. These results strongly suggest that a neuronal sialoglycoprotein mediates, directly or indirectly, the effect of MAG on neurite outgrowth. In this study, we would like to identify the sialic acid binding site on MAG and to assess its role in MAG's effect on neurite outgrowth.

The first clue to the identity of the sialic acid binding site on MAG was from the studies on two other siglec family members, CD22 and sialoadhesin. Through site-directed mutagenesis, residues involved in sialic acid binding of these two molecules were mapped to the GFCC'C" face of the N-terminal domain, the V-like domain, centered at arginine 97 in sialoadhesin and at arginine 130 in CD22 (Vinson et al., 1996; van der Merwe et al., 1996; Figure 5.1). All members of the siglec family share high sequence similarities in this area, and this arginine residue is conserved (Vinson et al., 1996). Therefore, this area is speculated to represent the binding site also in the other members of the siglecs. By aligning the sequence of the first Ig-like domains of MAG to sialoadhesin and CD22, it was noted that this arginine corresponds to arginine 118 of MAG (Kelm et al., 1994).

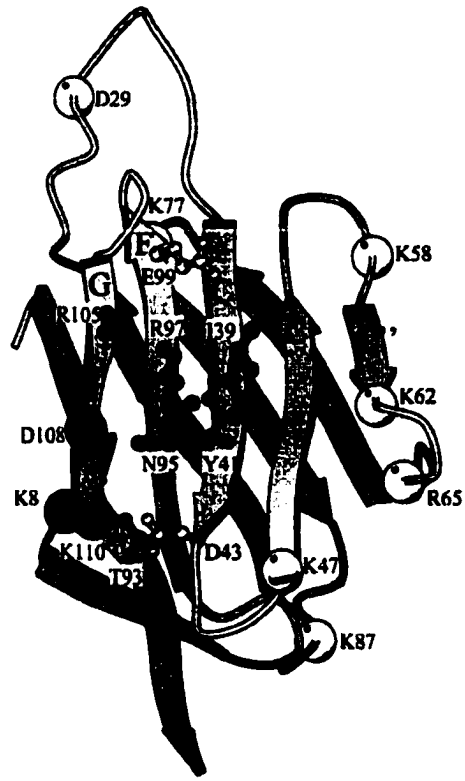


Figure 5.1 Ribbon diagram of the CD8α-based model of the sialoadhesin V-like domain. Residues that abolished sialic acid binding when mutated are shown in black. Those that had no effect are shown in white. Residues that occur in region of the model where sequence alignment is uncertain are shown only as C-α spheres, whereas those that occur in regions of greater certainty are represented with side chains. According to this model, residues involved in sialic acid binding of sialoadhesin were mapped to the GFCC'C" face of the N-terminal domain, the V-like domain, centered at arginine 97 (Vinson et al., 1996).

To identify the sialic acid binding site on MAG, arginine 118 was chosen as a candidate for site-directed mutagenesis. It was mutated either to an alanine (R118A) or to an aspartate (R118D), changing the positive charge to neutral or negative, respectively. The mutated

forms of MAG were expressed both by transfected Schwann cells and in soluble Fc-forms. Changes in sialic acid-dependent binding to neurons were tested. After characterizing the expression of wild-type and mutated MAG in Schwann cells, neurite outgrowth was conducted to see whether the neurite growth inhibitory effect of MAG was altered by mutation of arginine 118.

5.2 Results

5.2.1 The putative sialic acid binding site on MAG maps to R118 of the first Ig-like domain

When the first Ig-like domain of MAG, sialoadhesin and CD22 are aligned, an arginine corresponding to arginine 97 in sialoadhesin, arginine 130 in CD22 and arginine 118 in MAG appear to be conserved in these three proteins (Figure. 5.2).

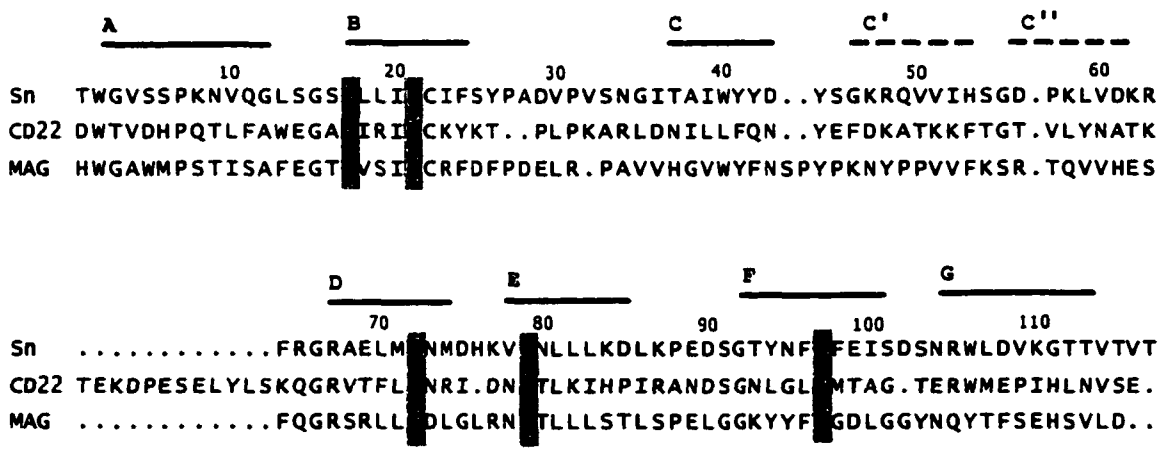


Figure 5.2 Alignment of the first Ig domain of MAG, sialoadhesin (Sn), and CD22. Predicted b-strands are labeled as A, B, C, C', C", D, E, and F. Amino acids involved in sialic acid binding of sialoadhesin and conserved in MAG are highlighted. The arrow refers to arginine 118 in MAG.

To determine if Arg118 in MAG is involved in sialic acid-dependent binding to neurons, site-derected mutagenesis was carried out and the nucleotides coding for this arginine were mutated to nucleotides coding for either alanine (R118A), a nondisruptive substitution, or aspartate (R118D), a disruptive substitution. The mutations were created both as MAG-Fc molecules, and as full-length L-MAG, which was then expressed in Schwann cells.

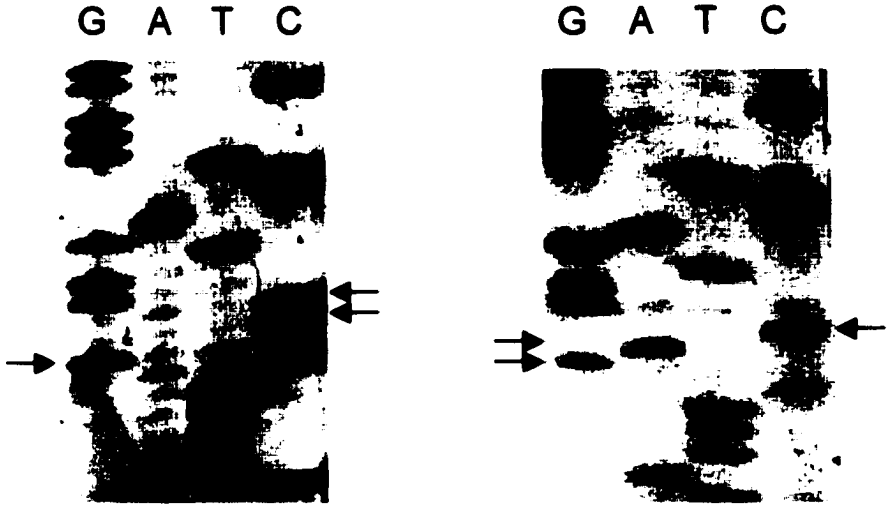


Figure 5.3 Dideoxy DNA sequencing to confirm the site-directed mutagenesis. Autoradiogram of sequencing gel shows mutation of amino acid position 118 in MAG from nucleotide coding for arginine "CGA" to nucleotide coding for (a) alanine "GCC" and (b) aspartic acid "GAC".

The mutations were confirmed by DNA sequencing. As shown in Fig. 5.2, after mutation, the wild type sequence coding for arginine, CGA, is changed to GCC that codes for alanine (Figure. 5.3a) in R118A cDNA, and to GAC that codes for aspartate (Figure. 5.3b) in R118D sequence.

5.2.2 Mutated MAGs maintain the native conformation

Before carrying out the neuron binding assay and neurite out growth assay, we first assessed whether the mutations had changed the native conformation of MAG. The ability of a conformation-dependent MAG monoclonal antibody (mAb513) to recognize R118A- and R118D-MAG-Fc was determined in an ELISA assay. Monoclonal antibody mAb513 recognizes the first three extracellular domains of MAG if they are folded in the native conformation (Poltorak et al., 1987). Moreover, we found that the recognition site of mAb513 on MAG must be close to, or overlap with, the sialic acid binding site of MAG, since the antibody was able to block the sialic acid-dependent binding of MAG to neurons (Tang et al., 1997b). As shown in Figure 5.4, when wild-type MAG-Fc, R118A- and R118D-MAG-Fc were immobilized on an ELISA plate, mAb513 bound to both the wild-type and mutated forms of MAG-Fc in a dose-dependent manner. More importantly, the characteristics of binding of antibody to the two mutated MAG-Fcs are indistinguishable from that of the wild type. This indicates that the affinity of the antibody is not changed by

these mutations and that the native conformation of MAG is most likely maintained in R118A- and R118D-MAG-Fc.

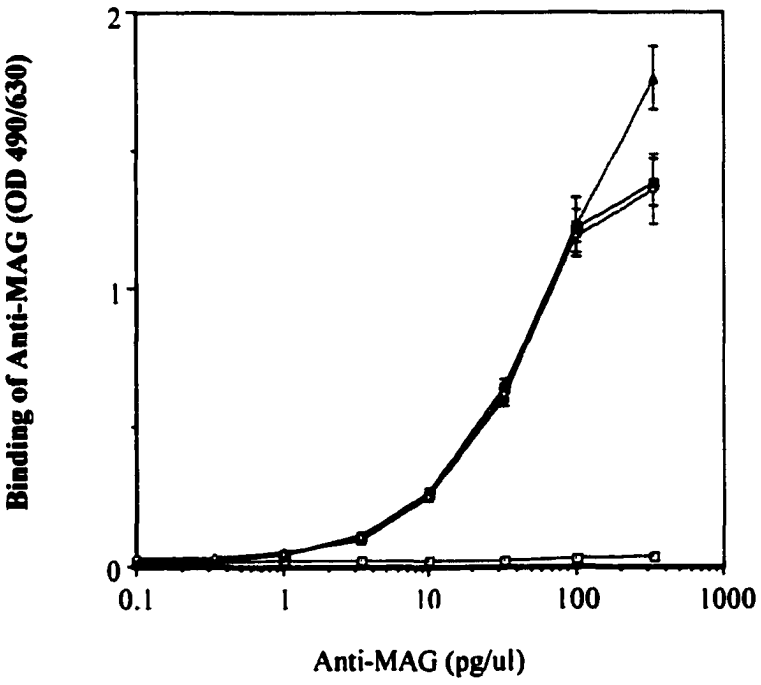


Figure 5.4 Binding of conformation-dependent MAG 513 monoclonal antibody to mutated MAG-Fc. The Fc chimeras were immobilized at a concentration of 1μg/ml on an ELISA plate already coated with anti-Fc antibody. MAG 513 monoclonal antibody was added at various concentrations and bound antibody was detected by ELISA. MAG-Fc (closed triangles), R118A-MAG-Fc (open triangles), R118D-MAG-Fc (closed squares), or MUC-Fc (open squares). Results represent three experiments (±SEM).

