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MAPPING THE NEURITE OUTGROWTH INHIBITORY DOMAIN OF MYELIN-ASSOCIATED GLYCOPROTEIN (MAG) AND BLOCKING THE INHIBITORY EFFECT OF MAG BY INTERLEUKIN-6

by

ZIXUAN CAO

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

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ABSTRACT

Mapping the Neurite Outgrowth Inhibitory Domain of Myelin-associated Glycoprotein (MAG) and Blocking the Inhibitory Effect of MAG by Interleukin-6

by

Zixuan Cao

Advisor: Professor Marie T. Filbin

Myelin-associated glycoprotein (MAG) is one of the potent CNS myelin-associated inhibitors of axonal growth after injury. MAG is a member of the Siglec protein family, a subgroup of the immunoglobulin superfamily that binds sialic acid residues. It has been established that sialic acid-dependent binding of MAG to neurons is not sufficient to inhibit axonal growth. Here, we demonstrate that the neurite outgrowth inhibition site is located on the fifth extracellular Ig-like domain of MAG, and that this region is distinct from the sialic-acid-binding site. First, we show that a truncated form of MAG, MAG(d3-5), is unable to perform sialic-acid-specific binding, yet retains the ability to inhibit neurite outgrowth. On the contrary, MAG(d1-3) can still bind to sialic acid residues, but has no effect on neurite extension. Furthermore, we constructed three chimeric proteins: Sn(d1-3)MAG(d4-5), Sn(d1-3)MAG(d4)Sn(d5) and Sn(d1-4)MAG(d5). Each of these chimeric proteins consists of only MAG Ig domain 4, 5 or both, the remaining Ig domains were replaced by the corresponding parts from the sialoadhesin protein. We found that Sn(d1-3)MAG(d4-5) and Sn(d1-4)MAG(d5) still inhibit axonal regeneration, whereas Sn(d1-3)MAG(d4)Sn(d5) can bind to sialic acid

residues but is unable to inhibit neurite outgrowth. These results indicated that the inhibition site on MAG is primarily located within extracellular domain 5. Based on these findings, a series of peptides derived from MAG Ig domain 5 have been tested in our neurite outgrowth assays. Preliminary investigations indicate that these peptides can function as antagonists to block MAG or CNS myelin-mediated neurite outgrowth inhibition *in vitro*.

The lack of axonal regeneration observed following injury to the adult mammalian CNS is attributable to several factors, including the myelin-associated inhibitors. It has been shown that increasing intracellular cAMP levels can abrogate the inhibitory effects of these molecules in a transcription-dependent manner. One of the genes whose expression is elevated by cAMP is the cytokine Interleukin-6, (IL-6). Here, we show that there is a robust increase in expression of IL-6 by cultured primary neurons after treatment with the cAMP analog, dbcAMP. When added to primary neurons in culture, IL-6 can overcome the inhibition of axonal growth by both MAG and myelin in a dose-dependent and transcription-dependent manner. In addition, following *in vivo* delivery of IL-6 via mini-osmotic pumps, DRG neurons are able to extend long neurites when subsequently grown on a monolayer of MAG-expressing CHO cells. Furthermore, blocking of the IL-6-associated signaling elements gp130 or JAKs, abrogate the IL-6-induced reversal of the MAG and myelin-mediated inhibition of neurite outgrowth. Taken together, this data suggests that IL-6 may be one of the regeneration-associated genes that play a role in the cAMP-induced enhancement of neurite outgrowth in the presence of MAG or myelin and it may also provide a potential therapeutic approach to encourage axonal regeneration following CNS injury.

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CHAPTER I

INTRODUCTION

1.1 Myelin-associated glycoprotein (MAG)

Myelin-associated glycoprotein (MAG) is a minor component of central and peripheral nervous system myelin. It is expressed specifically by myelin-forming glial cells and located restrainedly on the myelin surface at the internodes between the myelin and axolemma. As a cell adhesion protein, MAG was first demonstrated to function in myelination and the maintenance of myelin-axon interaction. But in recent years, more and more evidence has shown that MAG is also a bifunctional protein in axonal regeneration. It promotes axonal extension from young dorsal root ganglia neurons (DRG) yet inhibits neuronal regeneration from postnatal and adult neurons of many types. This characteristic of MAG classifies it as one of the major inhibitors, associated with myelin, that contribute to the overall lack of regeneration capacity in the adult mammalian CNS after injury.

1.1.1 Structural characteristics of MAG

MAG is a 100-kDa glycoprotein that belongs to the Immunoglobulin superfamily (Williams and Barclay 1988), located in the periaxonal membranes of myelin-forming oligodendrocytes and Schwann cells. MAG was first detected by the incorporation of radioactive sugar precursors into glycoproteins that were present in purified central nervous system (CNS) myelin fractions (Quarles, Everly et al. 1973). Three groups (Arquint, Roder et al. 1987; Lai, Brow et al. 1987; Salzer, Holmes et al. 1987) independently sequenced the cDNA for MAG from rat brain. Subsequently, they found that MAG is a integral membrane glycoprotein with a large extracellular exposed N-

terminal segment, a single transmembrane segment and a cytoplasmic C-terminal segment.

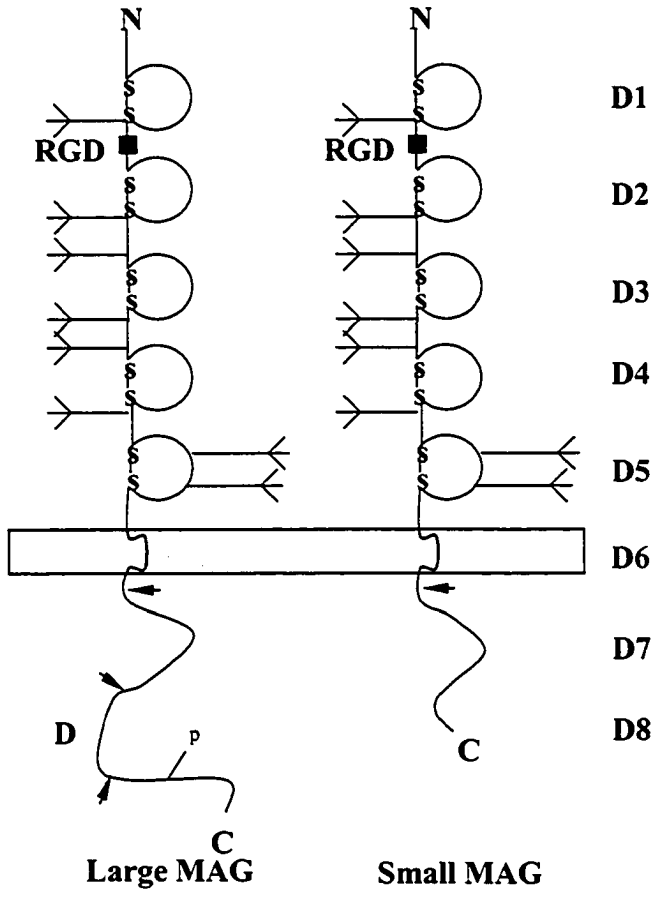


Figure 1.1: Schematic representation of two isoforms of MAG (Large MAG and Small MAG). The five extracellular Ig-like domains are indicated by the circles closed with disulfide bonds and the oligosaccharide chains by the branched structures. The Arg-Gly-Asp (RGD) sequence that may function in binding to integrin-type receptors on cells surfaces is indicated in domain D1. The hydrophobic transmembrane domain is D6. The two developmentally regulated isoforms are generated by alternative splicing of the same gene and only differ in the C-terminal cytoplasmic domains, D8. (Modified from Lai, 1987)

As shown in Figure 1.1, MAG protein exists in two isoforms containing 607 and 563 amino acids with the molecular masses of 72 and 67 kDa, respectively. Both MAG isoforms are generated by alternative splicing from a single gene. The MAG gene includes 13 exons, 10 of which encode the domains designated D0 to D8. The large form (L-MAG) and small form (S-MAG) of MAG are identical in their extracellular and transmembrane domains but different in cytoplasmic C-terminal domain. D0 is a 20-amino acid signal sequence that will be cleaved from the polypeptide during synthesis on the rough endoplasmic reticulum. D1 to D5 are five extracellular Ig-like domains that define MAG as a member of Immunoglobulin superfamily (Arquint, Roder et al. 1987; Lai, Watson et al. 1987; Salzer, Holmes et al. 1987). These domains, which contain primary amino acid sequence patterns that more closely resemble those in V regions of immunoglobulins but cysteine spacing characteristic of C regions, have been termed C2 domains (Williams and Barclay 1988) in contrast to C1 domains that resemble constant domains in both sequence and cysteine spacing. It has been demonstrated that C2 domains are of special feature for many neural adhesion proteins other than MAG (Williams and Barclay 1988). However, the first Ig like domain of MAG, D1, is interesting, for it has typical features of a C2 domain but is folded into a V conformation with 9 β-strands. This gives MAG an unusual intradomain disulfide bond between the second and third cysteines in D1 and an additional interdomain disulfide linkage between D1 and D2 (Salzer, Pedraza et al. 1990). D1 also contains an Arg-Gly-Asp (RGD) tripeptide that is known to be a binding site for some extracellular proteins to integrin-type receptors on cell surfaces (Ruoslahti and Pierschbacher 1986), suggesting that some aspects of MAG interactions with other cells could involve receptors in the integrin class.

However, because of its inaccessibility, it is unlikely that this RGD sequence mediates binding of MAG with integrins (Pedraza, Owens et al. 1990; Sadoul, Fahrig et al. 1990; Yim, Toda et al. 1995). D6 is a single transmembrane domain that contains a hydrophobic peptide sequence of 23 amino acids. It is demonstrated that one soluble, proteolytic fragment of MAG, which is truncated just before this domain, exists *in vivo* and is released from damaged myelin (Sato, Quarles et al. 1984; Yim and Quarles 1992; Moller 1996; Tang, Woodhall et al. 1997). D7 and D8 are two cytoplasmic domains of L-MAG, which is the principle form synthesized during the period of active myelination (Frail and Braun 1984; Lai, Brow et al. 1987; Salzer, Holmes et al. 1987; Tropak, Johnson et al. 1988). In the shorter isoforms of MAG, the sequence of D7 is identical to that of L-MAG, but D8 is replaced by a much shorter form containing only 10 amino acids.

In the rodent CNS, most of the MAG synthesized during active myelination is L-MAG, while both isoforms of MAG are present in adults. However, in the PNS, 95% or more of the MAG synthesized appears to be the shorter isoforms at all ages (Frail, Webster et al. 1985; Tropak, Johnson et al. 1988; Noronha, Hammer et al. 1989). Recently, a mutant mouse deficient in L-MAG has been generated, and data from qualitative and quantitative investigation of both CNS and PNS myelin reveals that L-MAG is critical for the formation of myelin in the CNS, whereas S-MAG is sufficient to maintain the integrity of PNS myelin (Fujita, Kemper et al. 1998).

1.1.2 MAG as a member of Siglec family

MAG was shown to belong to a subgroup of the Ig superfamily, termed Siglecs (Kelm, Brossmer et al. 1998). Siglecs are a group of cell surface Ig-like glycoproteins that share significant amino acid sequence similarity among their first four Ig-like domains. All members are sialic-acid binding proteins (Kelm, Brossmer et al. 1998). The founding members of the Siglec family are sialoadhesin (Siglec 1,marine macrophage-restricted cell surface molecule), CD22 (Siglec 2, B cell adhesion molecule), CD33 (Siglec 3, lymphoid differentiation antigen) and MAG (Siglec 4, myelin-associated glycoprotein) (Filbin 1995; DeBellard, Tang et al. 1996). These proteins share high sequence similarity in their extracellular regions. In the past few years, several human, ape and mouse members of the Siglec family have been identified through genomics and functional analysis. All are highly related to CD33 and share about 50-80% sequence similarity between each other. These new members are therefore described as "CD33-related siglecs" and considered as a separate subgroup from sialoadhesin, CD22 and MAG (Crocker 2002).

Although all the members of Siglecs bind to sialic acid, the specificity of binding differs for each. 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 O- or N-linked glycoconjugates (Kelm, Pelz et al. 1994; Cornish, Freeman et al. 1998). Since the binding affinity of MAG to sialic acid residues is low, MAG must be presented to a neuron as an aggregate before binding can be measured (Kelm, Pelz et al. 1994). This implies that MAG may cluster within the myelin periaxonal membrane and

interact with multiple sialic acid residues on the opposing axonal membrane to exert a stable binding and initiate a functional response *in vivo*.

1.1.3 Roles of MAG in formation and maintenance of myelin

MAG is expressed only by myelin-forming oligodendrocytes (CNS) and Schwann cells (PNS), and comprises about 1% and 0.1% of all myelin proteins in the CNS and PNS, respectively. In the adult CNS, MAG is located exclusively in the periaxonal oligodendroglial membranes of myelin internodes (Trapp, Andrews et al. 1989). This restricted distribution differs from that in adult PNS myelin where MAG is also detected in membranes of the paranodal loops, Schmidt-Lanterman incisures and mesaxons (Quarles 1989). Because of the particular location of MAG at the interface between the axon and myelin sheath, it suggests that MAG may play roles in myelin formation and stabilization (Bartsch 1996). *In vitro* cell culture experiments have provided evidence showing that MAG not only mediates interactions between nerve cells and myelin-forming glial cells (Poltorak, Sadoul et al. 1987; Sadoul, Fahrig et al. 1990), but also enhances survival of oligodendrocytes (Gard, Maughon et al. 1996), and plays a critical role in the initiation of myelination. When levels of MAG expression were experimentally reduced in Schwann cells via infection with retrovirus expressing MAG antisense RNA, the segregation and myelination of large caliber neurites in co-cultured dorsal root ganglion (DRG) neurons were impaired (Owens and Bunge 1991).

Using spontaneously occurring mutants and genetically engineered animals deficient in MAG, investigators have been able to further clarify the differential roles MAG plays in the CNS versus the PNS. During development in MAG deficient mice, a

significant delay in the formation of compact myelin in the optic nerves of young postnatal pups has been observed. The density of retinal ganglion cell axons surrounded by compact myelin was also dramatically reduced compared to age-matched control animals (Montag, Giese et al. 1994). The ultrastructure of compact myelin was unaffected in MAG-deficient mice, but a dilated periaxonal space and an abnormal formation of the periaxonal cytoplasmic collar have been reported (Li, Tropak et al. 1994; Montag, Giese et al. 1994). In addition, some axons in the mutant mice are concentrically surrounded by more than one myelin sheath (Bartsch, Montag et al. 1995). Moreover, in adult MAG-deficient mice, a substantial portion of oligodendrocytes undergo degenerative alterations in their distal processes, a pathological feature apparent either periaxonally or within compact myelin and is reminiscent of multiple toxic or immune-mediated demyelinating diseases, including multiple sclerosis (Rodriguez-Pena, Ibarrola et al. 1993; Lassmann, Bartsch et al. 1997). To conclude, it has been demonstrated that in the CNS MAG is involved in the initiation of myelination, formation of morphologically intact myelin sheaths, and long-tem maintenance of oligodendrocyte structure and myelin integrity. However, in the PNS, MAG only seems to be involved in the formation of morphologically intact myelin and long-term maintenance of myelin structure, but not in the initiation of myelination. This statement was based on data showing that in MAG-deficient mice, dilation of the periaxonal space and loss of the periaxonal cytoplasmic collar has been found in myelinated axons (Trapp, Quarles et al. 1984; Li, Tropak et al. 1994; Montag, Giese et al. 1994). In addition, degeneration of both Schwann cells and axons occurred more frequently, similar to what has been observed in the CNS (Fruttiger, Montag et al. 1995), but the speed of myelination in the

PNS of MAG mutants was no different than that observed in wild-type mice (Montag, Giese et al. 1994).

Taken together, the observations in both cell culture experiments and mutant mice are compatible with the identification of MAG as a cell adhesion molecule, a receptor that can transduce signals into the interior of myelin-forming glial cells, and a molecule that contributes to the cross talk between myelin-forming glial cells and axons.

1.1.4 Role of MAG as a bifunctional protein in neuronal regeneration

Myelin-associated glycoprotein was first demonstrated as a promoter of axonal outgrowth from neonatal dorsal root ganglion (DRG) neurons (Johnson, Abramow-Newerly et al. 1989). However, when grown on transfected Chinese hamster ovary (CHO) cells expressing MAG, both neonatal and adult cerebellar neurons extend neurites 70% shorter than those growing on control CHO cells (Mukhopadhyay, Doherty et al. 1994). Furthermore, evidence has shown that MAG can inhibit neurite outgrowth from all postnatal neurons studied to date including retinal, hippocampal, superior cervical ganglion, spinal cord, and DRG neurons older than PND3 (Mukhopadhyay, Doherty et al. 1994; DeBellard, Tang et al. 1996). This ability of MAG to inhibit axonal regeneration *in vitro* is not limited to expression by CHO cells, since significant inhibition of axonal regeneration has also been seen when neurons are grown on transfected Schwann cells expressing MAG (Shen, DeBellard et al. 1998). In addition, when used as a substrate for axonal outgrowth, both purified native MAG and recombinant MAG can inhibit axonal regeneration (McKerracher, David et al. 1994; Bartsch, Bandtlow et al. 1995; Li, Shibata et al. 1996). These results suggest that MAG can either promote or inhibit neurite

outgrowth depending on the age and type of neuron. Therefore, it is classified as a bifunctional molecule.

Moreover, a soluble, proteolytic product of MAG, dMAG, which consists of the entire extracellular domain and is released in abundance from isolated or damaged myelin, also potently inhibits neurite outgrowth (Tang, Woodhall et al. 1997). In addition, in a growth cone collapse study, about 60% of the growth cones of PND1 hippocampal neurons collapsed when they encountered polystyrene beads coated with recombinant MAG (Li, Shibata et al. 1996), whereas no such collapse was observed with denatured MAG. Together, these results confirmed that MAG is an inhibitory molecule rather than merely a non-permissive substrate for axonal regrowth.

MAG-deficient mice (MAG-/-), which were created by deletion of the MAG gene using homologous recombination (Li, Tropak et al. 1994; Montag, Giese et al. 1994), were used to address the inhibitory effect of MAG on axonal regeneration in CNS *in vivo*. However, there are some discrepancies in the results reported. One group reported that after spinal cord injury, not only more axons regenerated across the lesion site, but also longer axons were extended in the MAG-/- mice compared to MAG+/+ mice (Li, Shibata et al. 1996). They also reported that although there was no significant difference in the average length of axons between neurons grown on substrate of purified myelin from MAG-/- or MAG+/+ mice, but after fractionating the inhibitory activity of CNS myelin into several peaks by DEAE-chromotography (McKerracher, David et al. 1994), a more significant reduction (49%-55%) of inhibitory activity was observed in the first major peak corresponding to MAG than that observed in the whole MAG-/- myelin (Li, Shibata et al. 1996). Consistent with these results, further experiments also implicated that when

using CNS myelin prepared from MAG-/- mice as a substrate, the neurite outgrowth from cerebellar, retinal, or adult DRG neurons was twice as long as on MAG-containing myelin (DeBellard, Tang et al. 1996).

However, controversial results were obtained by another group using a different line of MAG-/- mice (Bartsch, Bandtlow et al. 1995). This group reported that they found no difference in axonal regeneration between MAG-/- and MAG+/+ mice after spinal cord injury. Furthermore, although they observed about 20% of improvement of neurite outgrowth from NG108 cells growing on MAG-/- myelin compared to normal myelin, there was no increase in axonal extension from cerebellar or adult DRG neurons from MAG-/- mice. On the other hand, adding IN-1 antibody onto neuronal cultures can partially reverse the inhibition of axonal outgrowth on both types of myelin extracts and also increase *in vivo* regeneration (Bartsch, Bandtlow et al. 1995).

Two possibilities may lead to this discrepancy. First, two lines of MAG-/- mice were independently created and used by these two research groups, these two mice lines might not be identical and may response differently after injury. Second, the methods of axonal regeneration analysis used by the two groups are different, which may also generate variations in their results. However, despite these discrepancies, two conclusions are confirmed by both teams. First, MAG contributes to the inhibitory effect of CNS myelin on axonal regenerations. Secondly, Some other myelin-associated molecules, except for MAG, contribute to the overall inhibitory effects of CNS myelin. Since then, two other myelin-associated molecules have been identified as inhibitors of axonal regeneration in the CNS other than MAG. Nogo, a member of reticulon family of proteins expressed as three different isoforms, has been cloned by three individual groups

(Chen, Huber et al. 2000; GrandPre, Nakamura et al. 2000; Prinjha, Moore et al. 2000); Among the three Nogo isoforms, Nogo-A, the full-length protein, is highly expressed by oligodendrocytes and localized in the CNS myelin. It has been shown that two regions in Nogo-A, the N-terminal and the extracellular 66-amino-acid region, have inhibitory effects to neurite outgrowth *in vitro* (Chen, Huber et al. 2000; GrandPre, Nakamura et al. 2000; Prinjha, Moore et al. 2000; GrandPre, Li et al. 2002). A third inhibitor identified was Oligodendrocyte-myelin glycoprotein (OMgp), which is also expressed on CNS myelin and can inhibit neurite outgrowth (Wang, Koprivica et al. 2002). All these major myelin-associated axonal regeneration inhibitors will be discussed in more detail later.

Despite conflicting findings concerning CNS nerve regeneration in the MAG-/- mice, studies on peripheral nerve regeneration in MAG mutant transgenic mice further verify that MAG is also an inhibitory component in peripheral myelin *in vivo* (Schafer, Fruttiger et al. 1996). Compared to the CNS, the PNS is considered to be permissive for axonal growth (Caroni and Schwab 1988; Caroni and Schwab 1988; Schwab and Bandtlow 1994). Axonal regeneration after injury in the PNS only takes place after myelin is removed via Wallerian degeneration and Schwann cells have down-regulated expression of all the myelin-associated proteins, including MAG, thus becoming permissive to regeneration (Fawcett and Keynes 1990). This is demonstrated in the C57BL/Ola mouse, a mutant mouse with delayed lesion-induced Wallerian degeneration, that exhibits poor peripheral axonal regeneration after injury. (Brown, Booth et al. 1991; Brown, Lunn et al. 1992). However, in MAG-/-:C57BL/Ola mice (created by crossing MAG-/- mice with C57BL/Ola mice), an improvement in regeneration from axons associated with myelinated fibers was observed (Schafer, Fruttiger et al. 1996). In a

separate study, transgenic mice that express MAG under the control of the p75 promoter were generated (De Bellard and Filbin 1999). In these transgenic mice, expression of MAG by Schwann cells is upregulated with endogenous p75 after PNS injury. Interestingly, PNS regeneration in these transgenic mice is greatly retarded compared to their wild type counterparts. These results suggest that MAG is not only an inhibitor of neurite outgrowth *in vitro*, but also an inhibitory component of myelin that contributes to the prevention of axonal regeneration in *vivo*.

1.2 Regeneration in the adult mammalian CNS

Axons in the mammalian CNS do not spontaneously regenerate following injury and consequently, there is little functional recovery afterward. This differs greatly from the response of injured axons in the adult PNS, which do regenerate following injury. At present, there are no clinical treatments available to stimulate regeneration of cut axons. But in the past decade, numerous studies have focused on finding the mechanisms that prevent axons in adult CNS from regrowing after injury and searching for therapeutic strategies to overcome them. With all the evidence collected to date, more light has been shed on these inhibitory mechanisms. The reasons fall into two major categories, which are the extrinsic unfavorable environment and the intrinsic growth capacity of neurons.

1.2.1 Extrinsic inhibitory environment in adult mammalian CNS

Althought it was demonstrated decades ago that axons in the adult mammalian CNS can not perform spontaneous regeneration after injury, more and more convincing results have indicated that adult CNS axons are not intrinsically incapable of

regeneration. When provided with a suitable environment such as peripheral nerve (Berry, Carlile et al. 1996) or embryonic tissue (Bregman, McAtee et al. 1997), injured CNS axons can extend for long distances beyond the lesion site. This brings up the possibility that the local environment of the adult CNS may be the factor that halts the injured axons from regenerating. Multiple factors in the environment of the adult CNS may contribute to this regenerative failure, such as a post-natal decline in growth-promoting molecules such like neurotrophic factors, formation of a glial scar which contains several inhibitory molecules as well as acting as a physical barrier to axonal growth, and the presence of myelin-associated inhibitors of axonal extension in the adult CNS (Figure 1.2).

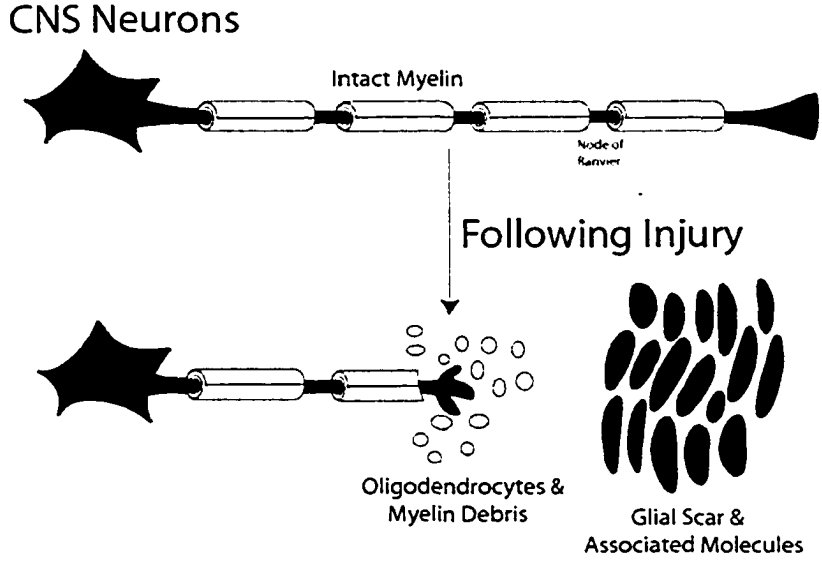


Figure 1.2 Schematic illustration of the CNS environment that neurons encounter after injury. Immediately after injury, a regenerating axon will encounter many myelin-associated molecules—expressed on the membrane surface or released after damage—that can inhibit axonal extension. In addition, the glial scar also contains several inhibitory molecules as well as acting as a physical barrier to axonal growth (Spencer, Domeniconi et al. 2003)

1.2.1.1 Myelin-associated inhibitors

It has long been recognized that adult mammalian CNS white matter (myelin) is inhibitory for axonal regeneration (Berry 1982). Consistent with this view are the observations that, first, lesioned embryonic spinal cord neurons can regenerate for a period that ends at the same time when myelination in the spinal cord begins (Keirstead, Hasan et al. 1992). Second, extensive regenerative growth from injured corticospinal tract fibers was observed in mice immunized with CNS myelin whereas no regeneration at all occurred in control mice (Huang, McKerracher et al. 1999). In the past few years, three molecules, Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp) have been identified as major inhibitory factors associated with adult CNS myelin (McKerracher, David et al. 1994; Mukhopadhyay, Doherty et al. 1994; Chen, Huber et al. 2000; GrandPre, Nakamura et al. 2000; Prinjha, Moore et al. 2000; Wang, Kim et al. 2002; Wang, Koprivica et al. 2002). Following their identification, substantial progress has been made towards the understanding of the nature of these myelin-associated inhibitors and the mechanisms of their action.

1.2.1.1.1 Nogo

Of the multiple inhibitory components identified in CNS myelin, Nogo is the most extensively studied. The study of Nogo started with the pioneering work of Martin Schwab and his colleagues. They first found that the inhibitory activity of myelin was enriched in two protein fractions of myelin extraction, with molecular weights of 35kDa (NI-35) and 250kDa (NI-250) (Caroni and Schwab 1988). A monoclonal antibody, termed IN-1, was raised against purified NI-250 which recognized both NI-35 and NI-

250 (Caroni and Schwab 1988). Application of IN-1 can not only neutralize the non-permissiveness of CNS myelin and oligodendrocytes thus improve neuronal extension *in vitro*, but can also enhance regrowth of corticospinal tract fibers *in vivo* (CST) with partial functional recovery (Caroni and Schwab 1988). However, the identity of the neuronal antigen(s) for IN-1 remained elusive until recently. Three groups have individually cloned the cDNA of a protein, termed Nogo, based on the peptide sequences derived from the purified bovine homologue of NI-250 (Chen, Huber et al. 2000; GrandPre, Nakamura et al. 2000; Prinjha, Moore et al. 2000). There are three major transcripts (Nogo-A, -B, and –C) originating from the Nogo gene by both alternative promoter usage and alternative splicing (Figure 1.3). Nogo-A appears to be NI-250 and the size of Nogo-B indicated that it might be NI-35. The three transcripts have a common carboxy-terminal domain of 188 amino acids, and this region is homologous to the reticulon protein family. The carboxyl portion of all Nogo isoforms contains two putative transmembrane domains, which are separated by an extracellular 66-amino-acid loop, termed Nogo-66 (GrandPre, Nakamura et al. 2000). Topological studies have implied that both the amino and carboxy termini of Nogo are likely to be intracellular, with only Nogo-66 expressed on the cell surface (GrandPre, Nakamura et al. 2000). Nogo-A is the only isoform that is specifically expressed in the CNS and is present on the cell surface of oligodendrocytes. It has two functional domains, Nogo-66 and Amino-Nogo. Both domains are thought to contribute to myelin-derived inhibition of neurite outgrowth. Nogo-66 specifically inhibits neurite extension, while the Amino-Nogo inhibits 3T3 fibroblast spreading, as well as blocking neurite growth (Chen, Huber et al. 2000; Prinjha, Moore et al. 2000; Fournier, GrandPre et al. 2001). The finding of the inhibitory

function of Amino-Nogo seems a bit perplexing, for structural studies suggest it be located on the cytoplasmic side of the membrane. This apparent paradox needs to be resolved and the answer may lay in the following two possibilities: conformational changes following injury which somehow expose the amino-terminal domain extracellularly; or disruption of myelin caused by injury releases the entire protein to be encountered with axons. The independent action of these two inhibitory domains suggests a synergistic effect on axon outgrowth.

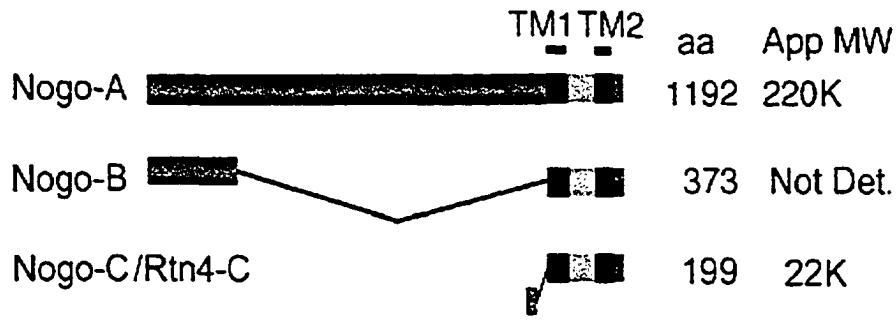


Figure 1.3: Schematic structure of Nogo isoforms. Three Nogo isoforms, Nogo-A, -B and –C are generated by alternative RNA splicing or promoter usage of a single gene. The region common to all three isoforms is the two transmembrane domains (TM1 and TM2) and the extracellular 66-amino-acid region between them (Chen, Huber et al. 2000).

1.2.1.1.2 Myelin-associated glycoprotein (MAG)

With a more detailed description of MAG in the first part of this chapter, the characteristics of MAG with regard to its function as a myelin-associated inhibitor of nerve regeneration will only be summarized briefly here.

MAG is a member of the immunoglobulin (Ig) superfamily, which contains five Ig-like domains in its extracellular sequence (Lai, Watson et al. 1987; Salzer, Holmes et al. 1987), and is present in both CNS and PNS myelin. MAG was first proved to be an

adhesion molecule that is required in the initiation of CNS myelination and maintenance of myelin-axon interaction in both the CNS and PNS (Bartsch 1996). In the last decade, it was revealed that MAG is also a bifunctional molecule with regard to neurite outgrowth, for it can either promote or inhibit neurite outgrowth depending on the age and type of neuron (McKerracher, David et al. 1994; Mukhopadhyay, Doherty et al. 1994; DeBellard, Tang et al. 1996). Moreover, a soluble, proteolytic product of MAG, dMAG, which consists of the entire extracellular domain and which is released in abundance from isolated or damaged myelin, also potently inhibits neurite outgrowth (Tang, Woodhall et al. 1997). Together, these results suggest that MAG is indeed an inhibitory molecule rather than merely a non-permissive substrate for axonal growth.

To study the function of MAG in inhibiting neuronal regeneration *in vivo*, MAG-deficient mice were generated and investigated by two groups. Conflicting results have been reported in which one group observed improved axonal regeneration in MAG-/- mice compared to wild type mice, while the other group found no difference (Li, Tropak et al. 1994; Montag, Giese et al. 1994). Nevertheless, despite the differences between the two mice lines and the methods each group used to measure the extent of regeneration from the injured axons, both groups observed an improvement in neurite length for NG108 cells grown on MAG-/- myelin substrate (Bartsch, Bandtlow et al. 1995; Li, Shibata et al. 1996). In addition, results from our lab also show an increase in neurite length of postnatal cerebellar, retinal, or adult DRG neurons when they were grown on MAG-/- myelin (DeBellard, Tang et al. 1996). These observations agree in that MAG contributes, at least partly, to the inhibitory activity of adult CNS myelin. Furthermore, studies with MAG-/-:C57BL/Ola mice and the transgenic p75 mice also implied that

MAG is an inhibitory molecule for axonal regeneration *in vivo* (Schafer, Fruttiger et al. 1996).

1.2.1.1.3 Oligodendrocyte-myelin glycoprotein (OMgp)

OMgp was first identified as a 440 amino-acid glycoprotein with a glycosylphosphatidylinositol (GPI) linkage anchored on the cell membrane of myelinating oligodendrocytes (Mikol, Rongnoparut et al. 1993; Habib, Marton et al. 1998). Only in very recent studies has OMgp been identified as another inhibitory component associated with CNS myelin.

In an initial purification procedure attempting to identify myelin-associated inhibitors, two major fraction of myelin protein was observed to have inhibitory activity, with MAG present in one of them (McKerracher, David et al. 1994). The Braun group further separated inhibitory proteins from the other fraction by PNA-agarose chromatography and identified OMgp as a potent inhibitor, first naming it Arretin based on its growth inhibitory properties (Kottis, Thibault et al. 2002). Simultaneously, the He group identified OMgp as an inhibitor by testing whether any GPI-anchored myelin proteins could prevent regeneration (Wang, Koprivica et al. 2002). OMgp was found to be highly enriched in Phospholipase C-released fractions of myelin and shown to have potent growth cone collapsing and neurite outgrowth inhibitory activities. The inhibitory activity of OMgp *in vitro* appears to be as potent as that of MAG and Nogo, and it also recognizes NgR as its neuronal binding receptor just as MAG and Nogo-66 do (Domeniconi, Cao et al. 2002; Liu, Fournier et al. 2002; Wang, Koprivica et al. 2002).

1.2.1.1.4 NgR-p75 is the functional receptor signaling complex shared by the three major myelin-associated inhibitors

The cloning of Nogo was an exciting movement in the field due to the intriguing results previously achieved with monoclonal antibody IN-1, in which both *in vitro* axonal outgrowth against myelin inhibition and *in vivo* anatomical and functional recovery following CNS injury had been achieved (Caroni and Schwab 1988; Schnell and Schwab 1990; Bregman, Kunkel-Bagden et al. 1995). Although it is clear that the IN-1 antibody must recognize several other proteins in spinal cord extracts other than Nogo (Spillmann, Bandtlow et al. 1998), Nogo's predominant contribution to myelin inhibition encouraged more studies focusing on the mechanisms of its action. Further insight into this question came with the cloning of a receptor for Nogo-66 (Fournier, GrandPre et al. 2001). The receptor was expression cloned using an alkaline phosphatase (AP) fusion protein assay. A functional active fusion protein, Nogo-66-AP was used to screen COS-7 cells transfected with pools of cDNA from mouse brain cDNA library. A novel 473 amino acid protein termed the Nogo-66 receptor (NgR) was then cloned (Figure 1.4).

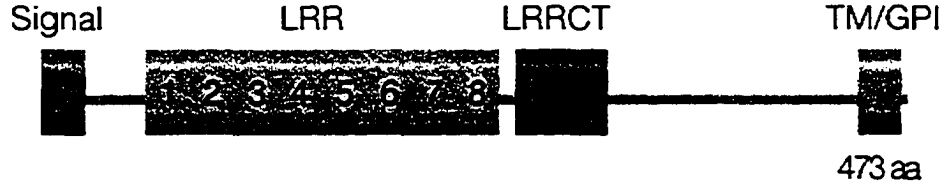


Figure 1.4: Structure of NgR. NgR contains a translocation signal sequence (Signal), eight Leucine-rich repeat motifs (LRR), an LRR carboxy-terminal motif (LRRCT), and a GPI anchor (Fournier, GrandPre et al. 2001)

NgR is a membrane surface protein with a signal sequence followed by eight leucine-rich-repeat (LRR) domains, an LRR carboxy-terminal flanking domain that is

cysteine rich, a unique region and a glycosylphosphatidylinositol (GPI) link that anchors the protein on the outer leaflet of plasma membrane. The distribution of NgR mRNA expression is predominant in the brain by a variety of CNS neurons, but undetectable in white matter or oligodendrocytes (Fournier, GrandPre et al. 2001), which is consistent with its function in regulating axonal regeneration and plasticity in the adult CNS. Notably, NgR mRNA has been found to be expressed in cerebral cortex pyramidal neurons and cerebellar Purkinje neurons, whose neuronal extension is inhibited by Nogo and myelin but can be restored by IN-1 or anti-Nogo-A antibody treatment (Schnell and Schwab 1990; Buffo, Zagrebelsky et al. 2000).

The ability of NgR to bind directly to Nogo-66 was demonstrated by a co-precipitation and its function as a receptor for Nogo-66 was shown in both gain of function and loss of function assays (Fournier, GrandPre et al. 2001). It has been shown that E12 chick DRG neurons respond strongly to Nogo-66 in a growth cone collapse assay (GrandPre, Nakamura et al. 2000). However, when these neurons were treated with phosphatidylinositol-specific phospholipase C (PIPLC) to remove all the GPI linked surface proteins including NgR, they no longer respond to Nogo-66. On the other hand, E7 chick retinal ganglion cell neurons (RGC) do not express NgR endogenously nor do they respond to Nogo-66 in growth cone collapse assay, these neurons became Nogo-66 sensitive after infection with recombinant HSV virus expressing NgR. Therefore, NgR is the neuronal receptor for Nogo and is capable of mediating both its binding and inhibitory activities.