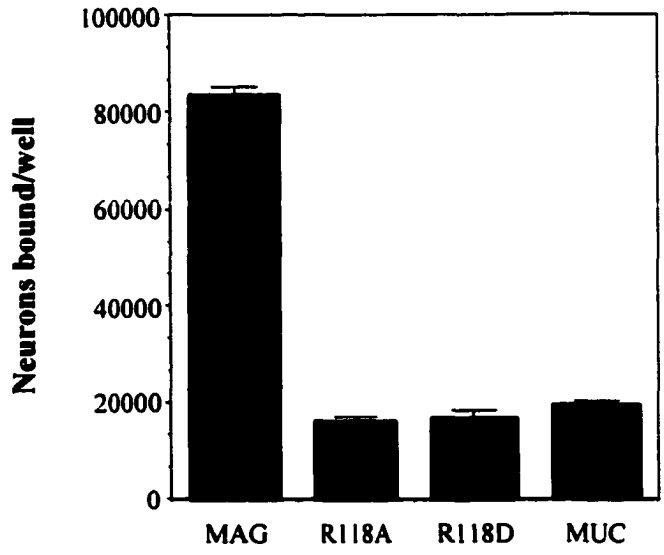


Figure 5.5 Binding of R118-mutated MAG to cerebellar neurons. Dissociated PND4 cerebellar neurons were allowed to bind to wild-type MAG-Fc (MAG), R118A-MAG-Fc (R118A), R118D-MAG-Fc (R118D) or MUC-Fc (MUC) immobilized at a concentration of 10 μg/ml, on a 96-well dish. 200,000 neurons added to each well and after incubation and washing, the number of cells bound was quatitated. Results are from three experiments, each with 10 samples and present the mean (±SEM)

5.2.3 Mutation of R118 abolishes sialic acid-dependent binding of MAG

To determine if MAG is involved in the sialic acid-dependent binding of MAG to neurons, a solid-phase binding assay was performed with wild type and mutated forms of MAG-Fc. For this assay, the Fc-chimeras were immobilized on a 96-well ELISA plate. Another five-

domain Ig family member, MUC18, fused to Fc, was used as a control chimera. A single-cell suspension of cerebellar neurons was incubated in these Fc-chimera-coated wells. It can be seen in Figure 5.5 that, consistent with the previous results (Tang et al., 1997b), when wild type MAG-Fc was coated at a concentration of 10 μg/ml, a significant number of neurons bound. In wells coated with the control chimera, MUC-Fc, only background binding was seen. In contrast, at the same concentration, neither R118A- nor R118D-MAG-Fc bound specifically to neurons. The number of neurons bound in these wells was the same as the background binding to the control MUC-Fc's wells. Based on recognition by the conformation-dependent MAG antibody, both mutated forms of MAG-Fcs retain the native conformation of MAG. Thus, the loss of neuronal binding by either a non-disruptive or a disruptive substitution at R118 indicates that this residue is critical for sialic acid-dependent binding of MAG to neurons.

5.2.4 Characterization of mutated MAG expression by Schwann cells

To test the effect of mutation at R118 in MAG on its inhibition of neurite outgrowth, mutated forms of MAG were introduced into CHO cells and Schwann cells. Neurite outgrowth assays were carried out and the inhibitory activity of mutated MAG was compared to that of wile type MAG. Studies with CHO cells were carried out by other researchers in our lab. In this study, we assessed the effect of this mutation by expressing the mutated MAG on Schwann cell surface. For this purpose, cDNAs encoding for the full-length wild

type or mutated MAG were transfected into Schwann cells via liposome-mediated transfection.

Following transfection, cells were grown in media containing the selective reagent G418 for about 2 weeks. Resistant colonies were pooled, live cells were immunofluorescently stained using mAb513 and then sorted by FACS for cells expressing relatively high levels of MAG. These cells were expanded and the expression of MAG was assessed by Western blotting and again by immunofluorescent staining of intact cells.

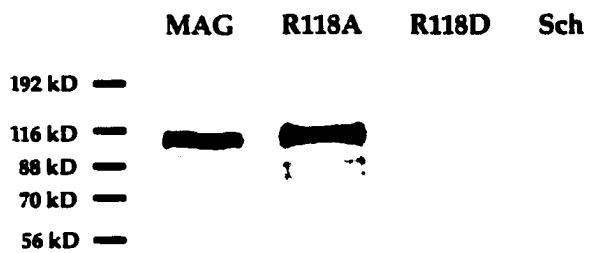


Figure 5.6 Western blotting of mutated forms of MAG expressed by transfected Schwann cells. Lysates of wild-type MAG-transfected Schwann cells (MAG), R118A-MAG-transfected Schwann cells (R118A), R118D-MAG-transfected Schwann cells (R118D), and control transfected Schwann cells (Sch) were separated by SDS-PAGE (8%) and immunostained for MAG. Each lane was loaded with 20 μg of protein. Molecular weight standards are shown on the left, from top to bottom, 198, 118, 88, 70, and 56 kD.

Figure 5.6 shows immunostaining of a Western blot of lysates of Schwann cells expressing wild type or mutated MAG. The amount of R118A-MAG and wild-type MAG expressed by Schwann cells are approximately equivalent. R118A-MAG and wild-type MAG expressed by Schwann cells have a molecular weight of about 100kD, similar to that of MAG expressed in CHO cells and in sciatic nerves. Therefore, they are most likely glycosylated to the same extent as MAG expressed in vivo.

Surprisingly, no MAG expression was detected in the lysate of R118D-MAG transfected Schwann cells after expansion. A small portion of transfected cells expressing a high level of R118D was initially obtained in the first FACS sorting. However, during the expansion, Western blots showed that expression of MAG in this cell population had decreased dramatically. For this reason, a second FACS sorting was carried out. Again, there were only a small number of Schwann cells expressing a high level of R118D-MAG. However, when the double FACS-sorted cells were expanded, Western blotting and immunofluorescence staining fail to detect any expression of MAG. These results suggested that either Schwann cells expressing a high level of R118-MAG stopped producing this mutated MAG after several passages, or they stopped proliferating. The former possibility is more likely correct as there was no evidence of dying cells in the cultures. The reason for the loss of this cell population is not clear.

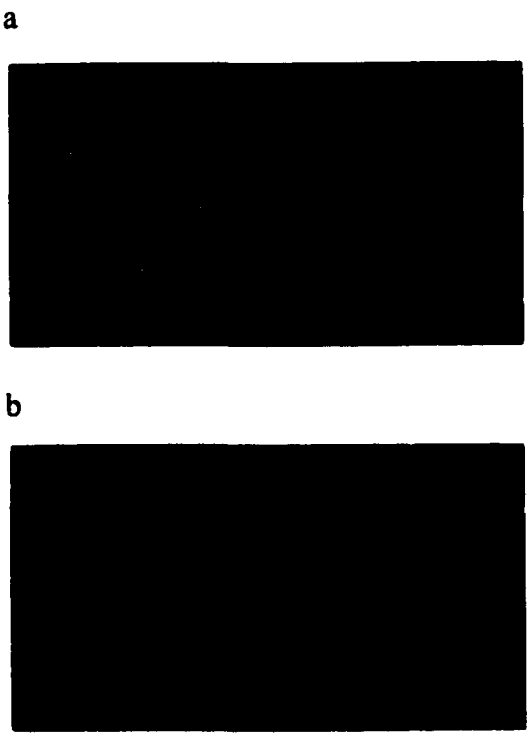


Figure 5.7 Detection of MAG on the surface of transfected Schwann cells by immunofluorescent staining. Live Schwann cells expressing wild-type MAG (a) or R118A-mutated MAG (b) were incubated with MAG 513 monoclonal antibody (5 μg/ml), then fixed with 4% paraformaldehyde and incubated with phycoprobe-conjugated, goat anti-mouse IgG (1:50).

To determine whether R118A-MAG reaches the surface of transfected Schwann cells, live cells were immunostained with mAb513. As shown in Figure 5.7, the Schwann cells expessing R118A-MAG and wild-type MAG were immunofluorescently stained and the staining was evenly distributed on the cell surface. As expected, no MAG was detected on the surface of R118D-transfected Schwann cells (data not shown). Hence, these results

indicate that both the wild type and R118A-MAG expressed by Schwann cells have reached the cell surface. Furthermore, immunostaining using mAb513 also confirms that R118A-MAG has maintained the native conformation of MAG when expressed in Schwann cells.

5.2.5 Mutated MAG expressed by Schwann cells retains its ability to inhibit neurite outgrowth

To test the effect of mutated MAG expressed by Schwann cells on neurite outgrowth, isolated cerebellar neurons were grown overnight on monolayers of wild-type or R118A-MAG expressing Schwann cells. Neurite length extended on these cells was compared. Untransfected Schwann cells, which do not express MAG, were also included as a negative control of this neurite outgrowth assay.

It is shown in Figure 5.8 that when grown on wild type MAG-expressing Schwann cells, neurite outgrowth of cerebellar neurons was inhibited by about 40%, which is similar to the infected MAG-expressing Schwann cells (see the previous chapter). Surprisingly, a similar inhibition of neurite outgrowth was also observed when neurons were grown on Schwann cells expressing R118A-MAG. This result suggests that although mutation at R118 abolishes the sialic acid-dependent binding of MAG to neurons, the ability of MAG to inhibit neurite outgrowth is not altered when the mutated MAG is expressed in Schwann cells.

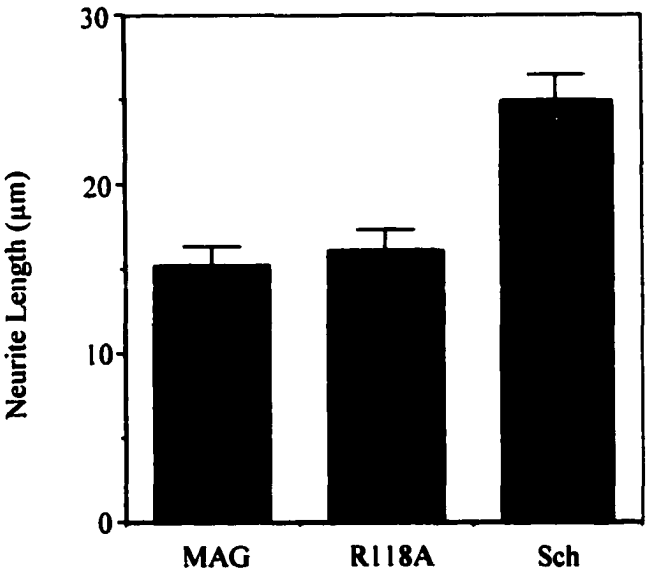


Figure 5.8 The effect of R118A mutated-MAG expressed by Schwann cells on neurite outgrowth from cerebellar neurons. Dissociated cerebellar neurons were cultured for 18 hr on confluent monolayers of wild-type MAG-expressing (MAG), R118A-MAG-expressing (R118A), or control Schwann cells not expressing MAG (Sch). Co-cultures were fixed and immunostained for GAP-43, and neurite length was measured. Results show the mean length of the longest neurite per cell (±SEM) for 100-200 individual neurons.

5.3 Conclusion and discussion

Through site-directed mutagenesis, an arginine at amino acid 118 in the first Ig-like domain of MAG was changed either to an alanine, a non-disruptive substitution, or to an aspartate, a disruptive substitution. This mutation does not appear to have lead to a loss of native conformation, since the conformation-dependent MAG monoclonal antibody, mAb513, binds with the same affinity to wild type and the two mutated MAG in their soluble Fc-chimeric forms. In addition, the same antibody also recognizes both wild type and R118A-MAG expressed by Schwann cells. The sialic acid-dependent binding of MAG-Fc to neurons were completely abolished not only by the disruptive mutation but also by the non-diruptive mutation, suggesting that R118 in MAG may be involved directly in sialic acid binding.

R118 in MAG was chosen as the candidate for site-directed mutagenesis because mutation of this conserved arginine in two other members of the family, sialoadhesin (R97) and CD22 (R130) completely abolished the sialic acid binding (Vinson et al., 1996; van der Merwe et al., 1996). The sialic acid binding site was previously mapped to the first N-terminal Ig-like domains, the V-like domains, of these two molecules using a series of domain deletion constructs (Nath et al., 1995). To identify which specific amino acids in this domain is responsible for this sialic acid binding, site-directed mutagenesis was carried out (Vinson et al., 1996; van der Merwe et al., 1996). For sialoadnesin, a total of 6 amino acid residues were found to be involved in the sialic acid binding (Vinson et al., 1996). Drastic changes (changing acidic amino acids to basic amino acids or visa versa) of any one of these six

residues abolished sialic acid-dependent binding to erythrocytes. These mutations should not have changed protein conformation, because the binding affinity of three monoclonal antibodies directed to two distinct epitopes of sialoadhesin was not altered. When superimposed onto a CD8α (whose structure is known)-based model of the V-like domain of sialoadhesin, these residues were seen to constitute a contiguous binding site clustered around the arginine 97 on the F-strand, forming a sialic acid binding pocket. When these residues were substituted with alanine, only mutation at arginine 97 disrupted the binding. A more conservative mutation of arginine 97 to lysine, which caused only slight changes in the side chain length, also completely abolished sialic acid-dependent binding. Thus, arginine 97 was suggested to be involved directly sialic acid recognition (Vinson et al., 1996). This arginine is conserved among members of siglec family. In CD22, it corresponds to arginine 130. Similarly, substitution of arginine 130 in CD22 to either glutamic or alanine completely abolished the sialic acid-dependent binding (van der Merwe et al., 1996). Therefore, it is suggested that this arginine is likely to be the key residue in mediating sialic acid-dependent binding of siglecs to cells.

It is noted that R118 in MAG is the first residue in the Arg-Gly-Asp (RGD) motif, which is known to be recognized by integrins (Ruosahti and Pierschbacher, 1987). However, previous studies suggested that this recognition is unlikely to be involved in the interaction between MAG and neurons (Pedraza et al., 1990; Sadoul et al., 1990), since an RGD-containing peptide did not interrupt binding of MAG-containing liposomes to neurons. In addition, antibodies against this sequence failed to recognize native MAG but bound only to denatured

MAG. This implies that the RGD sequence is not exposed at the surface of the molecule and consequently not accessible for interaction. The alignment with the CD8 structure supports this postulation and suggests that this conserved arginine is folded in the interior of the first Ig-domain. As the other two amino acids (Gly119 and Asp120) are not conserved within the sialoadhesin family, it is probable that only R118 in the RGD sequence is involved in sialic acid binding and mediating the binding of MAG to neurons.

It was shown previously that soluble MAG-Fc is dependent on sialic acid binding to inhibit neurite outgrowth (Tang et al., 1997b). In the current study, we show that mutation at R118 abolishes the sialic acid-dependent binding of MAG-Fc to neurons. We also found in neurite outgrowth assays that mutation of R118 completely abolished the ability of MAG-Fc to inhibit neurite outgrowth (Tang et al., 1997b). It was shown by others in our lab that when full-length MAG is expressed on the surface of CHO or Schwann cells, its ability to mediate sialic acid binding is also abolished by mutation of R118 (Tang et al., 1997b). However, we found in this study that R118-mutated MAG expressed by Schwann cells still inhibits neurite outgrowth to the same extent as wild-type MAG, even though it has lost its sialic acid binding capabilities. Similarly, the inhibitory effect of MAG expressed by CHO cells was not altered by mutation of R118 (Tang et al., 1997b). This is consistent with our previous finding that removal of all sialic acid residues from neurons before the neurite outgrowth assay, completely blocked the inhibition by MAG-Fc (Tang et al., 1997b), but not the inhibition by MAG expressed on the surface of CHO cells (DeBellard et al., 1996). In addition, sialoadhesin, expressed either by CHO cells or as a soluble Fc-chimeric form, does

not inhibit neurite outgrowth even though it binds to neurons in a sialic acid-dependent manner and recognizes the same sialic acid linkage as MAG (Tang et al., 1997b). Furthermore, a truncated form of MAG, MAG (d1-3)-Fc (which contains only the first three Ig-like domains of MAG), is able to mediate the same sialic acid-dependent binding to neurons (Kelm et al., 1994), but unlike full length MAG-Fc, is not able to inhibit neurite outgrowth. This suggests that although they contain the sialic acid binding site, the first three Ig-like domains are not sufficient for neurite outgrowth inhibition, and domains 4 and 5 are also required for this function. Together, these results indicate that sialic acid binding to neurons is, in itself, insufficient to induce inhibition. We suggest that another site on MAG, most probably involving domains 4 or 5, is required for neurite outgrowth inhibition.

The idea of two distinct, neuronal recognition sites on MAG is postulated (Fig. 5.8). arginine 118 binds to a neuronal sialoglycoprotein, and another site is the neurite inhibition effecting site. The binding to the sialoglycoprotein may be required to intitiate and/or to stabilize the interaction of the inhibition site to neurons. Mutation in the first site on MAG-Fc abolished the sialic acid-dependent binding to neurons, therefore the interaction of the second, neurite growth inhibition site to neurons can not be initiated or maintained. However, when MAG is expressed on the cell surface, loss of sialic acid-dependent binding may be compensated for by interaction of another adhesion molecule (s) on these cells. This adhesive interaction allows the neurite growth inhibition site on MAG to interact with neurons, so that neurite outgrowth is still inhibited even though the sialic acid-dependent binding is abolished.

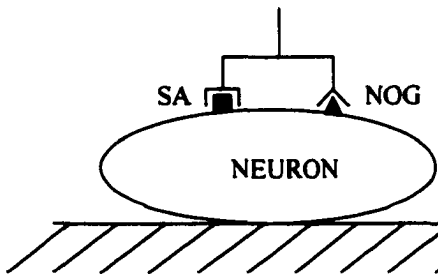
A two-site model has also been proposed for the interaction of P-selectin with its ligand PSGL-1 (Pouyani and Seed, 1995; Sako et al., 1995). P-selectin interacts with sialylated, fucosylated O-linked glycans attached to a variety of ligands (Varki, 1994). For interaction with PSGL-1 ligand, however, P-selectin also requires a sulfated tyrosine for high affinity binding. Consequently, two binding sites are proposed for the interaction of P-selestin with PSGL-1, one is carbonhydrate-dependent and one is amino acid dependent (Pouyani and Seed, 1995; Sako et al., 1995).

In conclusion, we have shown that arginine 118 is critical for sialic acid-dependent binding of MAG to neurons, and that sialic acid binding alone is insufficient to induce inhibition of neurite outgrowth. Therefore, we propose a model that MAG interacts with neurons via two sites, a sialic acid binding site and a neurite growth inhibiting site, which, by interacting with receptors on neurons, transduces signals to block neurite outgrowth.

Figure 5.9 Schematic diagram illustrating the model for the inhibitory effect of MAG on neurite outgrowth. There are two recognition sites on MAG, a sialic acid binding site (SA; square symbol) and a neurite outgrowth inhibition site (NOG; triangle symbol). When MAG is expressed by Schwann cells or by CHO cells, both sites engage the neuron and neurite outgrowth is inhibited (A). When MAG mutated at its sialic acid binding site is expressed by cells, another cell adhesion molecule (CAM) on the cell surface engages the neuron along with the neurite outgrowth inhibition site and neurite outgrowth is still inhibited (B). When soluble MAG-Fc is added to neurons, both the sialic acid binding site and the neurite outgrowth inhibition site engage the neuron and neurite outgrowth is inhibited (C). However, when MAG-Fc mutated at the sialic acid binding site is added to neurons, it can not bind to neurons and consequently the inhibition site can not engage and there is no inhibition of neurite outgrowth (D). In contrast, when MAG (d1-3)-Fc is added to neurons it binds via its sialic acid binding site but does not inhibit axonal growth because the inhibition site is absent.

A)

SOLUBLE MAG-Fc

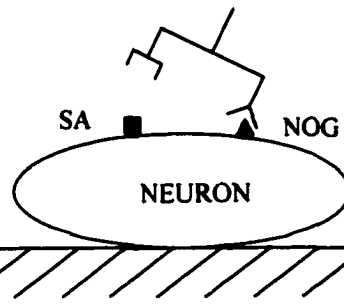


INHIBITION

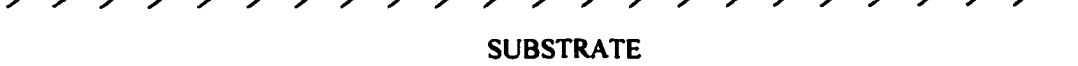
BUT NOT

B)

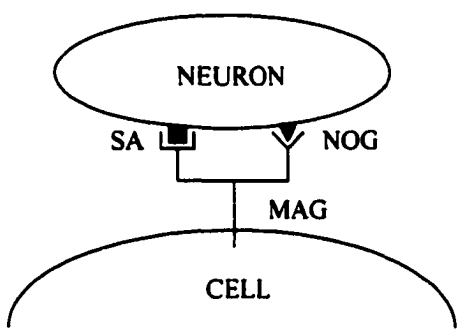
MUTATED MAG-Fc



NO INHIBITION

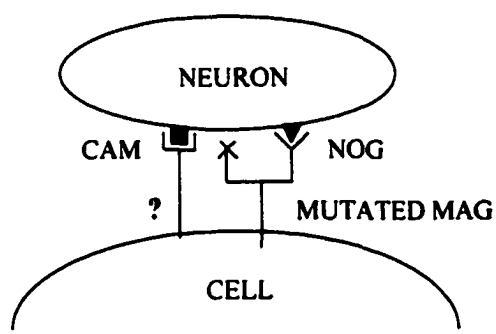


C)



INHIBITION

D)

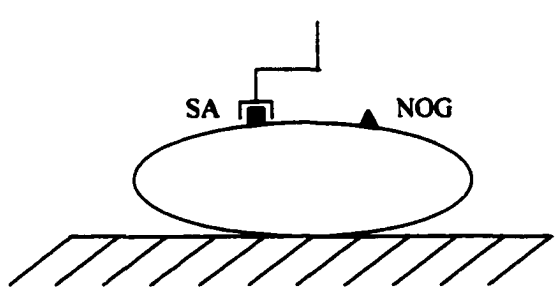


INHIBITION

NOR

E)

SOLUBLE MAG (d1-3)-Fc



NO INHIBITION

Chapter VI

Signal Transduction Mechanisms Underlying the Inhibition by MAG

6.1 Introduction

Since MAG was first shown as a potent inhibitor of neurite outgrowth in 1994, a lot of effort has been exerted to characterize its function in axonal regeneration in vivo and in vitro. The signal transduction pathway by which MAG exerts its effect, however, has not yet been addressed.

Possible clues have recently emerged with studies towards other inhibitory molecules. Central nervous system myelin, or its associated NI-35 inhibitor, was shown to induce neuronal growth cone collapse. This effect was completely blocked by the presence of pertussis toxin (PTX), which inactivates Gi or Go proteins. This suggests that myelin or its associated inhibitory proteins might induce growth cone collapse via stimulating a neuronal receptor that is coupled with PTX-sensitive G proteins (Igarashi et al., 1993). In separate studies, it was found that the growth cone collapse induced by CNS myelin also involved a rapid increase in intracellular calcium ($[Ca^{2+}]_i$) levels (Bandtlow et al., 1993). Both a release from intracellular stores and an influx from extracellular environment have been suggested to contribute to this Ca^{2+} level increase (Bandtlow et al., 1993; Moorman and Hume, 1993). It is generally thought that increases in intracellular calcium level may alter both actin and tubulin polymerization states (Schliwa et al., 1981; Yamamoto et al., 1983), and affect the activity of growth cone myosin (Bridgman and Dailey, 1989), as well as other cellular events, which might influence the cytoskeletal architecture of growth cones and lead to their arrest and collapse.

Similarly, calcium levels increases have also been shown to play a crucial role in the signaling mechanism of lysophosphatidic acid (LPA)-induced growth cone collapse and neurite retraction in PC12 cells. Blockade of plasma membrane Ca^{2+} channel with divalent cations Mn^{2+} or the use of Ca^{2+}-free media completely abolishes LPA-induced neurite retraction (Tigyi et al., 1996a). LPA-induced Ca^{2+} immobilization has been shown to be mediated via the phosphoinositide (PIP) pathway (Tigyi et al., 1996a). Inhibitors of phospholipase C (PLC) or protein kinase C (PKC) diminish both the LPA-induced Ca^{2+} immobilization and neurite retraction (Tigyi et al., 1996a). However, activation of phosphoinositide (PIP)-Ca^{2+} second messenger system does not by itself induce neurite retraction (Tigyi et al., 1996a). Therefore, this pathway is thought to play a permissive role in the morphological response. It was found that the Ras-like small GTP-binding protein Rho plays a key role in mediating LPA-induced neurite retraction. Inactivation of Rho by the ADP-ribosylation with the *C. botulinum* exoenzyme C-3 not only abolishes LPA induced neurite retraction but also induces a neuronal differentiation of PC12 cells (Tigyi et al., 1996b).

LPA as well as other molecules such as thrombin were also shown to induce neurite retraction in neuroblastoma cells (Jalink et al., 1994). The small G protein Rho is also essential in mediating these morphological changes. Myosin light chain kinase has been identified as a downstream target of activated Rho (Kimura et al., 1996), and when inhibited, blocks LPA induced neurite retraction (Jalink et al., 1994). In these cells, however, although a PIP-Ca^{2+} signaling cascade is similarly activated, it is not involved in the morphological

changes (Jalink and Moolenaar, 1992). These results suggest that the signal transduction pathways induced by the same molecule might be differentially regulated in different cell types or in cells under different physiological states.