Unlike the identification of the Nogo receptor, searching for the MAG receptor remained elusive for many years after MAG had been demonstrated as an inhibitor of

axon outgrowth in 1994, though many researchers have sought to answer this question. After the identification of MAG as a member of Siglec family (Kelm, Pelz et al. 1994), the hunt for its receptor has focused on the its sialic acid binding activity. Work from the Filbin group has shown that the first Ig-like domain of MAG, specifically the amino acid Arg118 within Ig domain 1, is responsible for its binding to sialic acid residues (DeBellard, Tang et al. 1996; Tang, Shen et al. 1997). But they also demonstrated that the sialic-acid-dependent binding activity of MAG is neither necessary nor sufficient for MAG to exert its inhibitory effect on neurons. Evidence came from the fact that if MAG, mutated on Arg118, was expressed as a cell membrane protein, it still inhibited neurite extension (Tang, Shen et al. 1997). Recently, gangliosides, in particular GD1a and GT1b, were proposed as function-mediating binding partners for MAG, because clustering of GT1b with an IgM antibody mimics the inhibitory effects of MAG (Vinson, Strijbos et al. 2001; Vyas, Patel et al. 2002). However, very recent discoveries indicated that this may not be the case. Two groups have both independently identified that NgR also serves as the functional receptor for MAG (Domeniconi, Cao et al. 2002; Liu, Fournier et al. 2002).

In a previous study, Filbin's group had shown that a soluble, chimeric form of MAG, MAG-Fc (which contains the extracellular domain of MAG fused with the Fc portion of human IgG), was able to specifically precipitate a number of neuronal surface proteins. One of them is about 80 kDa which is approximately the same molecular weight as NgR (De Bellard and Filbin 1999). Recently, they further revealed that this neuronal MAG-binding protein is indeed NgR, which functions as the MAG-binding receptor and is necessary for MAG to exert inhibition on neurite outgrowth. The evidence came from

several findings. First, the normal binding and inhibition of neurite outgrowth by MAG requires a GPI-linked protein, which is consistent with the GPI linkage profile of NgR. Second, direct association of MAG with NgR was assessed by co-immunoprecipitation and cell surface binding of MAG with either transfected CHO cell-expressed or neuronal endogenous NgR. In addition the binding between MAG and NgR was also shown to be sialic-acid-independent. Third, preventing the interaction between endogenous neuronal NgR and MAG by using excess soluble NgR as a competitor, blocking anti-NgR antibody or a truncated, dominant negative form of NgR, abrogated the neurite outgrowth inhibition by MAG and myelin in general (Domeniconi, Cao et al. 2002). Simultaneously, Strittmatter's group also reported the identification of MAG as a functional ligand of NgR using an expression-cloning strategy (Liu, Fournier et al. 2002). With the use of different binding and functional assays, they also observed the direct binding of MAG to NgR and the indispensability of NgR for the inhibitory activity of both MAG and CNS myelin. Even though there is still a minor discrepancy between these two groups with regard to whether MAG and Nogo-66 compete with each other for the binding site on NgR, it was clearly demonstrated that NgR signals axonal growth inhibition by MAG, and is a required component of MAG inhibitory signaling.

Interestingly, Wang et al. also identified NgR as an OMgp binding protein via an expression-cloning strategy at almost the same time. In addition to a direct binding between NgR and OMgp, they also revealed that NgR plays a functional role in the OMgp-mediated growth inhibition. This was shown by the fact that transfection of NgR into neurons that are normally unresponsive to OMgp, can make them sensitive to and be inhibited by OMgp (Wang, Koprivica et al. 2002).

Therefore, NgR mediates growth inhibition by MAG, Nogo, and OMgp, three structurally different proteins. Even more surprising is that all three myelin-derived growth inhibitory proteins bind NgR with high affinity, and blocking access to NgR removes sensitivity to all three growth inhibitory proteins (Fournier, GrandPre et al. 2001; Domeniconi, Cao et al. 2002; Liu, Fournier et al. 2002; Wang, Koprivica et al. 2002). These findings indicate the possibility of designing an NgR inhibitor that can block the effects of all the myelin-associated inhibitors at the same time and allow injured axons to regrow *in vivo*.

Since NgR is GPI-linked to the neuron surface and does not have an intracellular domain, it is reasonable to suspect the requirement of an additional transmembrane protein to transduce the NgR signal intracellularly. A previous study has already suggested that p75 may play a role in signaling in response to MAG (Yamashita, Higuchi et al. 2002). This group showed that neurons isolated from p75 -/- mice were no longer inhibited by MAG and that p75 expression co-localized with sites of MAG binding. Also, the interaction between MAG and p75 can be visualized by co-immunoprecipitation of endogenous p75 with MAG from mouse postnatal cerebellum. However, since no evidence so far has shown that MAG associates with p75 directly, these results suggest p75 may act as a signal-transducing element rather than a binding partner for MAG. Indeed, this postulation is strongly implied by the recent studies of two groups. Soon after the identification of NgR as the ligand binding receptor for the three major myelin inhibitors, Wang and colleagues (Wang, Kim et al. 2002) showed strong evidence that p75 not only performs direct association with NgR endogenously through its extracellular domain, but is also necessary for NgR-mediated neurite outgrowth inhibition induced by

each individual myelin inhibitor and by myelin in general. This group found that the inhibitory activities of each individual inhibitor or purified adult CNS myelin was significantly abolished if the NgR-p75 interaction was interrupted, using either competitive soluble p75-Fc protein or a dominant negative NgR which lacks the binding site for p75. In addition, Mu-ming Poo's group also observed similar results showing that p75 and NgR form a receptor complex which mediates the repulsive signaling by MAG (Wong, Henley et al. 2002). Taken together, with the previous demonstration that MAG elicits axonal outgrowth inhibition activity through activation of the small GTPase, Rho (Lehmann, Fournier et al. 1999), a new model could then be established: After CNS injury, myelin-associated inhibitors are exposed to injured neurons and bind to NgR. This ligand-receptor interaction leads to the association of p75 with NgR and the transduction of inhibitory signals downstream into the cell. By activation of Rho, this results in the inhibition of nerve regeneration (Figure 1.5).

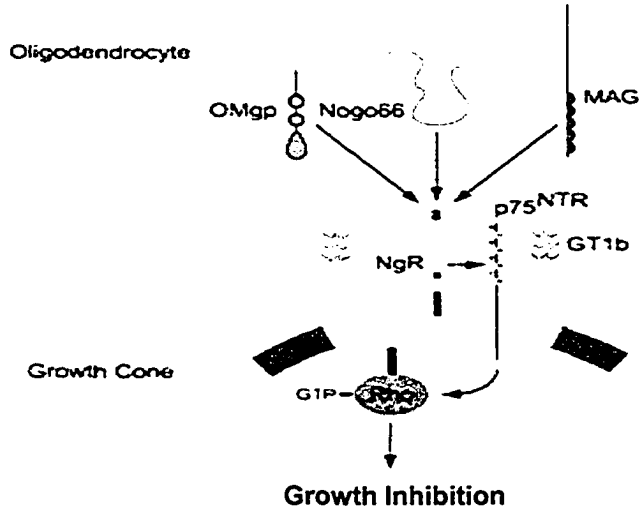


Figure 1.5: Schematic diagram showing signals induced by myelin-associated neurite growth inhibitors. MAG, Nogo66 and OMgp all bind with the Nogo66 receptor (NgR), which in turn interacts with p75. The activation of p75 transduces the inhibitory signals intracellularly, and through activation of Rho GTPase, inhibits neurite outgrowth (McKerracher and Winton 2002).

1.2.1.1.5 Other myelin-associated inhibitors

Other than Nogo, MAG and OMgp, there are also a number of other molecules associated with myelin that inhibit axonal regeneration. In the past few years, several axonal guidance molecules have been suggested to be involved in axonal regeneration as well. For example, collapsin-2, an inhibitory guidance cue active during development, was reported to be expressed by oligodendrocytes and present in both the gray and white matter in the spinal cord (Luo, Shepherd et al. 1995). In addition, netrins and slits, both of which are important guidance cues for axonal path-finding, have shown to repel spinal motor, olfactory and hippocampal axons and collapse their growth cones *in vitro* (Li, Chen et al. 1999; Nguyen Ba-Charvet, Brose et al. 1999). Moreover, Janusin/restrictin, a tenascin-related ECM molecule, is also found to be secreted mainly by oligodendrocytes and exhibits repulsive activity towards growth cones when used as a substrate *in vitro* (Pesheva, Spiess et al. 1989; Schachner, Taylor et al. 1994). Finally, chondroitin sulphate proteoglycans (CSPG), expressed by reactive astrocytes, which have been suggested previously to inhibit axonal outgrowth *in vitro* (Canning, Hoke et al. 1996), were also demonstrated to play a role in inhibiting axonal regeneration in the CNS after injury *in vivo*. It was recently reported that removal of glycosaminoglycan (GAG) chains from CSPGs *in vivo* resulted in improved regeneration and partial sensory recovery (Bradbury, Moon et al. 2002).

1.2.1.2 Glial scar

After spinal cord injury, damaged neurons are exposed not only to the myelin-associated inhibitory molecules, but also to a glial scar which forms within weeks of

injury by primarily reactive astrocytes (Reier, Perlow et al. 1983; Jakeman and Reier 1991). Electron microscopic observations reveal that after CNS injury, the processes of reactive astrocytes are tightly interlinked and form a mechanical barrier which prevents regenerating neurons crossing. Besides forming a physical obstacle, condensed reactive astrocytes also create a chemical barrier by expressing a number of inhibitory molecules including tanascin, keratin, semaphoring III and chondroitin sulfate proteoglycans (CSPG), all of which have been shown to inhibit axonal outgrowth *in vitro* (Letourneau, Condic et al. 1994; McKeon, Hoke et al. 1995; Canning, Hoke et al. 1996; Davies, Goucher et al. 1999; Pasterkamp, Giger et al. 1999). Thus, both the mechanical and biochemical changes that occur at the lesion site establish an inhibitory environment for regenerating neurons.

1.2.2 Intrinsic neuronal state

As described above, due to the myelin-associated inhibitory molecules that become exposed after spinal cord damage and the formation of a glial scar, the environment of the adult CNS spinal cord prevents injured axons from regenerating both mechanically and chemically. However, embryonic neurons transplanted into the injured spinal cord are able to regenerate over long distances (Bates and Stelzner 1993; Hasan, Keirstead et al. 1993). This suggests that embryonic neurons are intrinsically different from their adult counterparts and have a higher growth capacity that can overcome the inhibitory effect elicited by CNS myelin. Another example comes from the different responses of peripheral and central axonal branches of adult DRG neurons to injury. The peripheral branch of adult DRG axon regenerates after injury, whereas the central branch,

which courses in the dorsal columns of the spinal cord, does not (Ramon y Cajal 1928). In the attempts to enhance the intrinsic growth capacity of injured central branch, improved axonal extension after central branch injury was first achieved using peripheral nerve graft accompanied with a peripheral nerve lesion performed at the same time (Richardson and Issa 1984; Richardson and Verge 1986; Oudega, Varon et al. 1994). Then, more profound regeneration of lesioned dorsal column axons was reported by Woolf's group (Neumann and Woolf 1999) (Figure 1.6).

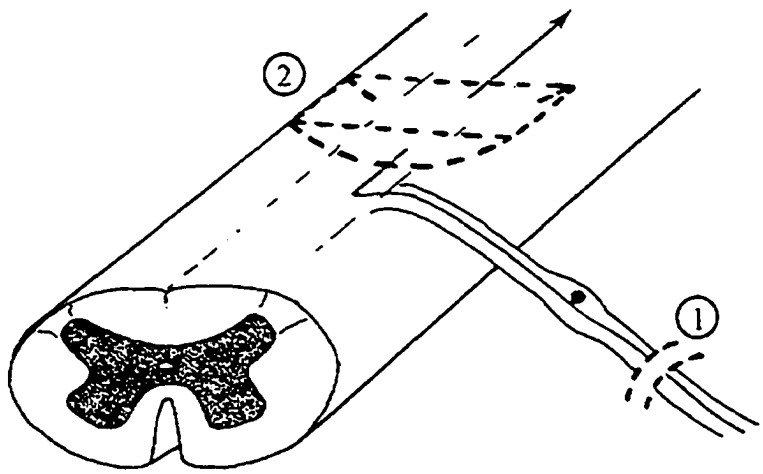


Figure 1.6: Schematic illustration of preconditioning peripheral branch lesion and subsequent central branch lesion in DRG nerves. Preconditioning lesions were created in the sciatic nerve (L4-L6) (1) either simultaneously with, or 1-2 weeks prior to, a dorsal column transection at T6-T7 (2). Regeneration occurred through the dorsal comumn lesion site if the preconditioning lesion preceded the dorsal column lesion by 1 or 2 weeks; regrowth occurred only into the lesion if the reconditioning lesion was inflicted at the same time; no regeneration occurred without a peripheral lesion. The dashed line shows the sites of lesions. (Filbin 1999)

This group demonstrated that transection of the sciatic nerve (conditioning lesion) resulted in regeneration of the subsequently lesioned dorsal column axons of the same DRG neurons, without the addition of any agents that block myelin or glial scar

inhibitors. Optimal regeneration of dorsal column axons was obtained when the lesion was performed one week after a sciatic nerve transection. Approximately 50% of the experimental animals showed substantial axonal extension into and beyond the lesion site, mostly through the gray matter but with considerable regeneration also observed in the white matter. This indicates that the conditioning lesion in the sciatic nerve increases the growth capacity of the DRG neurons, and allows the central branch to overcome the inhibition of the CNS environment. The question that now remains is what types of changes occur in the DRG neurons after a conditioning lesion that induce this increased growth capacity.

The elements that contribute to the intrinsic growth capacity of neurons have long been sought. Several studies have indicated that cytosolic cyclic nucleotide levels can dictate the neuronal response to guidance cues, neurotrophic factors, and myelin inhibitors (Lohof, Quillan et al. 1992; Kim and Wu 1996; Ming, Song et al. 1997; Song, Ming et al. 1997; Cai, Shen et al. 1999). We have demonstrated that neuronal cAMP levels are key elements in dictating the regenerative capacity of neurons. First, exposure of neurons to neurotrophins prior to encountering myelin inhibitors results in an abrogation of the inhibition. This "priming" effect is mediated by an elevation of neuronal cAMP levels, and can be abolished by addition of a PKA inhibitor during the priming procedure. Furthermore, artificial elevation of cAMP levels with an analogue of cAMP, dibutyryl cAMP, can also block the inhibition by MAG or myelin (Figure 1.7) (Cai, Shen et al. 1999). Also, it was found that endogenous levels of cAMP were high in embryonic and neonatal neurons and decreased with age. The switch of neuronal response to MAG from promotion to inhibition of neurite outgrowth, which marks the

developmental loss of regenerative capacity, parallels the developmentally regulated decrease in endogenous neuronal cAMP levels (Cai, Qiu et al. 2001). Finally, the potential roles that intracellular cAMP levels play in regulating neuronal growth capacity explains very well the mechanisms underlining the improved growth of dorsal column axons after a conditioning lesion (Qiu, Cai et al. 2002). Strong evidence has shown that 1 day after transection of the sciatic nerve, cAMP levels in the DRG were elevated by about three-fold as compared to uninjured control. This was accompanied by a significantly increased growth capacity of DRG neurons on MAG and myelin. One week after sciatic nerve transection, the cAMP levels return to control levels, but the growth of DRG neurons on MAG and myelin was even better than 1 day after injury. Moreover, direct injection of db-cAMP into the DRG without a peripheral lesion mimics the conditioning lesion effect on regeneration of injured dorsal column fibers, resulting in significant axonal regrowth into and beyond the lesion site.

In summary, the intrinsic growth state of the neuron dictates its response to the CNS environment and consequently regulates its ability to regenerate after injury. It has been established that endogenous cAMP levels play a key role in determining intrinsic growth capacity of neurons. Cyclic AMP levels are high in younger neurons but decrease with age. This decrease in cAMP levels parallels the developmental loss of the regenerative capacity of the neuron. In adult neurons, an elevation in cAMP results in increased growth capacity and overcomes the inhibition by MAG and myelin. Thus, manipulation of neuronal cAMP levels is likely to have significant therapeutic implications in improving nerve regeneration in the adult CNS after injury.

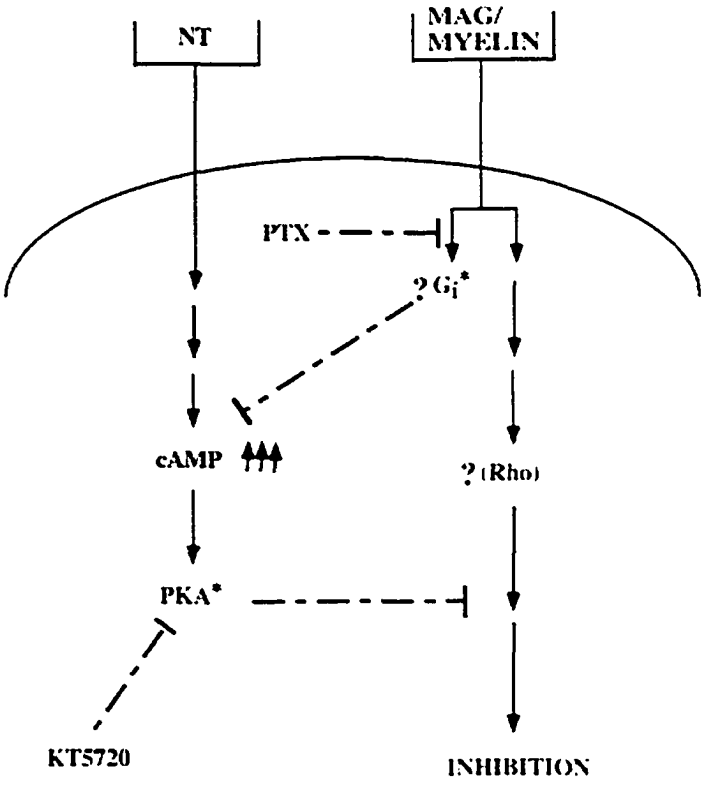


Figure 1.7: Model explain how priming with neurotrophins blocks inhibition by MAG or myelin. During priming, neurotrophins (NT) interact with a surface neuronal receptor inducing and increase in neuronal cAMP, which in turn activates protein kinase A (PKA*). The activity of protein kinase A can be inhibited specifically by KT5720. Activation of PKA or some unidentified downstream signal then blocks subsequent inhibition by MAG or myelin, perhaps by inactivating the small G protein, Rho. If, however, neurotrophin is added to the neuron at the same time as exposure to MAG or myelin, cAMP is prevented from increasing by MAG/myelin activation of a pertussis toxin (PTX)-sensitive G protein (Gi*) and so inhibition of axonal regeneration is not blocked. G protein activation by MAG or myelin has no direct effect on inhibition of axonal regeneration by MAG or myelin. (Cai, Shen et al. 1999)

1.2.3 Approaches to achieving long distance axon regeneration after CNS injury

Success in achieving long distance regeneration in the CNS of adult animals was first demonstrated many years ago using peripheral nerve grafts to replace the inhibitory CNS environment (Bray, Villegas-Perez et al. 1987; Aguayo, Rasminsky et al. 1991;

Berry, Carlile et al. 1996). Peripheral nerve grafts are living nerve tissue that contain Schwann cells which provide trophic supply and a favorable substrate for growth. In experiments where peripheral nerve grafts were used to replace cut optic nerves, not only did retinal ganglion cells extend axons the full length of the grafts (McKerracher, Vidal-Sanz et al. 1990), but also formed functional synapses with target cells which can be visualized by both electron microscopy and the recoding of post-synaptic responses following stimulation (Vidal-Sanz, Bray et al. 1987; Keirstead, Rasminsky et al. 1989; Vidal Sanz 1991). These studies have shed light on the potential for inducing long distance regeneration and reconnection in the CNS while providing an excellent model for further study. However, they are not likely to present a clinically practical therapy. The ideal strategy for repairing the injured CNS will allow axons to regrow directly on their native environment and form correct synaptic connection with target cells. Several studies in recent years have implicated some potentially useful methods to achieve this.

1.2.3.1 Blocking myelin-associated inhibitors with antibodies

Stimulation of regeneration in the CNS by blocking growth inhibitory molecules with antibodies was first demonstrated using IN-1 antibody to neutralize the inhibitory effect of CNS myelin after spinal cord and optic nerve injury (Schnell and Schwab 1990; Weibel, Cadelli et al. 1994). When hybridoma cells secreting IN-1 antibody were implanted into the brain to achieve a continuous supply of IN-1 antibody for the post-lesion period, the extension of injured corticospinal axons was greatly improved (Schnell and Schwab 1990). Moreover, axon regeneration in the spinal cord following IN-1 treatment has been shown to acquire partial functional recovery (Bregman, Kunkel-

Bagden et al. 1995). However, the number of regenerating axons was quite small and it is still unclear whether the functional recovery observed after spinal cord injury with IN-1 antibody treatment was due to regrowth and reconnection of injured axons or rearrangement of local circuitry by stimulating the sprouting of local uninjured axons (Buffo, Zagrebelsky et al. 2000). Nevertheless, the IN-1 antibody treatment certainly provided a useful method to partially overcome the inhibitory environment in the CNS and to encourage axonal regeneration. With the identification of more inhibitory components associated with CNS myelin, such as Nogo, MAG, OMgp and CSPG, a combination of antibodies against these inhibitors may have great potential in stimulating the regeneration of injured CNS axons.

Using a therapeutic vaccine approach, a novel immunization method avoiding autoimmune reactions, Huang and colleagues have achieved more remarkable long-distance regeneration in injured corticospinal tract fibers (Figure 1.8) (Huang, McKerracher et al. 1999). Prior to injury, animals were immunized over a 3-week period with a CNS myelin preparation to produce antibodies against all of the spinal cord-derived growth inhibitory molecules. About two thirds of all the injured spinal cord axons regenerated in the myelin-immunized mice, with a similar regeneration distances compared to IN-1 antibody-treated animals. With the success of the therapeutic vaccine approach, a future direction for this research has been indicated as to apply antibodies against CNS inhibitors by passive immunization at the time of lesion. This would allow antibodies to enter the lesion site immediately after injury, so that the injured axons will be able to grow with the blockade of myelin inhibition before the glial scar is formed.

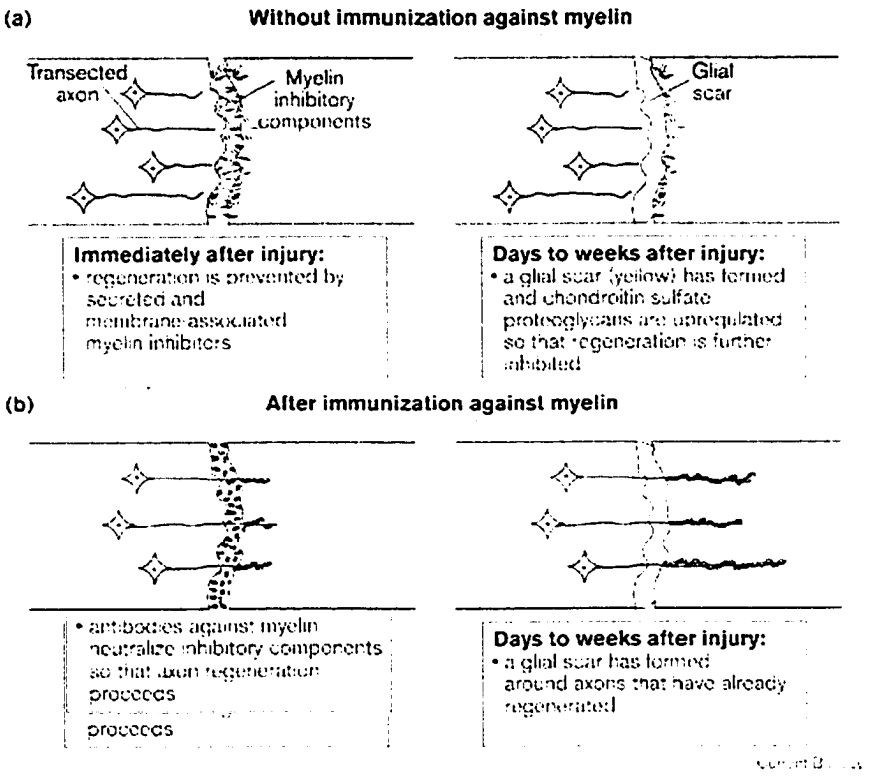


Figure 1.8: Improved axonal regeneration after immunization against CNS myelin. Axon regeneration in the spinal cord (a) without and (b) after immunization against myelin. As recently reported by Huang *et al* (Huang, McKerracher et al. 1999), immunization of mice against myelin allows axon regeneration over long distances; in the absence of immunization, no regeneration is seen. (Filbin 2000)

1.2.3.2 Switching the intrinsic state of neurons

Since the demonstration of CNS myelin as an inhibitor of axonal extension, strategies for improving nerve regeneration had long been focused on replacing this inhibitory substrate after injury. However, with a better understanding of the mechanisms by which the myelin-associated inhibitors prevent axonal regeneration and how the intrinsic neuronal state dictate the responses of neurons to those inhibitors, the injured neurons themselves became an alterative target for treatment after injury.

Many researchers have investigated the use of infusion pumps or gene therapy techniques to deliver growth factors to promote regeneration in the CNS, some of them

have achieved notable success (Blesch, Uy et al. 1999; Liu, Kim et al. 1999; Ramer, Priestley et al. 2000). However, results also showed that trophic factors may stimulate more of a local sprouting response rather than long distance regeneration (Diamond, Holmes et al. 1992; Mansour-Robaey, Clarke et al. 1994; Schnell, Schneider et al. 1994; Patel and McNamara 1995).

Now, the recent explosion in our understanding of the nature of the myelin-associated inhibitors and how they interact with neurons to exert their effect increases the possibilities for more therapeutic targets. The very recent finding that NgR and p75 function as a commonly shared neuronal receptor signaling complex for all of the three major myelin-associated inhibitors provided an exciting potential strategy, in which blocking the binding of ligands to NgR or blocking the interaction between NgR and p75 may result in overcoming the inhibitory activity by myelin. Indeed, *in vitro* experiments have already provided convincing evidence indicating that with either a blocking antibody or dominant negative form of NgR or p75, the signal transduction induced by inhibitors via the NgR-p75 receptor complex has been abrogated. These neurons are no longer inhibited by each individual inhibitor nor by myelin in general (Domeniconi, Cao et al. 2002; Liu, Fournier et al. 2002; Wang, Kim et al. 2002; Wong, Henley et al. 2002; Yamashita, Higuchi et al. 2002). These results suggest a direction of further research, which may focus on the designing of an agent to block either the binding or the activation of NgR-p75 receptor complex by inhibitory ligands and thus encourage nerve regeneration after CNS injury *in vivo*.

One approach that has succeeded in blocking the effects of all myelin-associated inhibitors simultaneously both *in vitro* and *in vivo*, is the modulation of Rho signaling.

Rho, a small GTPase, exists in an inactive (GDP bound) or an active (GTP bound) form, and the cycling between active and inactive states modulates signal pathways which regulate the cytoskeleton and cell motility (Hall 1998). McKerracher and colleagues have found that active Rho GTPase is required for the axonal regeneration inhibition by MAG or myelin (Lehmann, Fournier et al. 1999). This was shown by findings that when Rho is inactivated by C3 transferase-mediated ADP-ribosylation or by transfected dominant negative Rho, PC12 cells and retinal ganglion cells are able to grow on MAG or myelin substrates. These results, together with the discovery that all three major myelin inhibitors use NgR-p75 as receptor signaling complex, suggest that it is the interaction of the inhibitory ligands with the receptor complex that modulates Rho's activity. Hence, therapeutic approaches targeting the Rho signaling pathway may be able to block all the myelin inhibitors simultaneously. Two recent studies done by McKerracher's group have shown that this may indeed be the case. Inactivation of Rho or its downstream effector Rho-associated kinase (ROK) can induce improved axonal growth on inhibitory substrates *in vitro* and achieve limited nerve regeneration and functional recovery after CNS injury *in vivo* (Dergham, Ellezam et al. 2002; Winton, Dubreuil et al. 2002).

Another potential target for improving axonal regeneration is the intracellular second messenger, cAMP. It was previously shown that increasing the levels of cAMP can reverse the effects of MAG on both axonal extension (Cai, Shen et al. 1999) and growth cone turning (Song, Ming et al. 1998) *in vitro*. Furthermore, elevation of cAMP in vivo can not only improve subsequent axonal growth of neurons on inhibitory substrates *in vitro*, but also induce significant regeneration of spinal cord neurons after CNS injury *in vivo* (Cai, Qiu et al. 2001; Qiu, Cai et al. 2002). The mechanisms underlying the

effects of cAMP in overcoming myelin-mediated inhibition and encouraging neurite outgrowth is still under investigation, but it may involve expression of a group of growth-promoting molecules as well as cytoskeleton-associated regulators. To date, one of the downstream components of this signaling pathway has been identified, which is the synthesis of polyamines. It had been shown that overexpression of Arginase I or exogenous application of polyamines can mediate improved axonal regeneration on myelin substrates (Cai, Deng et al. 2002).

Taken together, these findings present a single theme. The binding of a single receptor complex to all three inhibitors initiates the activation of the same intracellular signaling pathway, so that mechanisms which block the inhibition of one individual inhibitor may result in overcoming the inhibition by all three and by myelin in general.

1.2.3.3 Cell transplantation

Since the establishment that the CNS environment is strictly non-permissive for the growth of adult neurons, many researchers have focused on using cellular graft transplantation to replace it after CNS injury. Some different cell transplants have great success in achieving significant regeneration, such as Schwann Cells (Paino and Bunge 1991; Xu, Guenard et al. 1995; Guest, Rao et al. 1997; Tuszynski, Weidner et al. 1998), fibrolblast expressing trophic factors (Tuszynski and Gage 1995; Nakahara, Gage et al. 1996; Blesch, Uy et al. 1999; Liu, Kim et al. 1999), fetal spinal cord transplants (Bregman, Kunkel-Bagden et al. 1993; Diener and Bregman 1998), macrophages (Lazarov-Spiegler, Solomon et al. 1996), embryonic stem cells (McDonald 1999) and olfactory ensheathing cells (OECs) (Li, Field et al. 1997; Ramon-Cueto, Plant et al. 1998;

Ramon-Cueto, Cordero et al. 2000). Transplantation of cells into the damaged adult spinal cord serves as a bridge across lesion cavities and provides a permissive substrate for injured axons to grow through. However, the environment provided by transplanted cells is so favorable that most neurons only extend axons within the grafts but never enter the inhibitory host tissue beyond. This lack of growth beyond the grafts was later solved by pumping neurotrophins — brain-derived neurotrophic factor (BDNF) or neurotrophic factor 3 (NT-3)- into the implanted embryonic tissue, which results in extended axonal regeneration within and out of the transplants into the host white matter beyond (Bregman et al. 2002). This is most likely due to the elevation of neuronal cellular cAMP levels by the neurotrophins (Cai, Shen et al. 1999). Another case where axons extend long distances out of the initial transplant region is bridging grafts that "capped" both ends of a Schwann cell-containing tube graft with OECs in order to smoothly transfer the transition from the PNS to the CNS (Ramon-Cueto, Plant et al. 1998). OECs are the principle glial cells of the olfactory system where axon growth occurs not only in response to injury but also as a normal physiological process throughout the lives of healthy individuals. OECs share properties with both Schwann cells and astrocytes. They not only provide a permissive environment themselves but also reduce the degree of glial scar formation, which together, allow more regenerating axons to grow into and out of the graft (Ramon-Cueto, Plant et al. 1998). Ramon-Cueto and colleagues have studied long term effectiveness of OEGs transplanted into the adult rat spinal cord after a complete spinal cord transection (Ramon-Cueto, Cordero et al. 2000), and demonstrated the regeneration of corticospinal, noradrenergic and serotonergic neurons. Moreover, they

have also observed a correlation between the extent of regeneration and the degree of functional recovery.

With our growing knowledge of the nature of axon regeneration and inhibition mechanisms, more strategies will be revealed by which further regeneration and functional recovery may be achieved after CNS injury. The effect of each of these strategies may be very limited, but it may be especially interesting and promising to combine several therapies and may result in a more substantial regeneration of injured axons. The next challenge to be overcome will then be to guide the regenerating axons to their appropriate postsynaptic target and to achieve successful functional recovery.

1.3 Interleukin-6 (IL-6)-type cytokines

Cytokines are small-polypeptide molecules that play important roles in the communication between cells of multicellular organisms. They are intercellular mediators regulating survival, growth, differentiation and effector function of many types of cells. They are also key players in the regulation of the immune response, particularly during infectious, inflammatory, neurological and endocrinological auto-immune diseases. Cytokines are expressed in very small amounts under normal physiological conditions, but can be synthesized and secreted rapidly after stimulation during various pathological conditions. The actions of cytokines can be auto-, para- or endo-crine, via specific cell-surface receptors on their target cells. In addition to the direct effects that cytokines exert on their target cells, they also affect the actions of other cytokines in either synergistic or antagonistic manners (Heinrich, Horn et al. 1998).

In recent years, a subgroup of cytokines, termed the interleukin-6-type cytokines, have received much attention in their production and function in central nervous system development, inflammation and diseases (Gadient and Otten 1997). Here, we will summarize information regarding this group of cytokines itself, its expression and function in the CNS and molecular signaling mechanisms that are involved in its neuronal effects.

1.3.1 IL-6-type cytokines and their receptors

The IL-6-type cytokines are a group of small polypeptide molecules characterized by a four-α-helix-boundle topology (Bazan 1990). They comprise interleukin-6 (IL-6), IL-11, leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and cardiotrophin (CT)-1 (Table 1.1). Except for CNTF and CT-1, all the other IL-6-type cytokines are secreted proteins synthesized with N-terminal signal peptides and released under both physiological and pathological conditions. CNTF and CT-1 are also released upon injury of their producer cells, but the release mechanism under normal conditions is still elusive (Taga and Kishimoto 1997).

All of the members of IL-6-type cytokines have common biological functions to promote neuronal survival or to prevent neuronal apoptosis. This redundancy in action may be due to the usage of a common signal transducing receptor, gp130, which has been shown to be essential for the survival of subgroups of motor and sensory neurons (Nakashima, Wiese et al. 1999). The receptor complex corresponding to each of the IL-6-type cytokines consists of two subunits, a ligand-binding subunit that mediates the differential responsiveness of cellular populations to each cytokine and a signal

transducing subunit, in this case the gp130 protein, to activate downstream signaling machinery (Figure 1.9).

Property	IL-6	IL-11	LIF	CNTF	CT-1	OSM
Number of amino acids	212	199	202	200	201	252
Molecular mass (kDa)	20.8	19.1	20	22.9	21.2	22.1
Potential N-glycosylation sites	2	0	6	0	0	2
Number of cysteine residues	4	0	6	1	2	5
Number of S-S bridges	2	0	3	0	?	2
mRNA size (kb)	1.3	1.5,2.5	1.8,4	1.0	1.7	2
Number of exons	5	5	3	2	3	3

Table 1.1: Biochemical properties of human IL-6-type cytokines. (Heinrich, Behrmann et al. 1998)

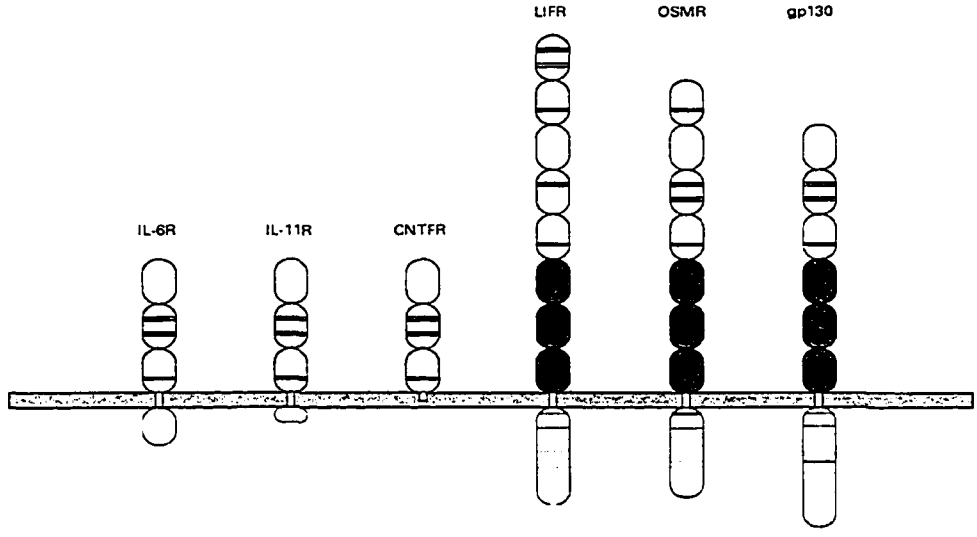


Figure 1.9: Receptors involved in IL-6-type cytokine signaling. Each of the IL-6-type cytokines, except for CT-1, has a membrane-bound receptor (IL-6R, IL-11R, CNTFR, LIFR, OSMR) responsible for ligand binding, and associates with another transmembrane protein gp130 for signal transduction. (Heinrich, Behrmann et al. 1998)

As illustrated in Figure 1.9, all the receptor subunits involved in IL-6-type cytokine signaling are membrane proteins with extracellular N-terminus and a single transmembrane domain, except for the CNTF receptor, which is a GPI-linked membrane surface protein. Upon receptor complex activation, IL-6, IL-11, CNTF, and possibly CT-1 first bind to their specific α-receptor subunits, namely IL-6R, IL-11R, CNTFR and an unknown receptor, respectively. After ligand binding, the complexes of cytokine and α-receptor are able to recruit the corresponding signal-transducing receptor subunit gp130 and form either gp130/gp130 homodimer (IL-6 and IL-11) or a gp130/LIFR heterodimer (CNTF and CT-1) (Taga and Kishimoto 1997). LIF binds directly to the LIFR with subsequent formation of the LIFR/gp130 heterodimer. OSM is the only family member known to bind directly to gp130, and then recruit either the OSMR or LIFR to form an activated receptor complex (Gearing, Comeau et al. 1992; Liu, Modrell et al. 1992; Sporeno, Paonessa et al. 1994; Thoma, Bird et al. 1994). Figure 1.10 illustrates the formation of receptor complex after ligand binding of IL-6-type cytokines.