Small G-protein Rho family members have recently been found to play important roles in modulating neurite growth and growth cone behavior. It has been reported that Collapsin-1 induced growth cone collapse in DRG neurons is mediated by Rac1 (Jin and Strittmatter, 1997). In the *in vivo* studies, expression of constitutively active Rac1 in transgenic mice led to a failure in axonal extension in the Drosophila and changes in dendrite morphology of mouse cerebellar Purkinjie cells (Luo et al., 1994; 1996).

Cyclic AMP-dependent activity has recently been shown to play a regulatory function in neurite growth and growth cone behavior. Elevation of intracellular cAMP levels by activation of adenylyl cyclase or by addtion of cAMP analogs attenuates the LPA-induced neurite retraction in PC12 cells in a dose-dependent manner (Tigyi et al., 1996b). Similar effects of cAMP are observed to regulate prostaglandin E_2 (PGE_2)-induced growth cone collapse and neurite retraction in PC12 cells (Katoh et al., 1996) and BDNF-induced growth cone collapse in Xenopus spinal neurons (Wang and Zheng, 1998). Interestingly, receptors for LPA and PGE_2 are both shown to be coupled with the pertussis toxin-sensitive Gi proteins, which, when activated, inhibits the increase of intracellular cAMP level (Tigyi et al., 1996b; Katoh et al., 1996). The activation of Gi does not by itself induce neurite retraction (Tigyi et al., 1996b; Katoh et al., 1996), however, it is more likely that its

activation provides certain protections for the morphological effect of LPA or PGE_2 by lowering cellular cAMP contents.

Taken together, neurite outgrowth and growth cone motility is regulated by complicated signaling mechanisms. Receptors for extracellular cues may activate several pathways at the same time and these pathways may fulfill different functions in the cellular response (Tigyi et al., 1996b). In the current study, an assessment of the underlying signaling mechanisms induced by MAG interaction with neurons is described.

6.2 Results

6.2.1 Inhibition of neurite outgrowth by MAG is independent of several known signaling pathways.

To determine the signaling mechanism underlying the inhibitory effect of MAG, several signaling pathways, which were previously suggested to participate in growth cone collapse and neurite retraction, were investigated. In order to assess if the inhibitory effect of MAG on neurite outgrowth is mediated by Ca^{2+}, several reagents which can either increase or decrease the intracellular Ca^{2+} levels were tested for their ability to reverse the inhibition by MAG. These included the L-type Ca^{2+} channel antagonist diltiazem, the N-type Ca^{2+} channel antagonists ω-Conotoxin, the cell membrane permeable Ca^{2+} chelator BAPTA/AM, and KCl, which causes Ca^{2+} influx into cells.

To examine the invovement of the phosphoinositide PIP-Ca^{2+} pathway, inhibitors of phospholipase C and protein kinase C, as well as an activator of protein kinase C, were tested. In addition, the functions of certain tyrosine kinases known to be involved in regulation of cytoskeleton reorganization were also examined in the inhibitory effect of MAG.

We also examined whether the effect of MAG is mediated via a heterotrimeric Gi protein, which is known to inhibit adenylyl cyclase activity. Pertussis toxin, which catalyzes ADP-

Table 1 The effects of a variety of reagents on the inhibition of neurite outgrowth by MAG. Dissociated cerebellar neurons were cultured on confluent monolayers of MAG-expressing of control CHO cells in the presence or absence of reagents with the concentrations indicated in the table. After 18 hr, the co-cultures were fixed and immunostained for GAP-43. The length of the longest neurite of each neuron was measured. The effect of the indicated reagent was determined by comparing the neurite length grown in the presence of this reagent to that grown in the control media.

Reagents	**Function of Reagents**	**Effect on Neurite Growth on MAG Cells**	**Effect on Neurite Growth on Control**
Diltiazem (1-100μM)	L-type Ca^{2+} channel antagonist	–	–
ω-Conotoxin (0.025-2.5μM)	N-type Ca^{2+} channel antagonist	–	–
BAPTA/AM (0-30μM)	Membrane permeable Ca^{2+} chelator	–	–
Kcl (40 mM)	Causing Ca^{2+} influx into cells.	–	–
Pertussis toxin (0-1μg/ml)	Inactivating Gi proteins	–	–
Rp-cAMP (150μM)	PKA inhibitor	–	–
Chelerythrine (0-650nM)	PKC inhibitor	–	–
4b-phorbol 12-myristate 13-acetate (PMA) (0-0.2μM)	PKC activator	–	–
Staurosporine (0-1μM)	ser/thr kinase inhibitor	–	–
Neomycin (0-50μM)	PLC inhibitor	–	–
Genistein (0-300μM)	tyrosine kinase inhibitor	–	–
Herbimycin (0-10μg/ml)	tyrosine kinase inhibitor	–	–
Lavendustin A (0-20μM)	tyrosine kinase inhibitor	–	–

ribosylation of the α subunits and inactivates Gi was included in the co-culture media. In addition, the functions of certain tyrosine kinases known to be involved in regulation of cytoskeleton reorganization were also examined in the inhibitory effect of MAG. Results were summarized in Table 1.

We found from all these experimental results that none of these reagents had any effect on inhibition of neurite outgrowth by MAG. This suggests that the signaling pathway through Ca^{2+}, PIP-Ca^{2+}, and some protein tyrosine kinases are not likely to be involved in transduction mechanisms underlying the inhibitory effect of MAG.

6.2.2 Elevation of cyclic AMP level prevents the inhibition of neurite growth by MAG

Next we investigated whether cAMP-dependent activity plays any role in the neurite growth inhibition by MAG. The effect of elevation of cyclic AMP levels was assessed. Cerebellar neurons from PND 7 rat pups were dissociated and plated on confluent monolayers of control and MAG-expressing CHO cells. A membrane permeable cyclic AMP analogue, dibutyryl-cAMP, was included in the co-culture media at a concentration of 0-10 mM. After overnight incubation, the co-cultures were fixed and stained, and the length of the longest neurite per neuron was measured. As illustrated in Fig.6.1, the average neurite length on MAG-expressing cells, in the absence of dibutyryl-cAMP, was 60% shorter than that on control cells. In contrast, when dibutyryl-cAMP was applied, neurite length on MAG-

expressing cells was increased greatly and was as long as that on control cells. The neurite length on control cells, however, was not significantly increased. Hence, the inhibitory effect of MAG was completely abolished by artificially elevating cAMP levels. This result raises the possibility that either MAG functions to decrease the intracellular cAMP levels and inhibits neurite outgrowth, or cAMP is involved in a different pathway which, when activated, can overcome the effect of MAG.

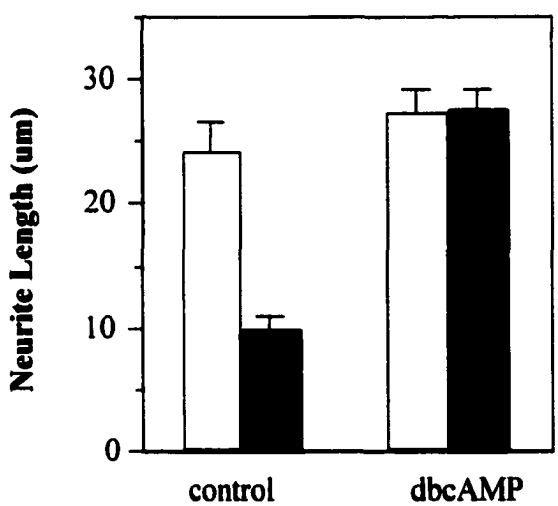


Figure 6.1 The effect of dibutyryl cAMP on neurite growth inhibition by MAG. Cerebellar neurons were plated onto monolayers of either MAG-expressing CHO cells (solid bars) or control cells (stippled bars) and cultured overnight in the presence or absence of dibutyryl cAMP at a concentration of 1 mM. The cultures were then fixed and immunostained for GAP-43, and the neurites were measured. Results show the mean length of the longest neurites (±SEM) for about 200 neurons.

6.2.3 Blocking of PKA activity does not affect neurite outgrowth on CHO cells

To assess the possible involvement of cAMP pathways in neurite inhibition by MAG, we blocked PKA activity with a PKA inhibitor, KT5720, to see whether this would produce an inhibitory effect on neurite outgrowth. Cerebellar neurons were grown on control or MAG-expressing CHO cells in media with or without 200nM of KT5720. After 18hr, neurite

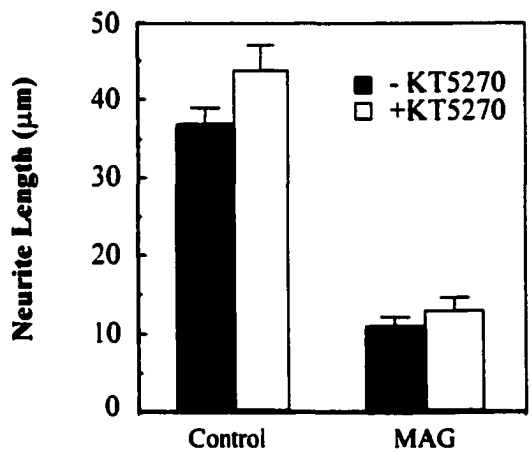


Figure 6.2 The effect of KT5270 on neurite outgrowth from cerebellar neurons. Cerebellar neurons were plated onto monolayers of either MAG-expressing CHO cells (solid bars) or control cells (stippled bars) and cultured overnight in the presence or absence of KT5270 at a concentration of 200 nM. The cultures were then fixed and immunostained for GAP-43, and the neurites were measured. Results show the mean length of the longest neurites (±SEM) for about 200 neurons.

length was measured (Fig. 6.2). It was found that on control CHO cells, cerebellar neurons extend long neurites regardless of the presence of KT5720. On MAG-expressing cells, neurite growth was equally inhibited with or without KT5720. No effect of KT5720 on neurite outgrowth was observed either on MAG-expressing cells or on control cells. This result suggests that a direct involvement of cAMP/PKA in MAG induced inhibition pathway is not likely. Therefore, the possibility that MAG inhibits neurite outgrowth through decreasing the cAMP levels in neuronal cells can be excluded.

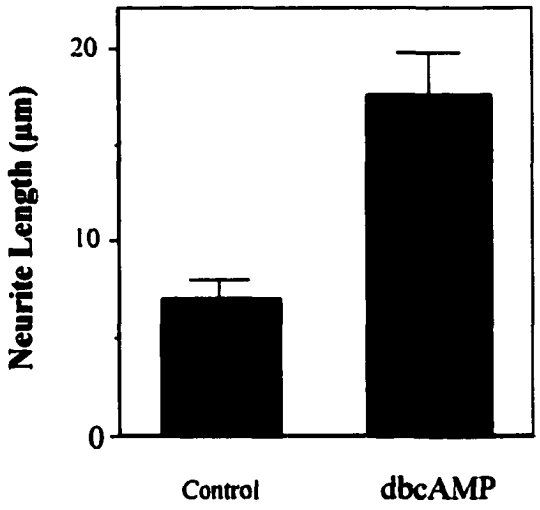


Figure 6.3 The effect of dibutyryl cAMP on neurite growth inhibition by CNS myelin. Cerebellar neurons were plated onto immobilized CNS myelin membranes and cultured overnight in the presence or absence of dibutyryl cAMP at a concentration of 1 mM. The cultures were then fixed and immunostained for GAP-43, and the neurites were measured. Results show the mean length of the longest neurites (±SEM) for about 200 neurons.

6.2.4 Elevation of cyclic AMP level increases neurite growth on CNS myelin

To test whether the elevation of cAMP level can also help to overcome the general inhibitory effect of CNS myelin, we cultured cerebellar neurons on freshly prepared CNS myelin, in media with and without the supplement of 1mM dibutyryl-cAMP. After overnight incubation, it was observed that in the presence of 1 mM dibutyryl-cAMP, cerebellar neurons extended significantly longer neurites on CNS myelin (Fig. 6.3). This suggests that increased cAMP levels also alleviate the general inhibition of CNS myelin on neurite outgrowth.

6.3 Conclusion and discussion

In this study, we have shown that elevation of cAMP levels completely blocks the inhibition of neurite growth by MAG. However, interfering with the cAMP pathway by the competitive agonist analogue of cyclic AMP, Rp-cAMP, or the PKA inhibitor, KT5720, does not by itself inhibit neurite outgrowth on CHO cells. Therefore, the possibility that MAG inhibits neurite outgrowth by directly decreasing the cAMP levels in neuronal cells can be excluded. Instead, it is more likely that cAMP is involved in a different pathway which crosstalks with the MAG induced pathway and that a low intracellular cAMP level may be a prerequisite for the manifestation of the inhibitory effect of MAG.

Similar results were also shown with DRG neurons of the PNS (Cai and Filbin, unpublished observations). Moreover, it was shown that neurons pretreated in 1 mM of dbcAMP were able to overcome the inhibitory effect of MAG even in the absence of dbcAMP in the culturing media (Cai and Filbin, unpublished observations). These results exclude the possibility that the extension of neurites on MAG cell surface is due to the change in MAG expression by cAMP. Together with the current results, these observations suggest that the response of neurons to MAG is regulated by the neuronal cAMP level.

These results are supported by recent studies on the effect of neurotrophins to block the neurite inhibition by MAG (Cai et al., 1999). It was found that neurons cultured overnight with BDNF or GDNF before exposure to MAG can overcome the neurite growth inhibition

by MAG. An increase the intracellular cAMP levels is observed during the neurotrophin priming and is suggested to be crucial for the blocking of MAG inhibition. If an antagonist of cAMP or PKA inhibitor is present during the priming, blocking of MAG inhibition is completely abrogated. Priming with neurotrophins for at least 6 hr prior to the exposure to MAG is necessary for the blocking of inhibition. Simultaneous addition of neurotrophins and exposure to MAG has no effect on cellular cAMP content, nor does it affect the neurite growth inhibition. These results are consistent with our observations and indicate that cAMP-dependent activity can modulate neurons response to MAG.

Interestingly, it is found in the same study that if neurons are exposed to MAG and neurotrophins simultaneously, but with the G protein inhibitor pertusis toxin, inhibition by MAG is blocked (Cai et al., 1999). It is also found that exposure to neurotrophins and MAG at the same time with pertusis toxin results in a significant increase in cellular cAMP levels, to the same extent as that induced by the neurotrophin priming. Therefore, it is likely that MAG activates a pertusis toxin-sensitive Gi protein, which in turn prevents the elevation of cAMP levels by neurotrophins. Blocking of inhibition will only be possible if neurons are primed in neurotrophins and cAMP is allowed to accumulate before the exposure to MAG. Thus, MAG not only induces a signaling pathway leading to the neurite growth inhibition, but also lowers cAMP levels by activating a Gi protein, which in turn potentiates its inhibition effect. Similar mechanisms have also been employed by some other factors that cause growth cone collapse and neurite retraction, such as lysophosphatidic acid (LPA) and prostaglandin E_2 (PGE_2)

We have also found in the current study that elevation of cAMP levels also prevents the neurite growth inhibition by CNS myelin. Consistent with this, Cai et al (1999) also showed that priming with neurotrophins blocks the inhibition effect of myelin and this blocking was abolished in the presence of PKA inhibitors (Cai et al., 1999). These results imply that either (1) the action of major myelin-associated inhibitors is blocked by the elevation of cellular cAMP levels or (2) that MAG is itself, the major contributor to the overall inhibition by myelin.

Recent studies have highlighted the importance of intracellular cAMP as a gating element in a number of different signaling pathways (Iyengar, 1996). It was recently reported that differences in cAMP-dependent activity in a neuron might result in opposite turning response of growth cones to the same guidance cue. A gradient of brain-derived neurotrophic factor (BDNF) normally triggers an attractive turning response of the growth cone of *Xenopus* spinal neurons in culture, but the same gradient induces repulsive turning of these growth cones in the presence of Rp-cAMP or a PKA inhibitor (Song et al., 1997). This cAMP-dependent switch of the turning response was also found for turning induced by netrin-1 (Ming et al., 1997), the axon guidance molecule secreted from the floor plate (Kennedy et al., 1994). Blocking cAMP-dependent activity switches the chemoattractant response to a repellent response. It was recently reported that MAG, when applied as a concentration gradient, also exerts chemorepellent effects on growth cones of *Xenopus* spinal neurons (Song et al., 1998). Interestingly, if cAMP levels were artificially increased, repulsion of growth cones by MAG was switched to attraction. This result is consistent with ours and

suggests that the signaling pathway induced by MAG is modulated by a cAMP-dependent activity.

The repulsive turning of growth cones is thought to result from a local, asymmetric collapse of growth cones (Fan and Raper, 1995). Recently, it has been found that cAMP-dependent activity also plays roles in regulating neuronal growth cone collapse. Acutely applied BDNF induces rapid growth cone collapse and neurite retraction of embryonic *Xenopus* spinal neurons that was isolated and cultured for 6 hr and that this collapsing effect is regulated by cAMP-dependent activity. Elevation of intracellular cAMP levels completely blocks the collapsing effect, whereas inhibition of PKA potentiates the collapsing action (Wang and Zheng, 1998). BDNF-induced growth cone collapse is only observed with neurons isolated and cultured for 6 hr but not those cultured for 24hr. Because the inhibition of PKA by Rp-cAMP restored the collaping response of neurons in 24 hr-cultures, it is suggested that embryonic Xenopus spinal neurons may up-regulate their endogenous cAMP-dependent activity during development in culture, leading to the blokade of their collapsing response to BDNF. Similarly, growth cone collapse and neurite retraction induced by LPA or prostaglandin E_2 (PGE_2) in PC12 cells are also shown to be gated by cAMP-dependent activity (Tigyi et al., 1996a; Katoh et al., 1996), where elevation of intracellular cAMP levels prevented growth cone collapse in a dose dependent manner. It was reported that MAG could induce growth cone collapse (Li et al., 1996). Whether this collapsing activity is also modulated by intracellular cAMP level is yet to be determined.

Based on our results, we propose a hypothesis for cAMP-dependent regulation of MAG induced neurite outgrowth inhibition (Fig. 6.4). MAG activates its neuronal receptor and in turn activates a downstream signaling cascade. Meanwhile, MAG also activates the Gi protein, which in turn inhibits adenylyl cyclase and prevents cAMP increase. Protein X is involved in this main pathway leading to the neurite growth inhibition. When intracellular cAMP level is low, X is in an unphosphorylated and active state. Elevation of cAMP in neurons activates PKA, which in turn phosphorylates the unknown protein "X". This phosphorylation inactivates X and therefore blocks the inhibition. Stimulation by neurotrophins will result in an increase in cellular cAMP levels. However, in the presence of MAG, this increase will be prevented as MAG activates a Gi pathway, although the inhibition pathway will not be affected. In contrast, if neurons are primed with neurotrophins before they contact MAG, cAMP levels will increase, and the inhibition pathway will then be modulated through the phosphorylation of protein X by PKA.

The identity of protein X, a downstream effector in the MAG inhibition pathway that is modulated by cAMP-dependent activity, is yet to be determined. Recently, the small GTP-binding protein Rho was reported to be critical in mediating cytoskeleton reorganization and thereby morphological changes in different types of cells (Bradke and Dotti, 1999; Gallo and Letourneau, 1998; Hall, 1998). PKA was reported to play a role in regulating the activity of RhoA. RhoA can be specifically phosphorylated by PKA at Ser-188. This phosphorylation does not affect the ability of RhoA to bind guanine nucleotides nor does it modify its intrinsic GTPase activity, but results in a decreased affinity for its downstream effectors (Dong et al.,

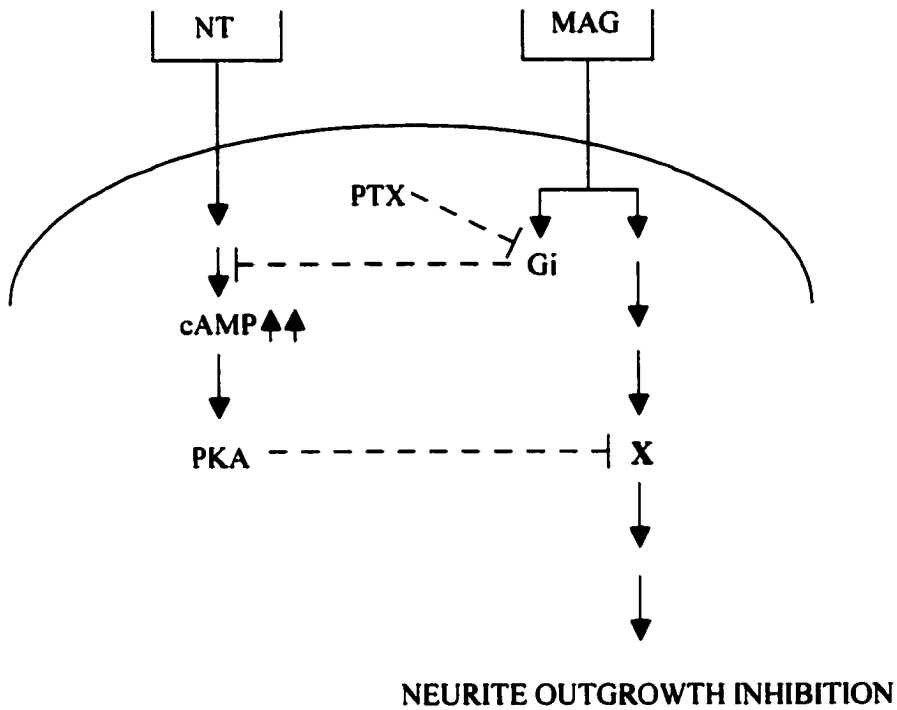


Figure 6.4 Schematic diagram showing the proposed model involved in the cAMP regulation of neurite outgrowth inhibition by MAG. Activation of neuronal receptors by MAG induces multiple intracellular signaling cascades and main pathway leading to neurite inhibition involves an unknown protein X whose activity can be modulated by cAMP-dependent protein activity. MAG also activates a pertusis toxin-sensitive Gi protein, which in turn inhibits adenylyl cyclase and prevents cAMP increase. This Gi pathway does not by itself cause neurite inhibition, but it protects the main inhibition pathway by preventing the increase of cAMP levels. During neurotrophin priming, neuronal cAMP levels are increased, which in turn activates PKA or some as yet unidentified downstream signal, and inactivates protein X in main neurite inhibition pathway. Inhibition by MAG will then be blocked. If however, neurotrophins are added at the same time as neurons are exposed to MAG, cAMP is prevented from increasing by MAG activation of Gi protein, the activity of X will not be affected, and neurite growth will still be inhibited by MAG.

1998), as well as in the translocation of membrane-associated RhoA into the cytosol and thereby separated from its downstream effectors (Lang et al., 1996). It was also reported that myosin light chain kinase (MLCK) is another target of PKA. Phosphorylation of MLCK by PKA decreases its affinity for Ca^{2+} and calmodulin and inhibits its activity (Tansey et al., 1994). Whether cAMP-dependent activity blocks the neurite inhibition by MAG through modulating the function of these proteins is now under investigation in our lab.

In summary, we have shown that cAMP-dependent activity is not directly involved in the neurite inhibition by MAG. However, it plays an important role in modulating neuronal response to MAG and myelin. Therefore, MAG, and perhaps other myelin-specific inhibitors, can be added to the growing number of molecules whose effect as either inhibitors/repellents or promoters/attractants of axonal growth can be determined by the endogenous levels of neuronal cAMP.