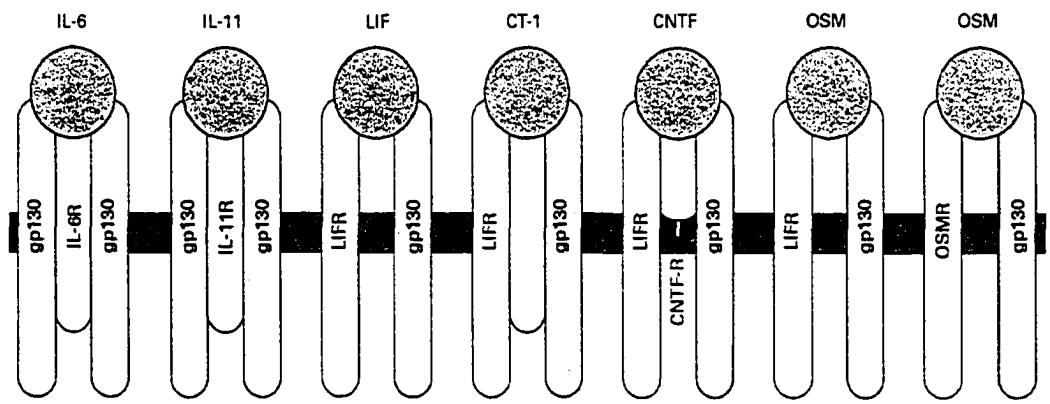


Figure 1.10: IL-6-type cytokine receptor complexes. (Heinrich, Behrmann et al. 1998)

Interestingly, all the identified ligand-binding α-receptors of IL-6-type cytokines, IL-6R, IL-11R and CNTFR, have been found to exist in both membrane-bound and soluble forms *in vivo* (Davis, Aldrich et al. 1993; Mullberg, Schooltink et al. 1993; Van Wagoner, Oh et al. 1999). The soluble form of cytokine receptors lack the transmembrane and cytoplasmic parts but still can bind to their specific ligands and form signaling receptor complex with gp130 as efficiently as their membrane-bound counterparts. Thus, the responsiveness of any given cell type to these cytokines is determined by either the expression of α-receptors or the presence of corresponding soluble receptors (Taga and Kishimoto 1997).

1.3.2 Expression of IL-6 in the central nervous system

Among all the IL-6-type cytokines, IL-6 is one of the best characterized. IL-6 is a 20 kDa small polypeptide expressed by various cell types and proved to have diverse biological functions. It's function as an immune and hematopoietic factor has been very well documented, such as driving the proliferation and differentiation of B cells (Muraguchi, Hirano et al. 1988), inducing the synthesis of acute phase proteins (Moshage 1997), and stimulating T cell maturation and activation (Takai, Wong et al. 1988). In the central nervous system, the expression of IL-6 had been detected in both pathological and normal conditions. In the brain, almost all cell types express IL-6, which include brain endothelial cells, glial cells as well as neurons. In situ hybridization has located IL-6 mRNA in many neuronal populations, such as hippocampal neurons, pyramidal neurons,

Purkinje cells, granular cells of the olfactory bulb and the cerebellum, as well as sensory and sympathetic neurons (Schobitz, Voorhuis et al. 1992; Gadient and Otten 1995).

In the intact brain, the levels of IL-6 remain very low. However, during brain injury, inflammation, and neuronal diseases, IL-6 levels become elevated. First, neurodegenerative diseases such as Parkinson and Alzheimer diseases have been reported to associate with an up-regulation of IL-6 as part of the inflammatory reactions (Strauss, Bauer et al. 1992; Muller and Ackenheil 1998). Moreover, after acute brain injury, IL-6 protein was also detected to be strongly up-regulated in cerebrospinal fluid (Hans, Kossmann et al. 1999). Likewise, an increase of IL-6 expression has been found in sensory neurons after axotomy of peripheral nerves (Murphy, Borthwick et al. 1999) and in motoneurons after spinal cord injury (Hayashi, Ueyama et al. 2000). Significantly elevated concentrations of IL-6 have also been reported in the cerebrospinal fluid and serum of patients after stroke (Tarkowski, Rosengren et al. 1995; Suzuki, Tanaka et al. 1999).

1.3.3 Regulation of IL-6 expression in glial cells and neurons

As mentioned above, IL-6 can be produced by multiple cell types in the nervous system, including neurons, astrocytes, microglia and macrophages, among which astrocytes may be the dominant source of this cytokine (Gruol and Nelson 1997). The expression of IL-6 by astrocytes can be regulated in many ways. First, proinflammatory factors such as interleukin-1β (IL-1β) and tumor necrosis factor- α (TNF-α) have been shown to induce IL-6 expression during inflammatory and diseased CNS conditions (Benveniste, Sparacio et al. 1990; Sawada, Suzumura et al. 1992; Sharif, Hariri et al.

1993), and with the administration of anti-inflammatory mediators such as isoproterenol (ISO) and dexamethasone, the induction of IL-6 by IL-1β and TNF-α were blocked (Benveniste, Sparacio et al. 1990; Grimaldi, Navarra et al. 1998; Nakamura, Johns et al. 1998). Second, it is postulated that neurotransmitters may also direct neuronal modulation of cytokine expression and trigger immune function, neuronal protection, and neuronal survival. For IL-6, the neuroactive peptides norepinephrine (NE), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating polypeptide (PACAP38), serotonin, adenosine, SP and calcitonin (CN) have all been shown to increase expression of IL-6 by astrocytes *in vitro*. It has been demonstrated that the mechanisms of how these neuropeptides regulate IL-6 expression are at multiple levels. Adenosine controls the expression of IL-6 at the transcriptional level (Schwaninger, Neher et al. 1997), while many other neuropeptides such as VT, VIP, NE, and PACAP induce IL-6 via cAMP accumulation (Grimaldi, Pozzoli et al. 1994; Benveniste, Huneycutt et al. 1995; Grimaldi, Florio et al. 1997; Kiriyama, Murayama et al. 1997). Third, several second messenger pathways like cAMP/PKA pathway and Ca^{2+}/PKC pathway have also been shown to increase IL-6 expression in astrocytes (Grimaldi, Pozzoli et al. 1994; Norris, Tang et al. 1994; Benveniste, Huneycutt et al. 1995; Grimaldi, Florio et al. 1997; Kiriyama, Murayama et al. 1997). Interestingly, synergistic induction of IL-6 is observed after activation of both cAMP/PKA and PKC pathways, indicating crosstalk between them (Benveniste, Huneycutt et al. 1995). Finally, the expression of IL-6 in astrocytes can also be auto-regulated or induced by other members of the IL-6 family of cytokines. Benveniste and colleagues have demonstrated that with the presence of the soluble IL-6 receptor, IL-6 itself can regulate expression of its own gene in human astrocytes (Oh,

Van Wagoner et al. 1998; Van Wagoner and Benveniste 1999). Also, both OSM and CT-1 have been shown to be able to stimulate high levels of IL-6 expression *in vitro* and *in vivo* (Van Wagoner and Benveniste 1999; Wallace, MacMaster et al. 1999; Bordet, Castelnau-Ptakhine et al. 2001; Bordet, Lesbordes et al. 2001). However, other members of the IL-6 family, LIF, CNTF and IL-11 have no significant effect on IL-6 productionn either alone or with other proinflammatory molecules.

Compared to the numerous studies dealing with the regulation of IL-6 production in glial cells, little is known about how it is modulated in neurons. Two proinflammatory factors, IL-1β and TNF-α, released from nerve stump after dorsal root ganglion axotomy, have been reported to stimulate the expression of IL-6 in cortical and sensory neurons (Murphy, Borthwick et al. 1999), just as how they function toward astrocytes. Further more, up-regulation of IL-6 in disease models of epilepsy and cerebral ischemia led to the hypothesis that excitotoxicity and neuronal activity can also induce this cytokine. Indeed, *in vitro* membrane depolarization and the glutamate agonist NMDA activate the neuronal expression of IL-6 (Ali, Nicole et al. 2000; Sallmann, Juttler et al. 2000). The mechanisms underlining the regulation of IL-6 in neurons by excitotoxicity or depolarization have not been fully understood. A collection of data has implied that the stimulatory effect of depolarization on IL-6 expression requires Ca^{2+} influx through L-type voltage-gated channels and subsequent activation of Ca^{2+}/calmodulin signaling pathway. The glucocorticoid response element (GRE) in the IL-6 promoter has been found responsive to depolarization, yet a novel Ca^{2+}-responsive transcription factor that can bind to this element still needs to be identified (Sallmann, Juttler et al. 2000; Suzuki, Tanaka et al. 2000).

Taken together, all the information we summarized above suggests that IL-6 produced by either glial cells or neurons can be regulated by a large variety of factors, which leads to synergistic increases in IL-6 secretion. This suggests the mechanisms by which high levels of IL-6 can be achieved in the CNS.

1.3.4 IL-6 has both beneficial and detrimental functions in the CNS

A number of studies have substantiated IL-6 as one of the early response cytokines that play important roles in the responses to many forms of injury. The function of IL-6 in the CNS is very complex, it behaves both as an neurotrophic molecule and a mediator of the acute phase reaction and inflammation. Most notably, expression of IL-6 in the brain is necessary for endotoxemia-induced fever and sickness behavior (Chai, Gatti et al. 1996; Bluthe, Michaud et al. 2000). It has been shown that IL-6 is associated with elevation of body temperature after focal cerebral ischemia, which is often encountered in stroke patients and leads to a considerable risk for more ischemic damages. In addition, IL-6 over-production is associated with the break down of the blood-brain barrier (Brett, Mizisin et al. 1995), angiogenesis and impaired learning (Campbell, Abraham et al. 1993; Heyser, Masliah et al. 1997). Moreover, both *in vitro* studies with cell cultures and *in vivo* studies of transgenic mice with deficiency or over-expression of IL-6 have revealed that IL-6 promotes astrocyte proliferaton, macrophage maturation and activation, as well as astrogliosis (Selmaj, Farooq et al. 1990; Campbell, Abraham et al. 1993; Fattori, Lazzaro et al. 1995; Penkowa, Moos et al. 1999). Finally, overexpression of IL-6 not only directly affects neuronal and glial cells, but also increases central production of other inflammatory cytokines, thus supports a

proinflammatory and destructive role of IL-6 when disregulated in the CNS (Di Santo, Alonzi et al. 1996).

On the other hand, a variety of studies provide evidence for IL-6 involvement in neuronal survival, protection, differentiation and regeneration (Hirota, Kiyama et al. 1996; Gadient and Otten 1997; Loddick, Turnbull et al. 1998; Marz, Heese et al. 1999). It was demonstrated that IL-6 stimulates neuronal differentiation of PC12 cells (Satoh, Nakamura et al. 1988), enhances the differentiation and survival of multiple neuronal types, including cholinergic, monoaminergic, and sensory neurons (Gadient and Otten 1997; Horton, Barlett et al. 1998), and protects primary neurons against glutamate toxicity (Ali, Nicole et al. 2000). In addition, systemic administration of IL-6 not only significantly reduces brain damage in the cerebral ischemic region (Loddick, Turnbull et al. 1998), but also enhances neuronal survival in the absence of nerve growth factor (NGF) in rat sympathetic neurons (Marz, Cheng et al. 1998). Further more, transgenic mouse models in which IL-6 and its soluble receptor are both overexpressed show accelerated regeneration of axotomized hypoglossal nerve (Hirota, Kiyama et al. 1996). Besides these, endogenous IL-6 has been shown to reduce the susceptibility of mice toward seizures (Penkowa, Molinero et al. 2001) and the learning disability after forebrain ischemia (Matsuda, Wen et al. 1996).

Taken together, these studies demonstrate that IL-6 is a multi-functional molecule in the CNS, so that the tight regulation of is expression is needed to maintain its beneficial functions and prevent its potentially detrimental effects.

CHAPTER II

METHODS AND MATERIALS

2.1 Cell culture

Permanently transfected MAG-expressing and control CHO cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technology) supplemented with 10% dialyzed Fetal Bovine Serum (FBS), praline (40mg/L), glycine (7.5mg/L), thymidine (0.73mg/L) and L-glutamine (0.29g/L) at 37°C in 7.3% CO_2. COS cells were maintained in DMEM supplemented with 10% gualified FBS under same conditions.

2.2 Isolation of primary neurons

For cerebella neurons, both cerebellums were taken from each animal at the age from postnatal day 1 (P1) to day 5 and dissociated in 6ml of 0.025% trypsin (Life Technology) by pipetting. 5ml DMEM containing 10% FBS was then added to terminate trypsinization. Cells were centrifuged at 1000 rpm for 5 min at 4°C and resuspended to a single-cell suspension in SATO (progesterone, 200nM; selenium, 224nM; putrescine 100μM; insulin, 4μg/ml; BSA, 0.35mg/ml; L-thyroxine, 0.4μg/ml; tri-iodo-thyronine, 0.34μg/ml) (Doherty *et al.*, 1990). For hippocampal neurons, cells were dissociated in the same manner and incubated in poly-L-lysine (16.6μg/ml, Sigma) pre-coated 24-well plates (6cm) at 37°C in 7.3% CO_2 overnight. Then cells were re-trypsinized off the plates and resuspended into single-cell suspension in SATO. For DRG neurons, ganglia were removed from animals aging at P5 and incubated in 6ml of SATO media containing of 0.025% trypsin and 0.15% collagenase type I (Worthington) for 60-90 min at 37°C. The ganglia were triturated and tryptonization was stopped by adding 5ml of DMEM containing 10% FBS, centrifuged at 1000 rpm for 5 min and resuspended in SATO (De Bellard *et al.*, 1996).

2.3 Neurite outgrowth assay on CHO cell monolayer

Monolayers of control and MAG-expressing CHO cells were grown to confluency in individual chambers of an 8-chamber tissue culture slide (Lab-Tek). Primary neurons were isolated and resuspended in Sato media as described previously (DeBellard, Tang et al. 1996). Then the neurite outgrowth assay was carried out by adding $5x10^4$ neurons to the immobilized myelin substrate or $2x10^4$ neurons to the CHO cell monolayers. Where indicated, dbcAMP (1mM), DRB at 5 μM, DFMO at 1mM to 5mM, Putrescine at 10 μM to 100 μM, BDNF at 200 ng/ml was added into the culture. After 16-18 hours of incubation, the neurons were fixed for 30 min with 4 % paraformaldehyde and permeabilized with ice-cold methanol for 2 min. The cells were then blocked for 30 min with DMEM containing 10% FCS and incubated overnight with a rabbit polyclonal antibody against the growth-associated protein GAP43 (1:4000, from R. Curtis and G. Wilkins Imperial College, London). Cells were washed three times with PBS-BSA (5%) and then incubated for 30 min at room temperature with a biotinylated donkey anti-rabbit IgG (1:500, Amersham, Arlington Heights, IL), washed three times, and then incubated with streptavidin-conjugated Texas Red (1:300, Amersham, Arlington Heights, IL) for 45 min. After three more washes, the slides were mounted in Permafluor (Immunon) and viewed with a fluorescence microscope. The length of the longest neurite for each GAP43-positive neuron for the first 180-200 neurons encountered when scanning the slide in a systematic manner was determined by using an Oncor image analysis program.

2.4 Neurite outgrowth assay on immobilized L1-Fc substrate

We have developed an assay in which the growth of isolated young cerebellar neurons on the substrate of the growth-promoting molecule L1 would be inhibited by the presence of wild type MAG-Fc (Tang, Shen et al. 1997). An 8-chamber slide (Lab-Tek) was first coated with 16.6μg/ml poly-L-lysine (Sigma) in sterile water for 1 hr at room temperature, then washed twice with freshly made 0.1M bicarbonate buffer. Wells were then incubated with anti-human Fc antibody (Sigma) 15μg/ml in 0.1M bicarbonate buffer for 2 hr at 37°C. After washing three times with DMEM, L1-Fc was immobilized onto anti-Fc by incubating 30μg/ml L1-Fc in the wells overnight at 37°C. Unbound L1-Fc was washed off with DMEM. Cerebellar granular neurons were suspended in Sato medium with 2% FBS into a concentration of $5x10^4$cells/well, and incubated with 25μg/ml of wild type or mutant MAG-Fc proteins at 37°C for 18 hours. The fixation and Staining procedures were the same as neurite outgrowth on CHO cell monolayer.

2.5 Myelin preparation

Myelin was purified from rat CNS white matter following the Norton's protocol (Norton and Poduslo 1973). After the final hypotonic shock, the membranes were centrifuged and resuspended in 10mM HEPES. Then the protein concentration of the preparation was determined (Biorad) and used immediately as a substrate in the neurite outgrowth assay.

2.6 Neurite Outgrowth on Immobilized Myelin

For myelin membranes, wells of an 8-chamber tissue culture slide (Lab-Tek) were coated with 16.6 μg/ml poly-l-lysine at room temperature for 1 hour. Rat CNS myelin at 0.5- 2.0μg total protein/well was dried overnight onto the coated wells and used as a substrate (Shen, DeBellard et al. 1998). $2x10^4$ cells/well of PND5 DRG or PND1 hippocampal neurons suspended in Sato medium with 2% FBS, and incubated in myelin-coated wells at 37°C for 24 hours. The fixation and Staining procedures were the same as neurite outgrowth on CHO cell monolayer.

2.7 Western blot analysis of expression of MAG by CHO cells

Cells (80-90% confluent) will be lysed in 0.5 M Tris-HCl (pH7.5) containing 2% SDS, 1 ug/ml chymotrypsin, 1 μg/ml pepstatin, and 1 ug/ml phenylmethylsulfonyl fluoride. The lysate can be homogenized by passage through a 23 gauge syringe. Protein concentration will be measured with a Bio-Rad kit before the addition of β-mercaptoethanol. The lysates should be incubated at 95°C for 5 min, after which they will be subjected to SDS-polyacrylamide gel (10%) electrophoresis. The proteins will be transferred to PVDF membrane and immunostained with monoclonal antibody against rat MAG for 1 hour at room temperature. For Sn(d1-3)MAG(4-5), Sn(d1-3)MAG(d4)Sn(d5), MAG(d3-5) and wild type MAG, we can use monoclonal antibody B11F7 which recognize denatured MAG Ig domain 4 as primary antibody (2.6μg/ml); For MAG(1-3), another monoclonal antibody 513 that recognize native rat MAG Ig domain 1-3 can be used as primary antibody (2μg/ml). For Sn(d1-4)MAG(d5), a combination of two monoclonal antibodies, 3D6 and SER4 (both 1μg/ml), which recognize Sialoadhesin Ig

domain 1 and 2/3, respectively, were used as primary antibodies. After washing membrane for three times with PBS-T, the membrane will be incubated in second antibody HRP-conjugated anti-mouse IgG (1:5000) at room temperature for 1 hour. After 3 times of washing with PBS-Tween, membrane will be illuminated with ECL western blotting detection reagent.

2.8 Sialic acid-dependent binding assay

Confluent monolayers of CHO cells expressing wild-type MAG, truncated or chimeric MAG, or control cells will be established over a 24-hour period in individual chambers of 8-well tissue culture slides (Lab-Tek). Monolayers will be treated for 1 hour with 100mU/ml neuraminidase (Calbiochem) at 37°C and washed twice with DMEM. Then 1×10^7 RBCs suspended in HEPES-DMEM will be added into each well. As in control, RBCs were incubated in PBS containing 100mU/ml neuraminidase at 37°C for 1 hour before added into wells. After 1 hour of incubation at 37°C, unbound erythrocytes will be washed off with DMEM and cells can be examined under a phase-contrast microscope and photographed.

2.9 Mutation, ligation and transformation

Three chimeric proteins, Sn(d1-3)MAG(d4-5), Sn(d1-4)MAG(5) and Sn(d13)MAG(d4)Sn(d5) can be synthesized by overlap extension PCR modified from "sticky feet"-directed mutagenesis (Clackson, 1989). PCR product of chimeric SnMAG cDNA fragment will be ligated and transformed into pCR-Script™ Amp SK(+) plasmid following pCR-Script™ Amp SK(+) Cloning protocol (Stratagene). After sequencing,

these chimeric SnMAG cDNA will be cut with restriction enzyme and subcloned into expression vector pcDNA3.1 containing a G418 resistance site.

2.10 Transfection

CHO cells will be transfected with 5 ug of DNA per 10 cm plate, following the protocol for stable transfection of adherent cells (SuperFect ™ Transfection; Qiagen). Remove medium containing transfection complex from the cells by gentle aspiration 4 hours after transfection, and wash cells 3-4 times with 4ml of PBS. Incubate cells in normal growth medium at 37°C with 5% CO_2 for 48 hr before changing into G418-selection medium. After 2-3 weeks of selection, the entire population of transfected cells was sorted by FACS for protein expression, and positive cells were maintained as single-cell clones. Colonies appears after ~3 weeks, and a number of clones will be picked, expended, and screened for expression of corresponding MAG chimera by Western blot analysis and cell surface immunofluorescence staining.

2.11 Immunostaining of MAG expressed on CHO cells

Cells are grown overnight in chambers of 8-well tissue culture slides coated with poly-L-lysine (Sigma) and fibronectin (Sigma) before fixed with 4% paraformaldehyde for 15 min at room temperature. Cells will then be washed with PBS three times and incubated with monoclonal antibody at 4°C overnight. For cells expressing SnMAG chimeric protein, two monoclonal antibodies SER4 and 3D6 (1μg/ml, from Dr. Paul Croker) against sialoadhesin Ig domain 2/3 and domain 1, respectively, can be used together as primary antibody, while for MAG(d1-3), wild type MAG expressing cells,

monoclonal antibody 513 can be used as primary antibody (2 μg/ml). MAG(d3-5) protein was detected by polyclonal anti-MAG antibody ECD (4 μg/ml). After washing with cDMEM for three times, cells will be incubated with Oregon green goat anti-mouse IgG at room temperature for 1 hour, then will be washed twice with PBS before being mounted with Gel-mount (Biomeda) and viewed with a Zeiss fluorescence microscope.

2.12 Immunoassay for IL-6

Dorsal root ganglia (DRG) and/or hippocampal neurons were isolated from P4-8 rat pups as previously described (DeBellard, Tang et al. 1996). 4 x 10^6 cells were plated onto poly-L-lysine-coated 96-well tissue culture plates and incubated for 24 hours at 37°C. The supernatant was collected and IL-6 levels were measured immediately via a competitive immunoassay kit (R&D Systems) according to the manufacturer's instructions. Each condition was repeated in quadruplicate for at least 3 separate experiments.

2.13 Preparation of Fc-chimeras

The pIG plasmid (Simmons, 1993) containing the cDNA for wild type MAG-Fc was constructed by fusing the extracellular domain of MAG to the Fc region of human IgG (Kelm et al., 1994; Doherty et al., 1995). To obtain Fc-chimeras of the mutated MAG cDNA, primers containing mutation site were synthesized (Invitrogen). Purified primers were then used to introduce corresponding mutation into wild type murine MAG-Fc plasmid by PCR according to the protocol from QuikChange Site-Directed Mutagenesis Kit (Stratagene). After PCR reaction, products were digested with DpnI

restriction enzyme to remove the template DNA before transform into MC1061/P3 supercompetent cells. The plasmids containing wild type or mutant MAG-Fc cDNA were transiently transfected into COS-1 cells with DEAE-dextran (Kelm et al., 1994). 70-80% confluent COS-1 cells were covered by the transfection solution with the following concentrations: 100ng Fc-chimera plasmids per $1.0x10^5$ cells, 250μg/ml DEAE-dextran, and 100μM chloroquine diphosphate in 10mM HEPES-buffered DMEM. The COS-1 cells were incubated in transfection solution at 37°C until the cells began to look vacuolated. The transfected COS-1 cells were osmotically shocked by PBS/10%DMSO for 2 min, washed twice with HEPES-buffered DMEM, incubated in DMEM with 1% IgG-depleted FBS for 8-10 days.

The chimeric proteins were purified by affinity chromatography using protein A Sephorose following transient expression of recombinant plasmid DNA in COS-1 cells. The media of transfected COS-1 cells were incubated with 0.5g protein A Sepharose per 400ml media, and slurry overnight at 4°C. The bound chimeric proteins were eluted from protein A Sepharose by 5ml 100mM glycine solution at pH3.0, then neutralized with 0.5ml 1M Tris buffer at pH8.0. The eluted proteins were concentrated to about 1-2mg/ml by Centricon-30 microcentrators (Amicon) via repeating centrifugation, sterilized by filtration with sterile Ultrafree-MC filter units (Millipore). Protein concentration was estimated using a Bio-Rad kit.

2.14 Solid-phase binding assay with immobilized Fc-chimeras

In order to maximize the chances of correct orientation of Fc-chimera, 15μg/ml anti-Fc antibody was first coated onto an Immulon-3 96-well ELISA plate (Dynatech) for

2 hr at 37°C in 0.1M bicarbonate buffer. Wild type and mutant MAG-Fc with increasing concentrations, 0.01-50μg/ml, were absorbed for 2 hr at 37°C to the coated plate. Prior to the binding assay, isolated neurons were vitally labeled with fluorescent dye, 10μM calcein AM (Molecular Probe) for 15 min at 37°C, then washed and resuspended in PBS. Then fluorescent-stained cerebellar neurons ($2x10^5$cells/well) were added into each well and incubated for 1 hr at room temperature. The plate was washed 2 to 4 times with PBS/0.25%BSA applied to each well under gravity. Bound cells were measured by FluorImager (Molecular Dynamics).

2.15 Neurite Outgrowth with *in vitro* IL-6 application

The neurite outgrowth assay on MAG-expressing cells or myelin was carried out as described previously (Mukhopadhyay, Doherty et al. 1994; Cai, Shen et al. 1999). Briefly, 5 x 10^4 isolated P1 hippocampal or P5 DRG neurons from rat pups were plated onto confluent monolayers of control and MAG-expressing CHO cells or purified myelin in 8-chamber tissue culture slides (Lab-Tek). Where indicated, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (20uM; Sigma), soluble gp130-Fc (2-50μg/ml, R&D Systems), anti-gp130 antibody (10-50μg/ml, R&D Systems), dibutyryl-cAMP (1mM; Calbiochem) or recombinant IL-6 (10-400ng/ml; R&D Systems) was added to the cultures. After 16–18 hr of incubation, the cultures were fixed for 30 min with 4% paraformaldehyde, permeabilized with ice-cold methanol, and immunostained with a rabbit polyclonal antibody against GAP43 (1:4000, from R. Curtis and G. Wilkins, Imperial College, London, United Kingdom). The slides were mounted in Permafluor (Immunon) and viewed under a fluorescence microscope. The length of the longest

neurite for each GAP43-positive neuron for the first 180–200 neurons encountered when scanning the slide in a systematic manner was determined using the Simple PCI image analysis program.

2.16 Isolation of total mRNA from cultured DRG neurons

DRG from P5 rat pups were isolated and 50 x 10^4/well neurons were plated onto purified myelin in 24-well tissue culture slides. Dibutyryl-cAMP (1mM, Calbiochem) was then added to the culture medium and the cells were incubated for 18 hours at 37°C with 7.3% CO_2. The cells were then washed once with PBS and removed from the plate via treatment with .025% trypsin (Gibco). RNA was prepared via the RNeasy RNA isolation kit (QIAGEN), according to the manufacturers instructions.

2.17 Intrathecal administration of recombinant rat IL-6

Recombinant rat IL-6 (R&D Systems) were diluted in calcium-magnesium free phosphate buffered saline (PBS-CMF), then fill into osmotic mini-pumps (Alzet) according to the instructions of the manufacturer. Minipumps were incubated in sterile saline solution (AmTech) at 37°C overnight or at least 6 hours. Rats at age of P20-P23 were anesthetized with isofluorane and inserted with one minipump in each animal. The spine of each animal was opened at L4-L6 through microsurgery and only the drug-delivering tip of each mini-pump was inserted into the space under dure membrane without injuring spinal cord. Operated animals were kept for two days before sacrificing and L4-L6 DRGs from each animal were isolated and underwent further neurite outgrowth procedures.

2.18 Subcutaneous administration of recombinant rat IL-6

Recombinant rat IL-6 were prepared and filled into osmotic mini-pumps as described above. Rats at age of P20-P23 were anesthetized with isofluorane, skin on the animal's back was shaved and sterilized with beta iodine and ethanol before incision was made. For each animal, one mini-pump was inserted under the skin and wound was closed with wound clips. Operated animals were kept two days before sacrificing and L4-L6 DRGs from each animal were isolated to undergo further neurite outgrowth assays.

CHAPTER III

THE SIALIC ACID DEPENDENT BINDING SITE OF MAG IS SEPARATE FROM ITS GROWTH INHIBITION SITE

3.1 Introduction

Myelin-associated glycoprotein (MAG) is a minor component of myelin, comprising only about 1% of the total protein in adult CNS and 0.1% in PNS myelin (Trapp 1990). The expression of MAG is preferentially located in the interface between myelinated axons and the periaxonal myelin membrane (Trapp, Andrews et al. 1989). The cloning of MAG was achieved by three groups (Arquint, Roder et al. 1987; Lai, Brow et al. 1987; Lai, Watson et al. 1987; Salzer, Holmes et al. 1987), and the results reveal that MAG is an integral membrane protein with 5 Ig-like domains, which comprise the N-terminal extracellular segment, a single transmembrane domain and a C-terminal cytoplasmic tail. Subsequently, MAG was identified as a member of the Siglec family of proteins, a subgroup of the Immunoglobulin superfamily, defined by their shared amino acid sequence similarity among the first 4-Ig like domains and the sialic-acid-dependent binding to their target cells (Kelm, Brossmer et al. 1998). Although all the members of the Siglec family bind to sialic acid, the specificity of binding is different for each. MAG has a low affinity binding for α 2,3-linked sialic acid residues attached to O-linked glycoconjugates (Kelm, Pelz et al. 1994). To date, the nature of MAG binding to the sialic acid residues is still elusive, those residues may act to assistant MAG encounter with neurons and allow it to exert its biological functions.

Since MAG is exclusively localized to the axon-myelin interface, it has been hypothesized to function in axon-glia interactions (Schachner and Bartsch 2000). Genetic depletion of MAG results in modest alterations in myelination, reduced axon caliber, reduced neurofilament spacing and phosphorylation, and progressive axonal degeneration (Li, Tropak et al. 1994; Montag, Giese et al. 1994; Fruttiger, Montag et al. 1995; Yin,

Crawford et al. 1998). These observations led to the conclusion that MAG is an important signaling molecule in axon-myelin interactions and is required for the maintenance of long-term axon-myelin stability. In addition to its role in the myelin-axon interaction, MAG has also shown to be one of the major molecules in myelin that inhibit axon regeneration after CNS injury (McKerracher, David et al. 1994; Mukhopadhyay, Doherty et al. 1994; Li, Shibata et al. 1996). This conclusion has been very well demonstrated by our lab using established in vitro neurite outgrowth assays (Mukhopadhyay, Doherty et al. 1994; Tang, Shen et al. 1997; Tang, Woodhall et al. 1997). Studies in our lab have provided strong evidence showing that MAG can inhibit neurite outgrowth from all postnatal neurons studied to date including retinal, hippocampal, superior cervical ganglion, spinal cord, cerebellar and DRG neurons older than PND3 (Mukhopadhyay, Doherty et al. 1994; DeBellard, Tang et al. 1996). This ability of MAG to inhibit axonal regeneration *in vitro* is not limited to their expression by CHO cells, since significant inhibition of axonal regeneration has also been seen when neurons were grown on transfected Schwann cells expressing MAG (Shen, DeBellard et al. 1998). In addition, when used as a substrate for axonal outgrowth, both purified native MAG and recombinant MAG can inhibit axonal regeneration (McKerracher, David et al. 1994; Bartsch, Bandtlow et al. 1995; Li, Shibata et al. 1996). Moreover, a soluble, proteolytic product of MAG, dMAG, which consists of the entire extracellular domain and is released in abundance from isolated or damaged myelin, also potently inhibits neurite outgrowth (Tang, Woodhall et al. 1997). These results reveal that MAG is indeed an inhibitory molecule for axonal regeneration rather than merely a non-permissive substrate for neuronal growth.

In studies with other members of the Siglec family, residues involved in the sialic acid-dependent binding of CD22 and sialoadhesin were mapped to the GFCC'C'' face of the NH_2-terminal domains, carried on Arg130 and Arg97, respectively (Vinson et al., 1996; van der Merwe et al., 1996). Alignment of the first Ig domain of MAG with CD22 and sialoadhesin reveals that this arginine is conserved in MAG at amino acid 118 (R118). When this Arg118 was mutated into either alanine or aspartic acid, sialic acid-dependent binding activity of MAG was abolished whether the mutant MAG was expressed in soluble form (MAG-Fc) or by transfected CHO cells or Schwann cells (Tang et al, 1997). This implied that Arg118 is the amino acid which is necessary for the sialic acid-dependent binding activity of MAG. However, this sialic acid binding activity itself is not sufficient to effect neurite outgrowth. Because both sialoadhesin and a truncated form of MAG-Fc (contains only the first 3 Ig-like domains of MAG) can bind to neurons in a sialic acid-dependent manner but cannot inhibit neurite outgrowth (Tang, Woodhall et al. 1997). In addition, although when Arg118 mutant MAG was expressed in a soluble form, the ability to inhibit neurite outgrowth was abolished. But when this mutant MAG was expressed on transfected CHO or Schwann cells, their ability to inhibit neurite outgrowth still remained. This implied that the sialic acid binding site is necessary for soluble MAG, but it is not necessary when MAG is expressed on a cell surface.

Based on these results, a two-site model has been proposed to illustrate the functional binding sites of MAG (Figure 3.1). According to this model, there are two distinct functional sites on MAG, a sialic acid-binding site and a neurite outgrowth inhibition site. The sialic acid-binding site is carried in the region of Arg118 on Ig domain 1, it plays an important role in mediating the sialic acid-dependent binding of

MAG to neurons. The inhibition site is located separately from the binding site, the fact that MAG(d1-3)-Fc can bind to neurons but does not inhibit neurite outgrowth implies that the inhibition site most likely resides within MAG Ig domain 4-5. When MAG is expressed as a soluble protein, its binding to neurons is completely dependent on the sialic acid-binding activity, so abolishing the binding site will completely block its inhibitory effect on neurite outgrowth. But when MAG is expressed on a cell surface, there might be some other adhesion molecules which can facilitate MAG's binding to neurons and, in this case, the loss of sialic acid binding site will not effect its inhibition of neurite outgrowth.

Hence, based on the observations above, the goal of the studies in this chapter is to illustrate whether the sialic-acid-dependent binding site is co-localized with the axonal regeneration inhibition site and, if not, whether the sialic-acid-dependent binding activity is indispensable for MAG to exhibit its inhibitory function.

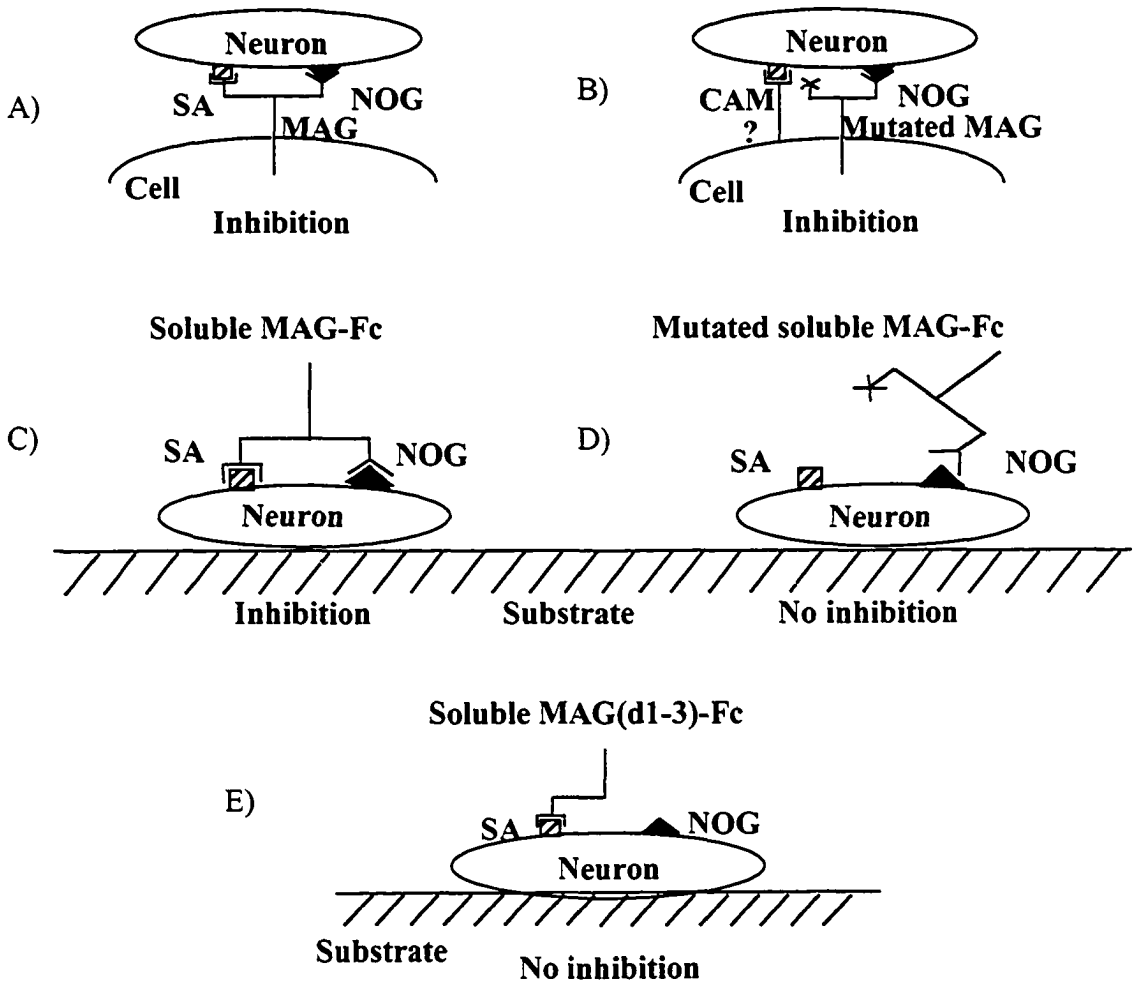


Figure 3.1 Two site model: The sialic acid binding site on MAG(R118) is critical for inhibition of axonal regeneration by soluble MAG-Fc but not MAG expressed by CHO cells. There are two recognition sites on MAG, a sialic acid binding site (SA; square symbol) and a neurite outgrowth inhibition site (NOG; triangular symbol). When MAG is expressed 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 in CHO cells, another cell adhesion molecule (CAM) on the CHO 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 and the inhibition sites 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 cannot bind to neurons and consequently the inhibition site cannot engage and there is no inhibition of neurite outgrowth (D). In contrast, when MAG (d1-3)-Fc is added to neurons in binds via its sialic acid binding site but does not inhibit axonal growth because the inhibition site is absent (Tang et al, 1997)

3.2 Results

3.2.1 Expression of truncated MAG(d1-3) and MAG(d3-5) by transfected CHO cells

In previous studies, we demonstrated that there are two distinct functional sites located on MAG. The sialic acid-binding site, Arg118 in Ig domain 1, is responsible for attaching MAG to the neuronal surface, and a second "inhibition site" which is responsible for the inhibition of neurite outgrowth (Tang, Shen et al. 1997). The sialic acid-binding site itself is not sufficient to inhibit neurite outgrowth and is unnecessary when MAG is expressed on cell surface. The fact that a truncated form of MAG-Fc, containing only the first three Ig domains of MAG, termed MAG(1-3)-Fc, can bind to neuron but does not inhibit neurite outgrowth implies that the inhibitory function of MAG requires Ig domain 4 and 5. In order to investigate our hypothesis, two truncated forms of MAG containing only Ig domain 1-3 and domain 3-5 were constructed and transfected into CHO cells (Figure 3.2)

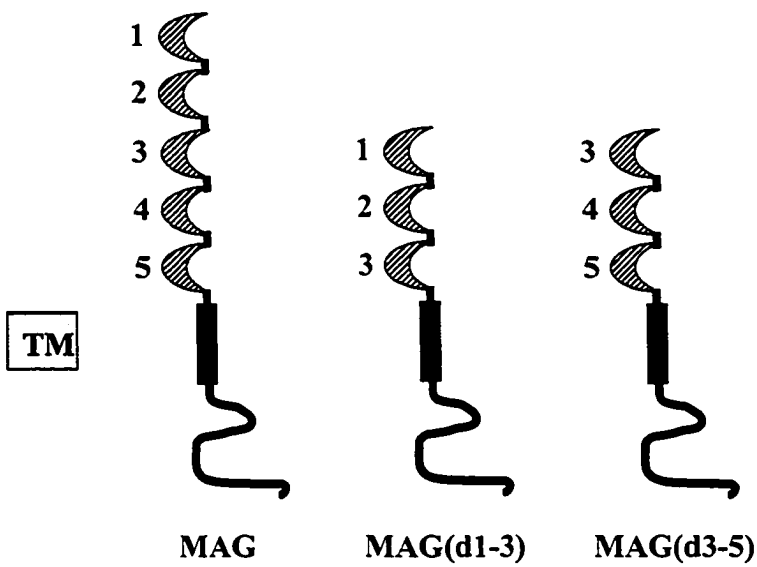


Figure 3.2 Schematic structure of truncated MAG. MAG(d1-3) contains the first three extracellular Ig like domains of MAG, and MAG(d3-5) contains Ig domains 3-5. Both of the two truncated forms retain intact transmembrane and cytoplasmic segments, so as to be expressed as integral membrane protein by transfected CHO cells. As a control, full length MAG was also transfected into CHO cells in the same way.