Chapter VII

References

Aguayo AJ, David S, Bray GM. (1981) Influences of the glial environment on the elongation of axons after injury: transplantation studies in adult rodents. J Exp Biol 95: 231-240

Alonso G, Privat A. (1993) Reactive astrocytes involved in the formation of lesional scars differ in the mediobasal hypothalamus and in other forebrain regions. J Neurosci Res 34(5): 523-538

Ard MD, Bunge MB, Wood PM, Schachner M, Bunge RP. (1991) Retinal neurite growth on astrocytes is not modified by extracellular matrix, anti-L1 antibody, or oligodendrocytes. Glia 4(1): 70-82

Argiro V, Johnson MI. (1982) Patterns and kinetics of neurite extension from sympathetic neurons in culture are age dependent. J Neurosci 2(4): 503-512

Argiro V, Bunge MB, Johnson MI. (1984) Correlation between growth form and movement and their dependence on neuronal age. J Neurosci 4(12): 3051-3062

Arquint M, Roder J, Chia LS, Down J, Wilkinson D, Bayley H, Braun P, Dunn R. (1987) Molecular cloning and primary structure of myelin-associated glycoprotein. Proc Natl Acad Sci U S A 84(2): 600-604

Bandtlow C, Zachleder T, Schwab ME. (1990) Oligodendrocytes arrest neurite growth by contact inhibition. J Neurosci 10(12): 3837-3848

Bandtlow CE, Schmidt MF, Hassinger TD, Schwab ME, Kater SB. (1993) Role of intracellular calcium in NI-35-evoked collapse of neuronal growth cones. Science 259: 80-83

Bahr M, Bonhoeffer F. (1994) Perspectives on axonal regeneration in the mammalian CNS. Trends Neurosci 17(11): 473-479

Barinaga M. (1994) Old protein provides new clue to nerve regeneration puzzle. Science 265: 1800-1801

Bartsch U, Kirchhoff F, Schachner M. (1989) Immunohistological localization of the adhesion molecules L1, N-CAM, and MAG in the developing and adult optic nerve of mice. J Comp Neurol 284(3): 451-462

Bartsch U, Bandtlow CE, Schnell L, Bartsch S, Spillmann AA, Rubin BP, Hillenbrand R, Montag D, Schwab ME, Schachner M. (1995) Lack of evidence that myelin-associated glycoprotein is a major inhibitor of axonal regeneration in the CNS. Neuron 15(6): 1375-1381

Bates CA, Meyer RL. (1997) The neurite-promoting effect of laminin is mediated by different mechanisms in embryonic and adult regenerating mouse optic axons in vitro. Dev Biol 181(1): 91-101

Bedi KS, Winter J, Berry M, Cohen J (1992) Adult rat dorsal root ganglion neurons extend neurites on predegenerated but not on normal peripheral nerves in vitro. Eur J neurosci 4: 193-200

Benowitz LI, Routtenberg A. (1997) GAP-43: an intrinsic determinant of neuronal development and plasticity. Trends Neurosci 20(2): 84-91

Berry M, Rees L, Hall S, Yiu P, Sievers J. (1988) Optic axons regenerate into sciatic nerve biografts only in the presence of Schwann cells. Brain Res. Bull. 20: 223-231

Beuche W, Friede RL. (1984) The role of non-resident cells in Wallerian degeneration. J Neurocytol 13(5): 767-796

Beuche W, Friede RL. (1986) Myelin phagocytosis in Wallerian degeneration of peripheral nerves depends on silica-sensitive, bg/bg-negative and Fc-positive monocytes. Brain Res 378(1): 97-106

Bjorklund A. (1992) Dopaminergic transplants in experimental parkinsonism: cellular mechanisms of graft-induced functional recovery. Curr Opin Neurobiol 2(5): 683-689

Bradke F, Dotti CG. (1999) The role of local actin instability in axon formation. Science 1999 283: 1931-1934

Bregman BS, Kunkel-Bagden E, Reier PJ, Dai HN, McAtee M, Gao D. (1993) Recovery of function after spinal cord injury: mechanisms underlying transplant-mediated recovery of function differ after spinal cord injury in newborn and adult rats. Exp Neurol 123(1): 3-16

Bregman BS, Kunkel-Bagden E, Schnell L, Dai HN, Gao D, Schwab ME. (1995) Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. Nature 378: 498-501

Bregman BS, McAtee M, Dai HN, Kuhn PL. (1997) Neurotrophic factors increase axonal growth after spinal cord injury and transplantation in the adult rat. Exp Neurol 148(2): 475-494

Bregman BS, Broude E, McAtee M, Kelley MS. (1998) Transplants and neurotrophic factors prevent atrophy of mature CNS neurons after spinal cord injury. Exp Neurol 149(1): 13-27

Bridgman PC, Dailey ME. (1989) The organization of myosin and actin in rapid frozen nerve growth cones. J Cell Biol 108(1): 95-109

Broude E, McAtee M, Kelley MS, Bregman BS. (1997) c-Jun expression in adult rat dorsal root ganglion neurons: differential response after central or peripheral axotomy. Exp Neurol 148(1): 367-377

Brown MC, Perry VH, Lunn ER, Gordon S, Heumann R. (1991a) Macrophage dependence of peripheral sensory nerve regeneration: possible involvement of nerve growth factor. Neuron 6(3): 359-370

Brown MC, Booth CM, Lunn ER, Perry VH. (1991b) Delayed response to denervation in muscles of C57BL/Ola mice. Neuroscience 43(1): 279-283

Brown MC, Lunn ER, Perry VH. (1992) Consequences of slow Wallerian degeneration for regenerating motor and sensory axons. J Neurobiol 23(5): 521-536

Brown MC, Perry VH, Hunt SP, Lapper SR. (1994) Further studies on motor and sensory nerve regeneration in mice with delayed Wallerian degeneration. Eur J Neurosci 6(3): 420-428

Cai D, Shen Y, De Bellard M, Tang S, Filbin MT. (1999) Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism. Neuron 22(1): 89-101

Cajal SR. (1928) In Regeneration and Degeneration in the Nervous System. Oxford University Press: Oxford

Caroni P, Savio T, Schwab ME. (1988a) Central nervous system regeneration: oligodendrocytes and myelin as non-permissive substrates for neurite growth. Prog Brain Res 78: 363-370

Caroni P, Schwab ME. (1988b) Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. J Cell Biol 106(4): 1281-1288

Caroni P, Schwab ME. (1988c) Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. Neuron 1(1): 85-96

Caroni P, Schwab ME. (1989) Codistribution of neurite growth inhibitors and oligodendrocytes in rat CNS: appearance follows nerve fiber growth and precedes myelination. Dev Biol 136(2): 287-295

Caroni P, Becker M. (1992) The downregulation of growth-associated proteins in motoneurons at the onset of synapse elimination is controlled by muscle activity and IGF1. J Neurosci 12(10): 849-861

Carpenter P, Sefton AJ, Dreher B, Lim WL. (1986) Role of target tissue in regulating the development of retinal ganglion cells in the albino rat: effects of kainate lesions in the superior colliculus. J Comp Neurol 251(2): 240-259

Chen DF, Schneider GE, Martinou JC, Tonegawa S. (1997) Bcl-2 promotes regeneration of severed axons in mammalian CNS. Nature 385: 434-439

Chen DF, Jhaveri S, Schneider GE. (1995) Intrinsic changes in developing retinal neurons result in regenerative failure of their axons. Proc Natl Acad Sci USA 92(16): 7287-7291

Chiquet M. (1989) Neurite growth inhibition by CNS myelin proteins: a mechanism to confine fiber tracts? Trends Neurosci 12(1): 1-3

Chong MS, Woolf CJ, Andrews P, Turmaine M, Schreyer DJ, Anderson PN. (1994) The downregulation of GAP-43 is not responsible for the failure of regeneration in freeze-killed nerve grafts in the rat. Exp Neurol 129(2): 311-20

Chong MS, Reynolds ML, Irwin N, Coggeshall RE, Emson PC, Benowitz LI, Woolf CJ. (1994) GAP-43 expression in primary sensory neurons following central axotomy. J Neurosci 14(7): 4375-4384

Clarke Le Gros WE. (1942) The problem of neuronal regeneration in the central nervous system. I. The influence of spinal ganglia and nerve fragments grafted into the brain. J. Anat. 77: 20-48

Clarke Le Gros WE. (1942) The problem of neuronal regeneration in the central nervous system. II. The insertion of the peripheral stump into the brain. J. Anat. 77: 251-259

Colello RJ, Schwab ME. (1994) A role for oligodendrocytes in the stabilization of optic axon numbers. J Neurosci 14(11 Pt 1): 6446-6452

Colello RJ, Pott U, Schwab ME. (1994) The role of oligodendrocytes and myelin on axon maturation in the developing rat retinofugal pathway. J Neurosci 14(5 Pt 1): 2594-2605

Collins F, Lee MR. (1982) Reversible developmental change in the ability of ciliary ganglion neurons to extend neurites in culture. J Neurosci 2(4): 424-430

Cohen J, Burne JF, Winter J, Bartlett P. (1986) Retinal ganglion cells lose response to laminin with maturation.Nature 322: 465-467

Cohen J, Burne JF, McKinlay C, Winter J. (1987) The role of laminin and the laminin/fibronectin receptor complex in the outgrowth of retinal ganglion cell axons. Dev Biol 122(2): 407-418

Cohen J, Nurcombe V, Jeffrey P, Edgar D. (1989) Developmental loss of functional laminin receptors on retinal ganglion cells is regulated by their target tissue, the optic tectum. Development 107(2): 381-387

Curtis R, Adryan KM, Zhu Y, Harkness PJ, Lindsay RM, DiStefano PS. (1993) Retrograde axonal transport of ciliary neurotrophic factor is increased by peripheral nerve injury. Nature 365: 253-255

Curtis R, Scherer SS, Somogyi R, Adryan KM, Ip NY, Zhu Y, Lindsay RM, DiStefano PS. (1994) Retrograde axonal transport of LIF is increased by peripheral nerve injury: correlation with increased LIF expression in distal nerve. Neuron 12(1): 191-204

de Curtis I, Quaranta V, Tamura RN, Reichardt LF. (1991) Laminin receptors in the retina: sequence analysis of the chick integrin alpha 6 subunit. Evidence for transcriptional and posttranslational regulation. J Cell Biol 113(2): 405-416

Daniloff JK, Levi G, Grumet M, Rieger F, Edelman GM. (1986) Altered expression of neuronal cell adhesion molecules induced by nerve injury and repair. J Cell Biol 103(3): 929-945

David S, Aguayo AJ. (1981) Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. Science 214: 931-933

David S, McKerracher L, Jackson D, Kottis V, Braun P. (1994) Neurite growth inhibitory activity in the mammalian PNS and CNS and its modulation by laminin. Soc Neurosci Abstr 20: 1296

Davies AM, Larmet Y, Wright E, Vogel KS. (1991) Coordination of trophic interactions by separate developmental programs in sensory neurons and their target fields. J Cell Sci Suppl 15:111-116

Davies AM. (1997) Neurotrophin switching: where does it stand? Curr Opin Neurobiol 7(1): 110-118

DeBellard ME, Tang S, Mukhopadhyay G, Shen YJ, Filbin MT. (1996) Myelin-associated glycoprotein inhibits axonal regeneration from a variety of neurons via interaction with a sialoglycoprotein. Mol Cell Neurosci 7(2): 89-101

Dent EW, Meiri KF. (1992) GAP-43 phosphorylation is dynamically regulated in individual growth cones. J Neurobiol 23(8): 1037-1053

Doherty P, Cohen J, Walsh FS. (1990) Neurite outgrowth in response to transfected N-CAM changes during development and is modulated by polysialic acid. Neuron 5(2): 209-219

Dong JM, Leung T, Manser E, Lim L. (1998) cAMP-induced morphological changes are counteracted by the activated RhoA small GTPase and the Rho kinase ROKalpha. J Biol Chem 273(35): 22554-22562

Dow KE, Guo M, Kisilevsky R, Riopelle RJ. (1993) Regenerative neurite growth modulation associated with astrocyte proteoglycans. Brain Res Bull 30(3-4): 461-467

Dow KE, Ethell DW, Steeves JD, Riopelle RJ. (1994) Molecular correlates of spinal cord repair in the embryonic chick: heparan sulfate and chondroitin sulfate proteoglycans. Exp Neurol 128(2): 233-238

Edwards AM, Braun PE, Bell JC. (1989) Phosphorylation of myelin-associated glycoprotein in vivo and in vitro occurs only in the cytoplasmic domain of the large isoform. J Neurochem 52(1): 317-20

Fan J, Raper JA. (1995) Localized collapsing cues can steer growth cones without inducing their full collapse. Neuron 14(2): 263-274

Fawcett JW, Housden E, Smith-Thomas L, Meyer RL. (1989) The growth of axons in three-dimensional astrocyte cultures. Dev Biol 135(2): 449-458

Fawcett JW, Keynes RJ. (1990) Peripheral nerve regeneration. Annu Rev Neurosci 13: 43-60

Fawcett JW. (1992) Intrinsic neuronal determinants of regeneration. Trends Neurosci 15(1): 5-8

Fawcett JW. (1997) Astrocytic and neuronal factors affecting axon regeneration in the damaged central nervous system. Cell Tissue Res 290(2): 371-377

Freeman SD, Kelm S, Barber EK, Crocker PR. (1995) Characterization of CD33 as a new member of the sialoadhesin family of cellular interaction molecules. Blood 85: 2005-2012

Fruttiger M, Montag D, Schachner M, Martini R. (1995) Crucial role for the myelin-associated glycoprotein in the maintenance of axon-myelin integrity. Eur J Neurosci 7(3): 511-515

Funakoshi H, Frisen J, Barbany G, Timmusk T, Zachrisson O, Verge VM, Persson H. (1993) Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. J Cell Biol 123(2): 455-465

Gallo G, Letourneau PC. (1998) Localized sources of neurotrophins initiate axon collateral sprouting. J Neurosci 18(14): 5403-5414

Goodman CS. (1996) Mechanisms and molecules that control growth cone guidance. Annu Rev Neurosci 19: 341-377