To obtain a stable truncated-MAG-expressing cell line, the entire pool of transfected cells was immunostained with anti-MAG antibodies and cells with high anti-MAG immunoreactivity were sorted by Fluorescent Activated Cell Sorting (FACS) and cultured as single-cell clones. Since both of these two proteins retain intact transmembrane and cytoplasmic domains, they are also expressed as integral membrane proteins on cell surface, just as is wild-type MAG. The expression of protein by transfected cells was first characterized by western blot analysis. Here we used mouse monoclonal antibody 513 that reacts with MAG Ig domains 1,2, and 3 to detect protein MAG(d1-3), and mouse monoclonal antibody B11F7 whose epitope resides in MAG Ig domain 4 to detect protein MAG(d3-5) (Figure 3.3). As shown, clones we selected do have strong expression of the proteins. Among all these positive clones, #6, #9, #12 of MAG(d1-3) and #1, #4, #8 of MAG(d3-5) were selected to undergo further binding activity and neurite outgrowth inhibition analysis.

Cell surface immunofluorescent staining was than carried out to investigate the surface-expression of the proteins by the transfected CHO cells (Figure 3.4). Monoclonal antibody 513 was used for characterization of wild type MAG and MAG(d1-3) proteins whereas polyclonal antibody ECD was used for the detection of MAG(d3-5). Results show strong surface expression of both truncated MAG mutants and wild type MAG by CHO cells, while there is no MAG protein expression detected in control transfected cells.

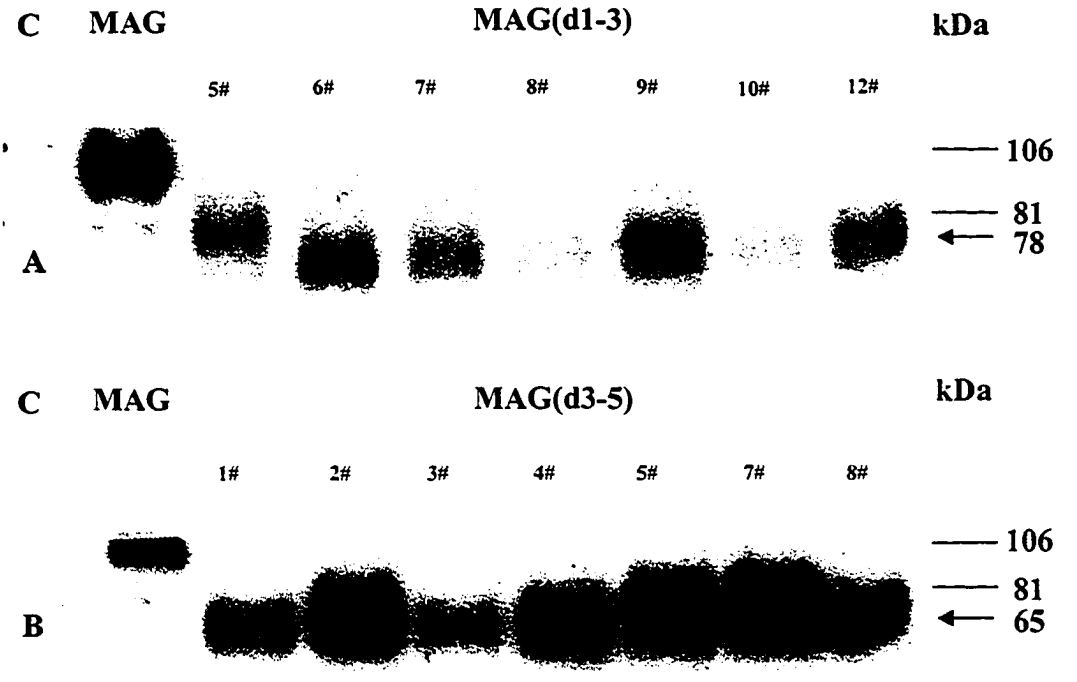


Figure 3.3 Western blot analysis for expression of MAG(d1-3) (panel A), and MAG(d3-5) (panel B) each from 7 transfected CHO single-cell clones; wild type MAG from transfected CHO cells (MAG21); and control transfected cells (R2). 10μg of total protein were loaded per lane. Arrow shows the bands representing MAG(d1-3) and MAG(d3-5), respectively. In panel A, mouse anti-MAG monoclonal antibody 513 was used as the primary antibody and samples were prepared under non-reducing conditions. In panel B, mouse anti-MAG monoclonal antibody B11F7 was used and samples were boiled and prepared with β-mercaptoethanol. HRP-conjugated goat anti-mouse IgG was used as a secondary antibody in both of the experiments.

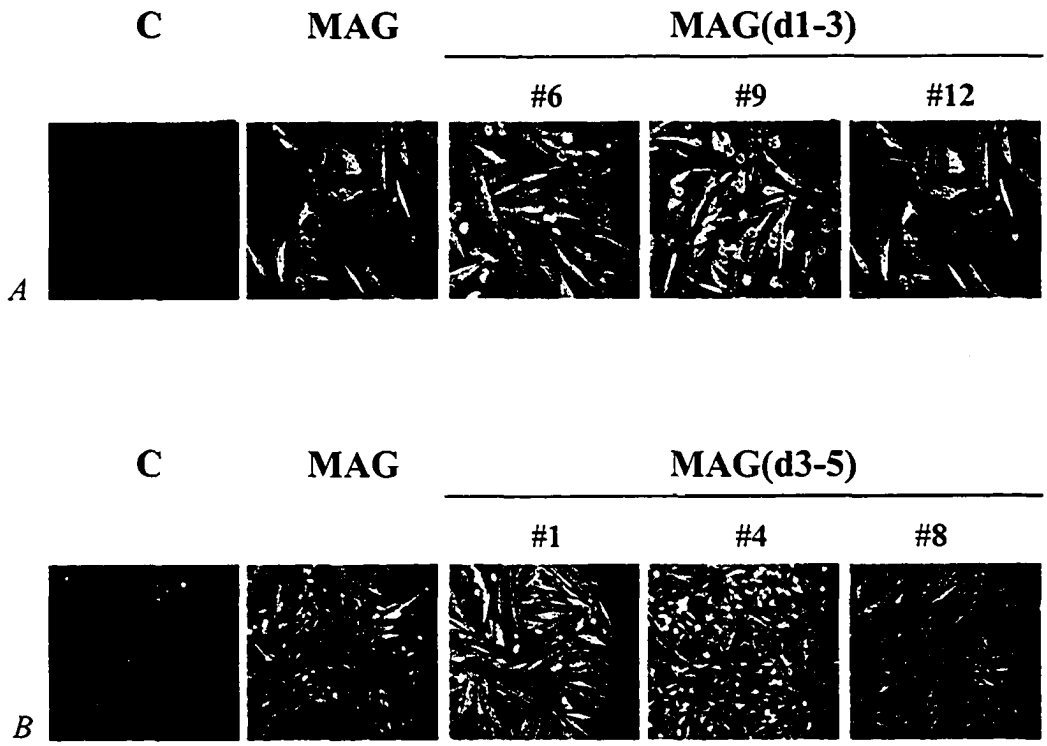


Figure 3.4 Surface immunoflurescence staining of CHO cell-surface expression of wild-type and truncated MAG. Wild type MAG and MAG(d1-3) are stained with the anti-MAG monoclonal antibody 513 (panel A). Wild-type MAG and MAG(d3-5) stained with anti-MAG polyclonal antibody ECD (panel B).

3.2.2 CHO cells expressing MAG(d3-5), but not those expressing MAG(d1-3), lack the sialic-acid-dependent binding activity yet still inhibit neurite outgrowth.

The sialic acid-dependent binding activity of the truncated MAG mutants were then assessed via a binding assay using human erythrocytes. Because human erythrocytes contain well-characterized O- and N-linked glycans, they provide a useful model system to study sialic-acid-dependent interactions. (Figure 3.5) The results show that MAG(d1-3), which contains the sialic-acid-binding site on Ig domain 1, is able to bind with erythrocytes in a sialic acid-dependent manner. Whereas MAG(d3-5), in which the first Ig domain responsible for the recognition of sialic acid residues is deleted along with Ig domain 2, has lost its sialic-acid-dependent binding activity (Figure 3.6). When erythrocytes were desialyated by incubation with neuraminidase before addition to the CHO cell monolayer, neither MAG(d1-3) nor MAG(d3-5) exhibit binding activity. This confirms that the binding between MAG(d1-3)-expressing CHO cells and erythrocytes that we observed is sialic-acid-dependent (Figure 3.7). The effect of these truncated MAG proteins on axonal extension was assessed using our neurite outgrowth assay with dissociated post-natal cerebellar neurons (Figure 3.8). Results confirm that CHO cell-expressed MAG(d1-3), with its intact sialic-acid-dependent binding activity, fails to inhibit neurite outgrowth from neurons grown on a monolayer of these cells. On the other hand, MAG(d3-5), which is deficient in binding to neuronal surface sialic acid residues, still inhibits neurite extension to a similar extent as wild-type MAG (Figure 3.9).

Consistent with the previous observations, our findings demonstrate that there are two distinct functional sites on MAG. The sialic-acid-dependent binding site, located on Arg118 on Ig domain 1, is neither necessary nor sufficient for MAG-mediated inhibition

of neurite outgrowth, whereas the neurite outgrowth inhibition site, carried within the 3-5 Ig like domains, is alone sufficient to inhibit neurite extension, when expressed as an integral membrane protein on the cell surface.

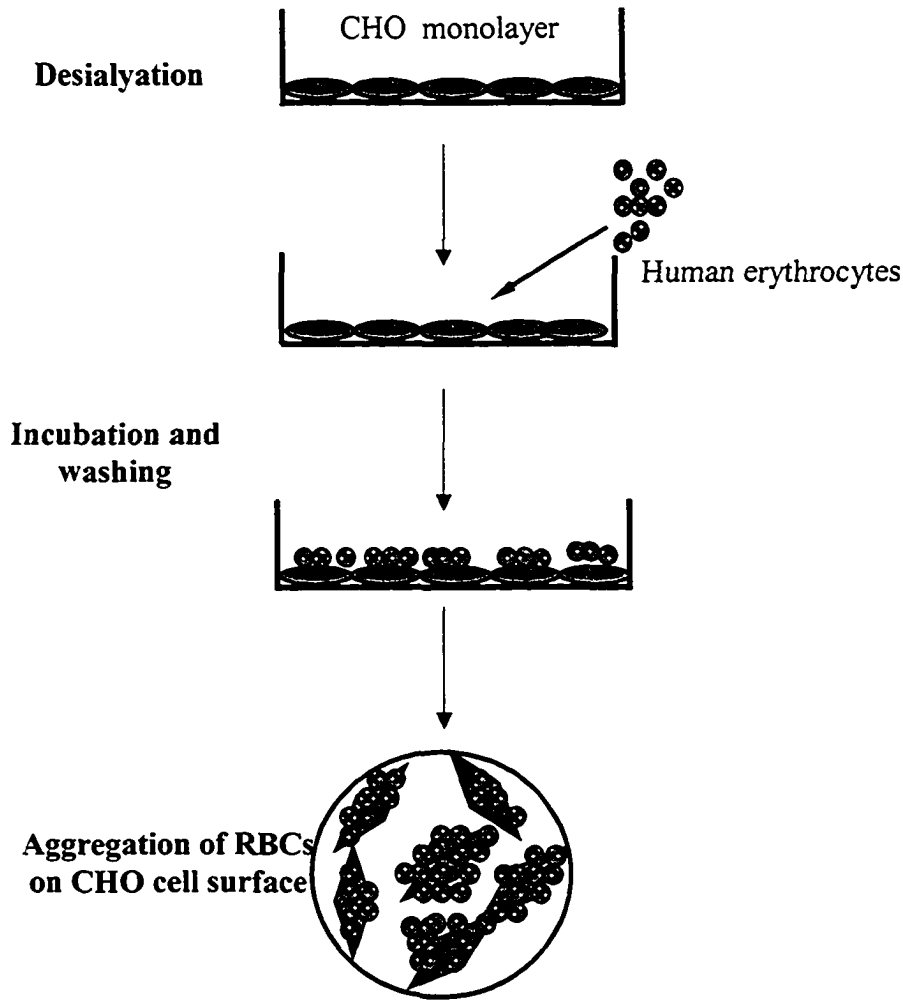


Figure 3.5 Schematic diagram of sialic-acid-dependent binding assay. Confluent CHO cell monolayers expressing potential sialic acid binding surface protein was pretreated with neuraminidase to remove the influence of *cis* sialoglycoconjugates. They were incubated together with fresh human erythrocytes containing abundant O- or N- linked glycoconjugates. After sufficient washing, all the non-specific binding was removed and the interactions between CHO surface proteins and glycoconjugates was visualized by the aggregation of erythrocytes on top of a CHO cell monolayer.

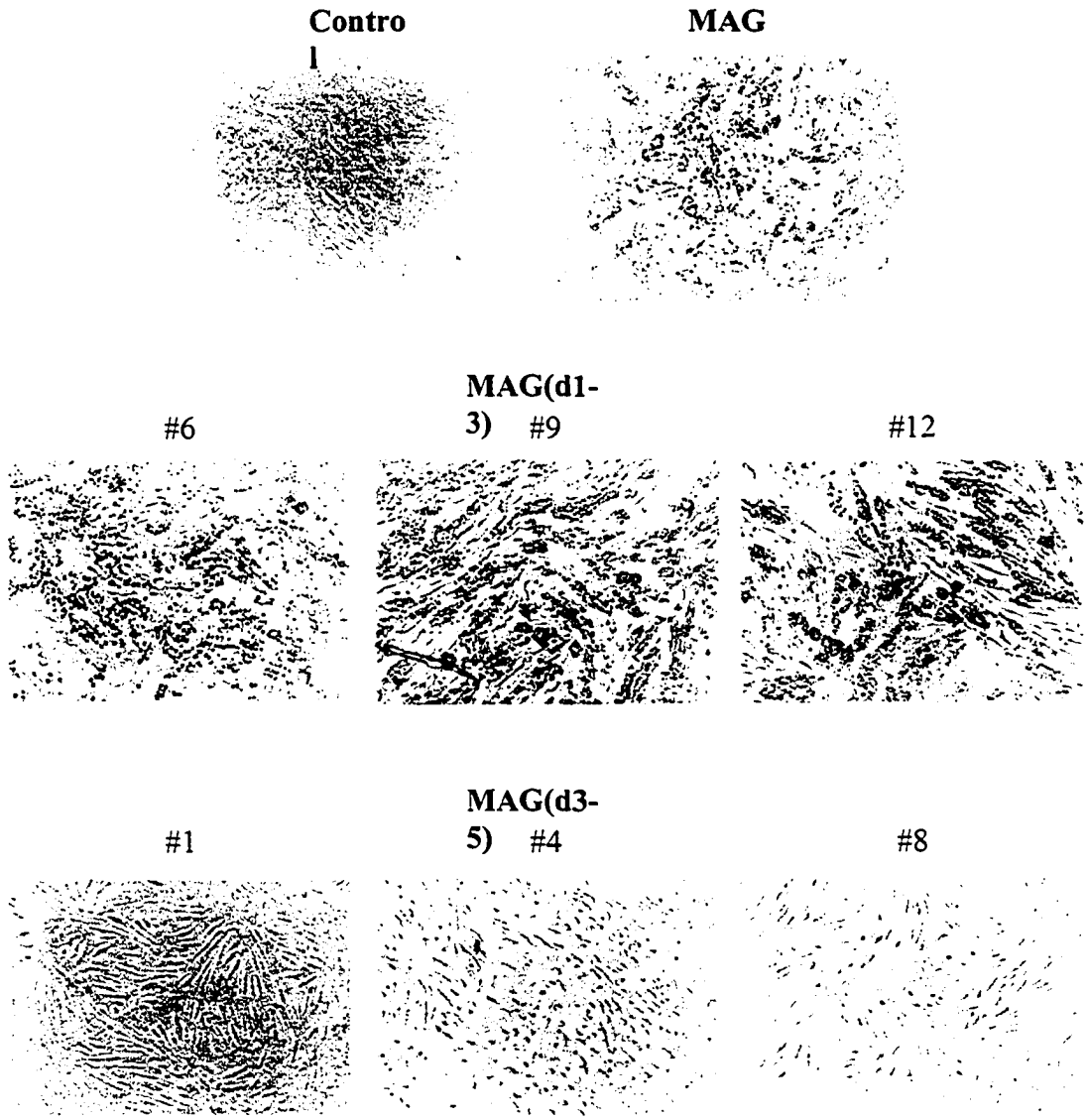


Figure 3.6 Binding abilities of MAG(d1-3) and MAG(d3-5) expressed by transfected CHO cells to sialic acid-containing human erythrocytes. Confluent monolayers of control CHO, full length or truncated MAG expressing CHO cells were first treated with 100mU/ml neuraminidase to remove the *cis* sialic acid residues. Then they were incubated with native human erythrocytes for 1 hour at 37°C. Three single-cell clones of each truncated MAG were selected to carry out this binding assay, clones #6,9,12 expressing MAG(d1-3), and clones #1,4,8 expressing MAG(d3-5). Binding of cell surface proteins to sialic acid-containing glycoconjugates is visualized by the formation of "rosette"-like erythrocytes clusters on top of the monolayers. Results reveal a significant binding of full length MAG and MAG(d1-3) constructs to erythrocytes, while no binding was observed with control cells and MAG (d3-5) expressing cells.