Gordon-Weeks PR. (1989) GAP-43--what does it do in the growth cone? Trends Neurosci 12(10): 363-365

Green PJ, Walsh FS, Doherty P. (1997) Signal transduction mechanisms underlying axonal growth responses stimulated by cell adhesion molecules. Rev Neurol (Paris) 153(8-9): 509-514

Hall A. (1998) Rho GTPases and the actin cytoskeleton. Science 279: 509-514

He Q, Dent EW, Meiri KF. (1997) Modulation of actin filament behavior by GAP-43 (neuromodulin) is dependent on the phosphorylation status of serine 41, the protein kinase C site. J Neurosci 17(10):3515-3524

Ide C, Osawa T, Tohyama K. (1990) Nerve regeneration through allogeneic nerve grafts, with special reference to the role of the Schwann cell basal lamina. Prog Neurobiol 34(1): 1-38

Igarashi M, Strittmatter SM, Vartanian T, Fishman MC. (1993) Mediation by G proteins of signals that cause collapse of growth cones. Science 259: 77-79

Iwashita Y, Kawaguchi S, Murata M. (1994) Restoration of function by replacement of spinal cord segments in the rat. Nature 367: 167-170

Iyengar R. (1996) Gating by cyclic AMP: expanded role for an old signaling pathway. Science 271: 461-463

Jalink K, Moolenaar WH. (1992) Thrombin receptor activation causes rapid neural cell rounding and neurite retraction independent of classic second messengers. J Cell Biol 118(2): 411-419

Jalink K, van Corven EJ, Hengeveld T, Morii N, Narumiya S, Moolenaar WH. (1994) Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. J Cell Biol 126(3): 801-810

Jelsma TN, Aguayo AJ. (1994) Trophic factors. Curr Opin Neurobiol 4(5): 717-725

Jessen KR, Mirsky R. (1991) Schwann cell precursors and their development. Glia 4(2): 185-194 Johnson PW, Abramow-Newerly W, Seilheimer B, Sadoul R, Tropak MB, Arquint M, Dunn RJ, Schachner M, Roder JC. (1989) Recombinant myelin-associated glycoprotein confers neural adhesion and neurite outgrowth function. Neuron 3(3): 377-385

Jin Z, Strittmatter SM. (1997) Rac1 mediates collapsin-1-induced growth cone collapse. J Neurosci 17(16): 6256-6263

Johnson AR. (1993) Contact inhibition in the failure of mammalian CNS axonal regeneration. Bioessays 15(12): 807-813

Kapfhammer JP, Schwab ME. (1994) Inverse patterns of myelination and GAP-43 expression in the adult CNS: neurite growth inhibitors as regulators of neuronal plasticity? J Comp Neurol 340(2): 194-206

Katoh H, Negishi M, Ichikawa A. (1996) Prostaglandin E receptor EP3 subtype induces neurite retraction via small GTPase Rho. J Biol Chem 271(47): 29780-29784

Keirstead HS, Hasan SJ, Muir GD, Steeves JD. (1992) Suppression of the onset of myelination extends the permissive period for the functional repair of embryonic spinal cord. Proc Natl Acad Sci USA 89(24): 11664-11668

Keirstead HS, Dyer JK, Sholomenko GN, McGraw J, Delaney KR, Steeves JD. (1995) Axonal regeneration and physiological activity following transection and immunological disruption of myelin within the hatchling chick spinal cord. J Neurosci 15(10): 6963-6974

Kelm S, Pelz A, Schauer R, Filbin MT, Tang S, de Bellard ME, Schnaar RL, Mahoney JA, Hartnell A, Bradfield P, Crocker PR. (1994) Sialoadhesin, myelin-associated glycoprotein and CD22 define a new family of sialic acid-dependent adhesion molecules of the immunoglobulin superfamily. Curr Biol 4(11): 965-972

Kelm S, Schauer R. (1997) Sialic acids in molecular and cellular interactions. Int Rev Cytol 175: 137-240

Kelm S, Brossmer R, Isecke R, Gross HJ, Strenge K, Schauer R. (1998) Functional groups of sialic acids involved in binding to siglecs (sialoadhesins) deduced from interactions with synthetic analogues. Eur J Biochem 255(3): 663-672

Kennedy TE, Serafini T, de la Torre JR, Tessier-Lavigne M. (1994) Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell 78(3): 425-435

Lai C, Brow MA, Nave KA, Noronha AB, Quarles RH, Bloom FE, Milner RJ, Sutcliffe JG. (1987) Two forms of 1B236/myelin-associated glycoprotein, a cell adhesion molecule for postnatal neural development, are produced by alternative splicing. Proc Natl Acad Sci U S A 84(12): 4337-4341

Lang P, Gesbert F, Delespine-Carmagnat M, Stancou R, Pouchelet M, Bertoglio J. (1996) Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. EMBO J 15(3): 510-519

Mansour-Robaey S, Clarke DB, Wang YC, Bray GM, Aguayo AJ. (1994) Effects of ocular injury and administration of brain-derived neurotrophic factor on survival and regrowth of axotomized retinal ganglion cells. Proc Natl Acad Sci U S A 91(5): 1632-1636

Martini R, Schachner M. (1986) Immunoelectron microscopic localization of neural cell adhesion molecules (L1, N-CAM, and MAG) and their shared carbohydrate epitope and myelin basic protein in developing sciatic nerve. J Cell Biol 103(6 Pt 1): 2439-2448

Martini R, Schachner M. (1988) Immunoelectron microscopic localization of neural cell adhesion molecules (L1, N-CAM, and myelin-associated glycoprotein) in regenerating adult mouse sciatic nerve. J Cell Biol 106(5): 1735-1746

McKerracher L, Essagian C, Aguayo AJ. (1993a) Temporal changes in beta-tubulin and neurofilament mRNA levels after transection of adult rat retinal ganglion cell axons in the optic nerve. J Neurosci 13(6): 2617-2626

McKerracher L, Essagian C, Aguayo AJ. (1993b) Marked increase in beta-tubulin mRNA expression during regeneration of axotomized retinal ganglion cells in adult mammals. J Neurosci 13(12): 5294-5300

McKerracher L, David S, Jackson DL, Kottis V, Dunn RJ, Braun PE. (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron 13(4): 805-811

McMillan MK, Thai L, Hong JS, O'Callaghan JP, Pennypacker KR. (1994) Brain injury in a dish: a model for reacive gliosis. Trens Neurosci 17: 138-142

Meiri KF, Gordon-Weeks PR. (1990) GAP-43 in growth cones is associated with areas of membrane that are tightly bound to substrate and is a component of a membrane skeleton subcellular fraction. J Neurosci 10(1): 256-266

Meiri KF, Saffell JL, Walsh FS, Doherty P. (1998) Neurite outgrowth stimulated by neural cell adhesion molecules requires growth-associated protein-43 (GAP-43) function and is associated with GAP-43 phosphorylation in growth cones. J Neurosci 18(24): 10429-10437

Meyer M, Matsuoka I, Wetmore C, Olson L, Thoenen H. (1992) Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA. J Cell Biol 119(1): 45-54

McKerracher L, Essagian C, Aguayo AJ. (1993b) Marked increase in beta-tubulin mRNA expression during regeneration of axotomized retinal ganglion cells in adult mammals. J Neurosci 13(12): 5294-5300

McKerracher L, David S, Jackson DL, Kottis V, Dunn RJ, Braun PE. (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron 13(4): 805-811

McMillan MK, Thai L, Hong JS, O'Callaghan JP, Pennypacker KR. (1994) Brain injury in a dish: a model for reacive gliosis. Trens Neurosci 17: 138-142

Meiri KF, Gordon-Weeks PR. (1990) GAP-43 in growth cones is associated with areas of membrane that are tightly bound to substrate and is a component of a membrane skeleton subcellular fraction. J Neurosci 10(1): 256-266

Meiri KF, Saffell JL, Walsh FS, Doherty P. (1998) Neurite outgrowth stimulated by neural cell adhesion molecules requires growth-associated protein-43 (GAP-43) function and is associated with GAP-43 phosphorylation in growth cones. J Neurosci 18(24): 10429-10437

Meyer M, Matsuoka I, Wetmore C, Olson L, Thoenen H. (1992) Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA. J Cell Biol 119(1): 45-54

Meyer-Franke A, Barres B. (1994) Axon myelination. Myelination without myelin-associated glycoprotein. Curr Biol 4(9): 847-850

Ming GL, Song HJ, Berninger B, Holt CE, Tessier-Lavigne M, Poo MM. (1997) cAMP-dependent growth cone guidance by netrin-1. Neuron 19(6): 1225-1235

Miragall F, Kadmon G, Husmann M, Schachner M. (1988) Expression of cell adhesion molecules in the olfactory system of the adult mouse: presence of the embryonic form of N-CAM. Dev Biol 129(2): 516-531

Montag D, Giese KP, Bartsch U, Martini R, Lang Y, Bluthmann H, Karthigasan J, Kirschner DA, Wintergerst ES, Nave KA. (1994) Mice deficient for the myelin-associated glycoprotein show subtle abnormalities in myelin. Neuron 13(1): 229-246

Moorman SJ, Hume RI. (1993) ω-Conotoxin prevents myelin-evoked growth cone collapse in neonatal rat locus coeruleus neurons in vitro. J Neurosci 13 (11): 4727-4736

Moorman SJ, Hume RI. (1994) Contact with myelin evokes a release of calcium from internal stores in neonatal rat oligodendrocytes in vitro. Glia 10(3): 202-210

Morrow DR, Campbell G, Lieberman AR, Anderson PN. (19993) Differential regenerative growth of CNS axons into tibial and peroneal nerve grafts in the thalamus of adult rats. Exp Neurol 120(1): 60-69

Moss DJ, Fernyhough P, Chapman K, Baizer L, Bray D, Allsopp T. (1990) Chicken growth-associated protein GAP-43 is tightly bound to the actin-rich neuronal membrane skeleton. J Neurochem 54(3): 729-736

Moya KL, Benowitz LI, Jhaveri S, Schneider GE. (1988) Changes in rapidly transported proteins in developing hamster retinofugal axons. J Neurosci 8(12): 4445-4454

Mukhopadhyay G, Doherty P, Walsh FS, Crocker PR, Filbin MT. (1994) A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron 13(3): 757-767

Nath D, van der Merwe PA, Kelm S, Bradfield P, Crocker PR. (1995) The amino-terminal immunoglobulin-like domain of sialoadhesin contains the sialic acid binding site. Comparison with CD22. J Biol Chem 270(44): 26184-26191

Nicholls J, Saunders NR. (1996) Regeneration of immature mammalian spinal cord after injury. Trends Neurosci 19(6): 229-234

Owens GC, Bunge RP. (1989) Evidence for an early role for myelin-associated glycoprotein in the process of myelination. Glia 2(2): 119-128

Owens GC, Boyd CJ, Bunge RP, Salzer JL. (1990) Expression of recombinant myelin-associated glycoprotein in primary Schwann cells promotes the initial investment of axons by myelinating Schwann cells. J Cell Biol 111(3): 1171-1182

Owens GC, Bunge RP. (1991) Schwann cells infected with a recombinant retrovirus expressing myelin-associated glycoprotein antisense RNA do not form myelin. Neuron 7(4): 565-575

Pedraza L, Owens GC, Green LA, Salzer JL. (1990) The myelin-associated glycoproteins: membrane disposition, evidence of a novel disulfide linkage between immunoglobulin-like domains, and posttranslational palmitylation. J Cell Biol 111(6 Pt 1): 2651-2661

Perry VH, Brown MC, Gordon S. (1987) The macrophage response to central and peripheral nerve injury. A possible role for macrophages in regeneration. J Exp Med 165(4): 1218-1223

Pouyani T, Seed B. (1995) PSGL-1 recognition of P-selectin is controlled by a tyrosine sulfation consensus at the PSGL-1 amino terminus. Cell 83(2): 333-343

Quarles RH, Everly JL, Brady RO. (1973) Evidence for the close association of a glycoprotein with myelin in rat brain. J Neurochem 21(5): 1177-1191

Quarles RH, Barbarash GR, Figlewicz DA, McIntyre LJ. (1983) Purification and partial characterization of the myelin-associated glycoprotein from adult rat brain. Biochim Biophys Acta 757(1): 140-143

Quarles RH, Trapp BD. (1984) Localization of myelin-associated glycoprotein. J Neurochem 43(6): 1773-1777

Robinson M, Adu J, Davies AM. (1996) Timing and regulation of trkB and BDNF mRNA expression in placode-derived sensory neurons and their targets. Eur J Neurosci 8(11): 2399-2406

Rossi F, Jankovski A, Sotelo C. (1995) Differential regenerative response of Purkinje cell and inferior olivary axons confronted with embryonic grafts: environmental cues versus intrinsic neuronal determinants. J Comp Neurol 359(4): 663-677

Rudge JS, Pasnikowski EM, Holst P, Lindsay RM. (1995) Changes in neurotrophic factor expression and receptor activation following exposure of hippocampal neuron/astrocyte cocultures to kainic acid. J Neurosci 15(10): 6856-6867

Ruoslahti E, Pierschbacher MD. (1987) New perspectives in cell adhesion: RGD and integrins. Science 238: 491-497

Sadoul R, Fahrig T, Bartsch U, Schachner M. (1990) Binding properties of liposomes containing the myelin-associated glycoprotein MAG to neural cell cultures. J Neurosci Res 25(1): 1-13

Saffell JL, Walsh FS, Doherty P. (1994) Expression of NCAM containing VASE in neurons can account for a developmental loss in their neurite outgrowth response to NCAM in a cellular substratum. J Cell Biol 125(2): 427-436

Saffell JL, Williams EJ, Mason IJ, Walsh FS, Doherty P. (1997) Expression of a dominant negative FGF receptor inhibits axonal growth and FGF receptor phosphorylation stimulated by CAMs. Neuron 18(2): 231-242 (Published erratum appears in Neuron (1998) 20(3): 619)

Sako D, Comess KM, Barone KM, Camphausen RT, Cumming DA, Shaw GD. (1995) A sulfated peptide segment at the amino terminus of PSGL-1 is critical for P-selectin binding. Cell 83(2): 323-331

Salzer JL, Bunge RP. (1980) Studies of Schwann cell proliferation. I. An analysis in tissue culture of proliferation during development, Wallerian degeneration, and direct injury. J Cell Biol 84(3): 739-752

Salzer JL, Holmes WP, Colman DR. (1987) The amino acid sequences of the myelin-associated glycoproteins: homology to the immunoglobulin gene superfamily. J Cell Biol 104(4): 957-965

Salzer JL, Pedraza L, Brown M, Struyk A, Afar D, Bell J. (1990) Structure and function of the myelin-associated glycoproteins. Ann N Y Acad Sci 605:302-12

Sanes JR, Engvall E, Butkowski R, Hunter DD (1990) Molecular heterogeneity of basal laminae: isoforms of laminin and collagen IV at the neuromuscular junction and elsewhere. J Cell Biol 111(4): 1685-1699

Savio T, Schwab ME. (1989) Rat CNS white matter, but not gray matter, is nonpermissive for neuronal cell adhesion and fiber outgrowth. J Neurosci 9(4): 1126-1133

Savio T, Schwab ME. (1990) Lesioned corticospinal tract axons regenerate in myelin-free rat spinal cord. Proc Natl Acad Sci USA 87(11): 4130-4133

Schachner M. (1994) Neural recognition molecules in disease and regeneration. Curr Opin Neurobiol. 4(5): 726-734.

Schafer M, Fruttiger M, Montag D, Schachner M, Martini R. (1996) Disruption of the gene for the myelin-associated glycoprotein improves axonal regrowth along myelin in C57BL/Wlds mice. Neuron 16(6): 1107-1113

Scherer SS, Salzer JL. (1995) Axon-Schwann cell interactions. In: Glial Cell Development. Oxford University Press: Oxford

Schliwa M, Euteneuer U, Bulinski JC, Izant JG. (1981) Calcium lability of cytoplasmic microtubules and its modulation by microtubule-associated proteins. Proc Natl Acad Sci U S A 78(2): 1037-1041

Schneider-Schaulies J, Kirchhoff F, Archelos J, Schachner M. (1991) Down-regulation of myelin-associated glycoprotein on Schwann cells by interferon-gamma and tumor necrosis actor-alpha affects neurite outgrowth. Neuron 7(6): 995-1005

Schnell L, Schneider R, Kolbeck R, Barde YA, Schwab ME. (1994) Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. Nature 367: 170-173

Schreyer DJ, Skene JH. (1993) Injury-associated induction of GAP-43 expression displays axon branch specificity in rat dorsal root ganglion neurons. J Neurobiol 24(7): 959-970

Schwab ME, Schnell L. (1989) Region-specific appearance of myelin constituents in the developing rat spinal cord. J Neurocytol 18(2): 161-169

Schwab ME. (1993) Inhibitors of neurite growth. Annu Rev Neurosci 16: 565-595

Schwab ME, Bartholdi D. (1996) Degeneration and regeneration of axons in the lesioned spinal cord. Physiol Rev 76(2): 319-370

Schwegler G, Schwab ME, Kapfhammer JP. (1995) Increased collateral sprouting of primary afferents in the myelin-free spinal cord. J Neurosci 15(4): 2756-2767

Shewan D, Berry M, Bedi K, Cohen J. (1993) Embryonic optic nerve tissue fails to support neurite outgrowth by central and peripheral neurons in vitro. Eur J Neurosci 5(7): 809-817

Shewan D, Berry M, Cohen J. (1995) Extensive regeneration in vitro by early embryonic neurons on immature and adult CNS tissue. J Neurosci 15(3 Pt 1): 2057-2062

Skene JH, Virag I. (1989) Posttranslational membrane attachment and dynamic fatty acylation of a neuronal growth cone protein, GAP-43. J Cell Biol 108(2): 613-624

Sketelj J, Bresjanac M, Popovic M. (1989) Rapid growth of regenerating axons across the segments of sciatic nerve devoid of Schwann cells. J Neurosci Res 24(2): 153-162

Song HJ, Ming GL, Poo MM. (1997) cAMP-induced switching in turning direction of nerve growth cones. Nature 388: 275-279 (Published erratum appears in Nature (1997) 389: 412)

Song H, Ming G, He Z, Lehmann M, McKerracher L, Tessier-Lavigne M, Poo M . (1998) Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. Science 281: 1515-1518

Stoll G, Trapp BD, Griffin JW. (1989) Macrophage function during Wallerian degeneration of rat optic nerve: clearance of degenerating myelin and Ia expression. J Neurosci 9(7): 2327-2335

Tanaka E, Sabry J. (1995) Making the connection: cytoskeletal rearrangements during growth cone guidance. Cell 83(2): 171-176

Tang S, Woodhall RW, Shen YJ, deBellard ME, Saffell JL, Doherty P, Walsh FS, Filbin MT. (1997a) Soluble myelin-associated glycoprotein (MAG) found in vivo inhibits axonal regeneration. Mol Cell Neurosci 9: 333-346

Tang S, Shen YJ, DeBellard ME, Mukhopadhyay G, Salzer JL, Crocker PR, Filbin MT. (1997b) Myelin-associated glycoprotein interacts with neurons via a sialic acid binding site at ARG118 and a distinct neurite inhibition site. J Cell Biol 138(6): 1355-1366

Tansey MG, Luby-Phelps K, Kamm KE, Stull JT. (1994) Ca(2+)-dependent phosphorylation of myosin light chain kinase decreases the Ca2+ sensitivity of light chain phosphorylation within smooth muscle cells. J Biol Chem 269(13): 9912-9920

Tetzlaff W, Kobayashi NR, Giehl KM, Tsui BJ, Cassar SL, Bedard AM. (1994) Response of rubrospinal and corticospinal neurons to injury and neurotrophins. Prog Brain Res 103: 271-286

Tigyi G, Miledi R. (1992) Lysophosphatidates bound to serum albumin activate membrane currents in Xenopus oocytes and neurite retraction in PC12 pheochromocytoma cells. J Biol Chem 267(30): 21360-21367

Tigyi G, Fischer DJ, Sebok A, Marshall F, Dyer DL, Miledi R. (1996a) Lysophosphatidic acid-induced neurite retraction in PC12 cells: neurite-protective effects of cyclic AMP signaling. J Neurochem 66(2): 549-558

Tigyi G, Fischer DJ, Sebok A, Yang C, Dyer DL, Miledi R. (1996b) Lysophosphatidic acid-induced neurite retraction in PC12 cells: control by phosphoinositide-Ca2+ signaling and Rho. J Neurochem 66(2): 537-548

Trapp BD, Andrews SB, Cootauco C, Quarles R. (1989) The myelin-associated glycoprotein is enriched in multivesicular bodies and periaxonal membranes of actively myelinating oligodendrocytes. J Cell Biol 109(5): 2417-2426

Trapp BD. (1990) Myelin-associated glycoprotein. Location and potential functions. Ann N Y Acad Sci 605: 29-43

Turnley AM, Bartlett PF. (1998) MAG and MOG enhance neurite outgrowth of embryonic mouse spinal cord neurons. Neuroreport 9(9): 1987-1990

van der Merwe PA, Crocker PR, Vinson M, Barclay AN, Schauer R, Kelm S. (1996) Localization of the putative sialic acid-binding site on the immunoglobulin superfamily cell-surface molecule CD22. J Biol Chem 271(16): 9273-9280

Varki A. (1994) Selectin ligands. Proc Natl Acad Sci U S A 91(16): 7390-7397

Varki A. (1997) Sialic acids as ligands in recognition phenomena. FASEB J 11(4): 248-255

Vaudano E, Campbell G, Anderson PN, Davies AP, Woolhead C, Schreyer DJ, Lieberman AR. (1995) The effects of a lesion or a peripheral nerve graft on GAP-43 upregulation in the adult rat brain: an in situ hybridization and immunocytochemical study. J Neurosci 15(5 Pt 1): 3594-3611

Vidal-Sanz M, Bray GM, Villegas-Perez MP, Thanos S, Aguayo AJ. (1987) Axonal regeneration and synapse formation in the superior colliculus by retinal ganglion cells in the adult rat. J Neurosci 7(9): 2894-2909

Vinson M, van der Merwe PA, Kelm S, May A, Jones EY, Crocker PR. (1996) Characterization of the sialic acid-binding site in sialoadhesin by site-directed mutagenesis. J Biol Chem 271(16): 9267-9272

Voyvodic JT. (1989) Target size regulates calibre and myelination of sympathetic axons. Nature 342: 430-433

Walsh FS, Doherty P. (1996) Cell adhesion molecules and neuronal regeneration. Curr Opin Cell Biol 8(5): 707-713

Walsh FS, Doherty P. (1997) Neural cell adhesion molecules of the immunoglobulin superfamily: role in axon growth and guidance. Annu Rev Cell Dev Biol 13: 425-456

Wang Q, Zheng JQ. (1998) cAMP-mediated regulation of neurotrophin-induced collapse of nerve growth cones. J Neurosci 18(13): 4973-4984

Wictorin K, Brundin P, Gustavii B, Lindvall O, Bjorklund A. (1990) Reformation of long axon pathways in adult rat central nervous system by human forebrain neuroblasts. Nature 347: 556-558

Wictorin K, Brundin P, Sauer H, Lindvall O, Bjorklund A (1992) Long distance directed axonal growth from human dopaminergic mesencephalic neuroblasts implanted along the nigrostriatal pathway in 6-hydroxydopamine lesioned adult rats. J Comp Neurol 323(4): 475-494

Williams AF, Barclay AN. (1988) The immunoglobulin superfamily--domains for cell surface recognition. Annu Rev Immunol 6: 381-405

Williams EJ, Furness J, Walsh FS, Doherty P. (1994) Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. Neuron 13(3): 583-594

Xu XM, Guenard V, Kleitman N, Aebischer P, Bunge MB. (1993) A combination of BDNF and NT-3 promotes supraspinal axonal regeneration into Schwann cell grafts in adult rat thoracic spinal cord. Exp Neurol 134(2): 261-272

Xu XM, Guenard V, Kleitman N, Bunge MB. (1995) Axonal regeneration into Schwann cell-seeded guidance channels grafted into transected adult rat spinal cord. J Comp Neurol 351(1): 145-160

Yamamoto H, Fukunaga K, Tanaka E, Miyamoto E. (1983) Ca2+- and calmodulin-dependent phosphorylation of microtubule-associated protein 2 and tau factor, and inhibition of microtubule assembly. J Neurochem 41(4): 1119-1125

Ye JH, Houle JD. (1997) Treatment of the chronically injured spinal cord with neurotrophic factors can promote axonal regeneration from supraspinal neurons. Exp Neurol 143(1): 70-81

Yin X, Crawford TO, Griffin JW, Tu Ph, Lee VM, Li C, Roder J, Trapp BD. (1998) Myelin-associated glycoprotein is a myelin signal that modulates the caliber of myelinated axons. J Neurosci 18(6): 1953-1962