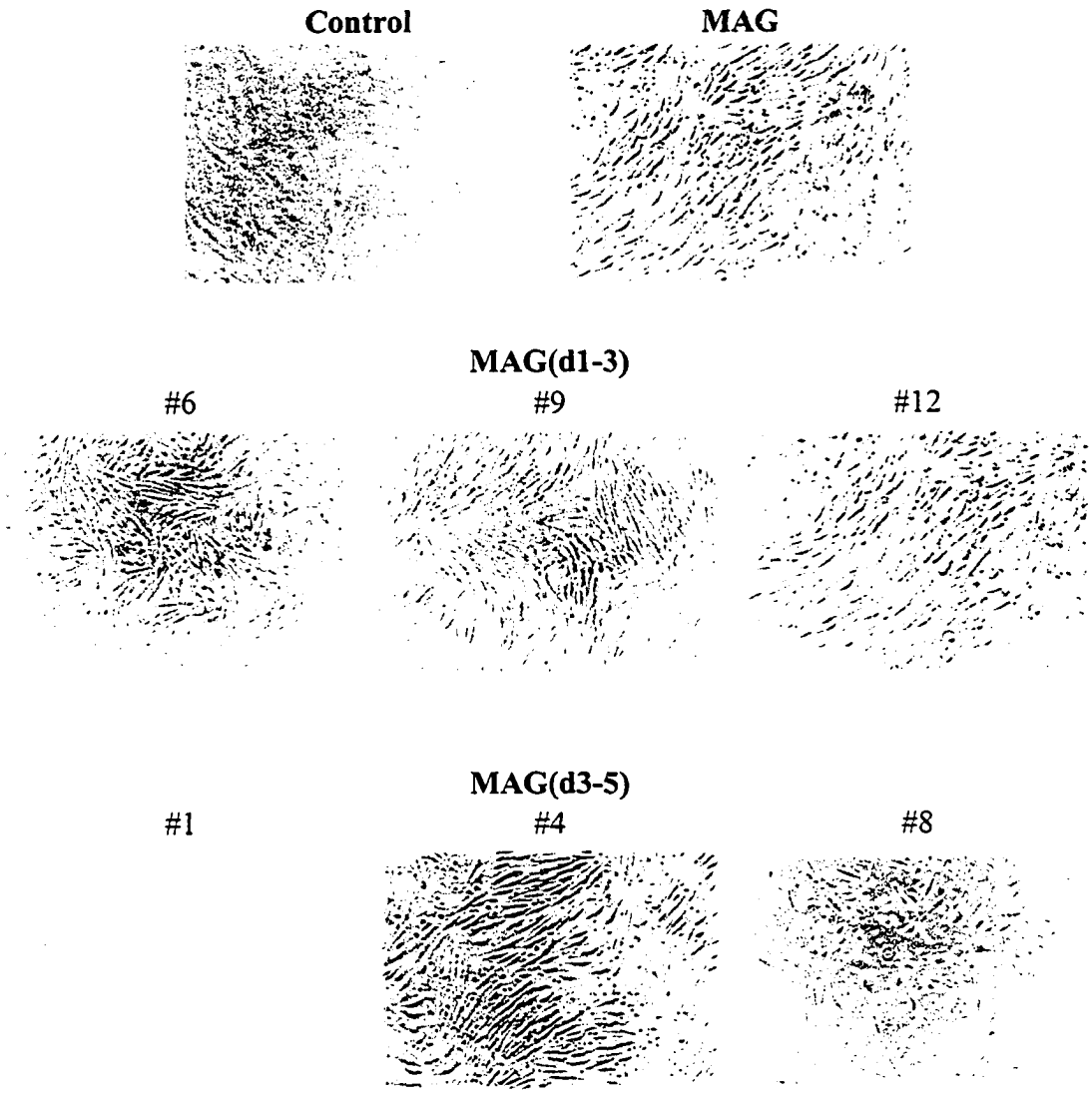


Figure 3.7 The binding of full length MAG and MAG(d1-3) to human erythrocytes is sialic-acid-dependent. In order to demonstrate whether the interactions we observed previously between CHO-expressed MAG, or MAG(d1-3) and erythrocytes is sialic acid dependent, human erythrocytes were pretreated with 100mU/ml neuraminidase before being applied to the CHO monolayers. As we see here, all the bindings between either full length MAG or MAG(d1-3) and erythrocytes were completely abolished by neuraminidase treatment, which indicates the existence of the sialic-acid-dependent binding activity in MAG(d1-3) but not in MAG(d3-5).

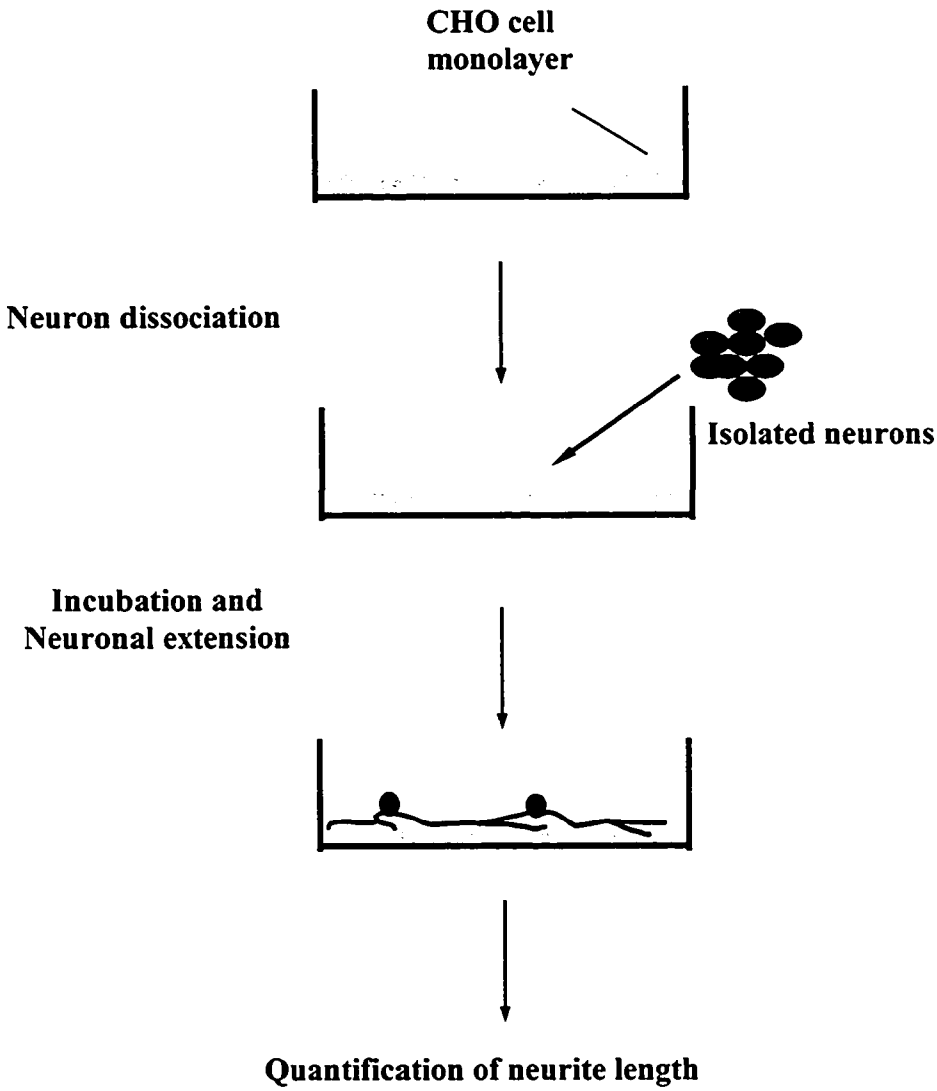


Figure 3.8 Schematic diagram of the *in vitro* neurite outgrowth assay. A CHO cell monolayer expressing MAG proteins was prepared one day before the assay. Primary neurons were then isolated and dissociated before plating onto the CHO monolayer. This CHO-neuron co-culture was maintained at 37°C for 18 hours before fixation and staining with anti-GAP43 antibody. The average length of neurites from 200 randomly selected GAP-43 positive neurons was quantified.

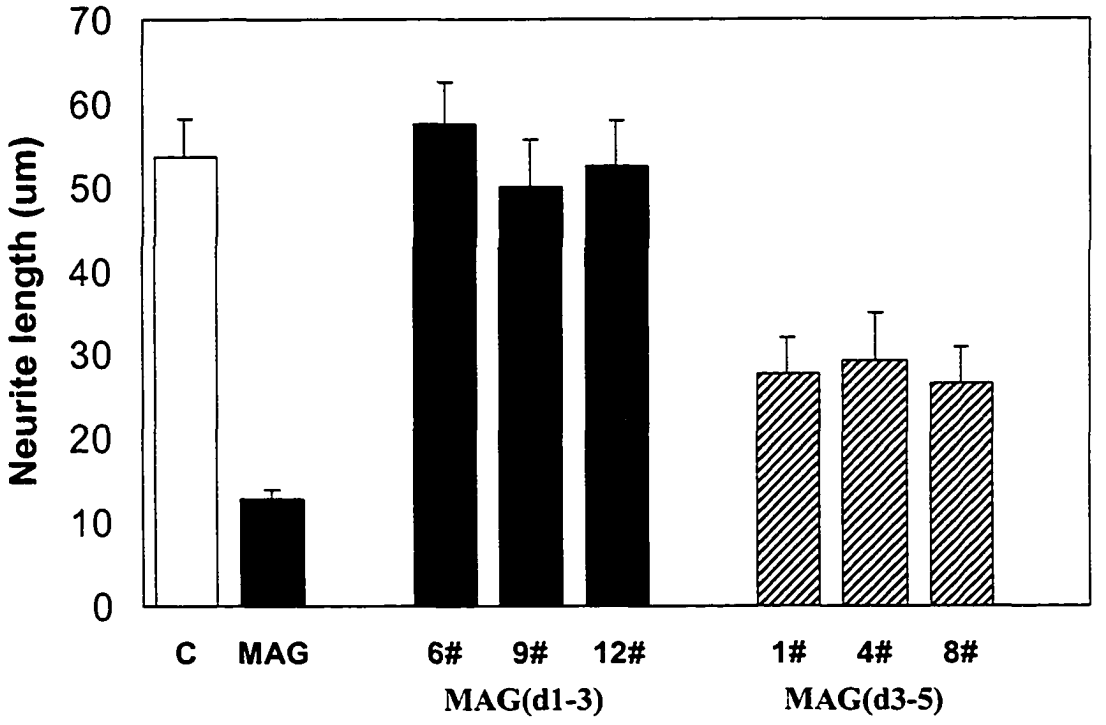


Figure 3.9 The effect of CHO cell-expressed MAG(d1-3) and MAG(d3-5) on neurite outgrowth from post-natal cerebellar neurons. Dissociated cerebellar neurons from PND3 rats were cultured for 18 hr on confluent monolayers of control CHO cells (C), wild-type MAG expressing cells (MAG) and truncated MAG(d1-3)- and MAG(d3-5)-expressing cells (numbers indicate different single cell clones of transfected cells). The co-cultures were fixed and stained for GAP-43, and the longest neurite of each GAP-43 positive neuron was measured. Results show that MAG(d1-3)-expressing cells do not inhibit neurite extension from these neurons, whereas MAG(d3-5)-expressing cells inhibit neurite extension to about 50% of the neurite length from neurons grown on control cells.

3.3 Discussion

Here, we report that the site characterized for the sialic acid-dependent binding activity of MAG, which localizes to the amino acid Arg118 in the first extracellular Ig domain, is distinct from the neuronal inhibition site. A truncated form of MAG, MAG(d1-3), contains the sialic acid binding activity but exhibits no inhibitory effect on neurite outgrowth. However, MAG(d3-5), another truncated form of MAG that lacks this binding site, does not perform the sialic acid-dependent binding yet still inhibits neurite outgrowth to about 50% of control neurite length when expressed as a membrane protein. These results suggest that there are indeed two functional sites on MAG proteins, a sialic acid recognition site and a neurite outgrowth inhibition site, which are distinctly located. This finding is not surprising considering the general profile of Siglecs. It is well known that each member of the Siglec family has a distinct binding preference for specific types of sialic acid and also for specific types of glycoconjugates linkages. This sialic acid-binding specificity may help the Siglec proteins in recognition and communication with their target cells. However, despite all the identified Siglec members that share very similar sialic-acid-binding specificities, they often have very different biological functions (Crocker 2002). One such example is Sialoadhesin, an adhesion molecule restricted to macrophages, and is involved in the interactions between developing myeloid cells in the bone marrow and lymphocytes in spleen and peripheral lymph nodes (Van Den Berg 1992). Sialoadhesin can also bind to neurons via sialic acid, in a linkage similar to that for MAG, but does not inhibit neurite outgrowth (Tang, Shen et al. 1997). This suggests that the sialic acid recognition site on Siglec proteins may only function in a general way for binding of proteins to their target cells, whereas the biological effects

of such an interaction are carried out by other parts of the protein. New evidence confirming this conclusion has recently emerged with the identification of NgR as the functional receptor mediating neurite outgrowth inhibition by MAG (Domeniconi, Cao et al. 2002; Liu, Fournier et al. 2002). Both groups have reported that NgR interacts with MAG in a high-affinity, sialic-acid-independent manner and, as a result of the binding, downstream signaling is induced which leads to a MAG-induced inhibition of axonal regeneration.

Even though the sialic-acid-dependent binding of MAG to neuronal surface glycoconjugates is neither necessary nor sufficient for inhibition of axonal growth, it still plays a very important role in the efficiency of MAG-mediated neuronal inhibition. Our lab demonstrated that, when sialic acid residues were removed from postnatal cerebellar neurons by treatment with neuraminidase, the inhibition of neurite outgrowth by wild-type MAG was attenuated significantly (DeBellard, Tang et al. 1996). The reduced potency of inhibition by MAG(d3-5) proteins, compared with the full length wild type MAG proteins, as we presented here, also indicates that the loss of the sialic-acid-dependent binding impairs the inhibitory effect of MAG. In recent years, evidence has accumulated suggesting that the interaction between MAG and neuronal gangliosides, especially the two major brain gangliosides, GT1b and GD1a, is important for the neurons's response to inhibition by MAG (Vinson, Strijbos et al. 2001; Vyas, Patel et al. 2002). It has been observed that neurite outgrowth inhibition is reduced by addition of soluble GT1b and GD1a to neurons plated on MAG-expressing CHO cells, and multicovalent clustering of GT1b and GD1a can mimic the inhibitory effect of MAG on neurite outgrowth (Vinson, Strijbos et al. 2001; Vyas, Patel et al. 2002). In addition,

neurons from transgenic mice engineered to lack certain gangliosides are not inhibited by MAG (Vyas, Patel et al. 2002). Yamashita and colleagues also found that the ganglioside GT1b can interact with p75, the signal transducing partner associated with NgR to mediate neurite outgrowth inhibition by Nogo-66, OMgp and MAG (Wang, Kim et al. 2002; Wong, Henley et al. 2002; Yamashita, Higuchi et al. 2002).

Together with our data reported here, these findings suggest a model whereby the sialic-acid-binding site on the first Ig domain of MAG recognizes specific sialoglycoproteins and sialoglycolipids localized to neuronal membrane rafts, and the interactions between them might help to cluster MAG with NgR, p75 and Rho, thus stabilizing the formation of the functional ligand-receptor complexes. Consequently, the engagement of MAG to its receptor complex will promote the binding of the inhibition site located within the last 2 Ig domains, to NgR and thereby induce downstream signaling through p75 and Rho, resulting in the inhibition of axonal regeneration.

CHAPT IV

MAPPING OF THE MAG INHIBITION SITE ON IMMUNOGLOBULIN LIKE DOMAIN 5

4.1 Introduction

As we described in the previous chapter, two distinct sites reside separately in MAG, and each of them can perform its function independently. The sialic acid recognition site is carried on amino acid Arg118 in the first Ig-like domain, and is responsible for the binding of MAG to specific sialic acid-linked glycoconjugates attached on the surface of target cells (Tang et al, 1997). This sialic acid-dependent binding site alone is not sufficient to inhibit axonal extension. Another active site, termed the "inhibition site", is located somewhere between Ig-like domains 3 and 5, and is responsible for the growth inhibitory properties of MAG toward neurite outgrowth. Results from the previous chapter have shown that when presented to neurons, this inhibition site alone can prevent neurite outgrowth to the same extent as full length MAG, provided that other cell adhesion molecules are present to assist the association of MAG with its neuronal receptor, regardless of the presence or absence of sialic-acid binding site. Since MAG is one of the major myelin-associated inhibitory molecules that contribute to the absence of axonal regeneration in the mammalian CNS after injury, this finding looks very promising. It is tempting to speculate that blocking this potent neuronal growth inhibitory site on MAG may provide a valuable therapeutic approach to improve axonal regeneration in mammalian CNS *in vivo*. Hence, the next question to be answered is where is this inhibitory site precisely located on MAG.

In order to identify which of the last three Ig-like domains of MAG indeed carries this inhibition site, three chimeric forms of MAG: Sn(d1-3)MAG(d4,5), Sn(d1-3)MAG(d4)Sn(d5) and Sn(d1-4)MAG(d5) were constructed by dissociating individual MAG Ig domains 3, 4 and 5 from each other and replacing the remaining domains with

their counterparts from Sialoadhesin. Sialoadhesin was the first members of the Siglec family to be identified (Kelm, Pelz et al. 1994). It is an adhesion molecule restricted to macrophages and mediates interactions with developing myeloid cells in the bone marrow and lymphocytes in the spleen and peripheral lymph nodes (Crocker, Mucklow et al. 1994). It has been shown that Sialoadhesin recognizes α 2,3-linked sialic acids attached to N- or O-linked glycoconjugates, which is similar to that recognized by MAG. However, unlike MAG, Sialoadhesin does not inhibit axonal regeneration (Tang, Shen et al. 1997). Thus, replacing the first 3 Ig-like domains in MAG with Sialoadhesin domains 1-3 will not introduce any steric effect on neurite outgrowth but will help the chimeric proteins to remain the normal length and sialic acid binding activity of wild type MAG. This strategy was accomplished using an "overlap extension PCR" strategy modified from "sticky feet"-directed mutagenesis.

"Sticky feet"-directed mutagenesis is a polymerase chain reaction (PCR) based technique for precisely swapping large fragments of DNA between two genes. In principle, long double stranded DNA fragments, derived by PCR, are equipped with "sticky feet" at the fusion site and can be used as efficient mutagenic primers to construct hybridized cDNA irrespective of the location of restriction sites (Clackson and Winter 1989). In our experiments, we used a long primer containing "sticky feet" at the Sialoadhesin-MAG (Sn-MAG) fusion site, generated by PCR, to amplify a large fragment of chimeric double-stranded cDNA and insert it into the wild type Sialoadhesin or MAG cDNA sequence (Figure 4.1).

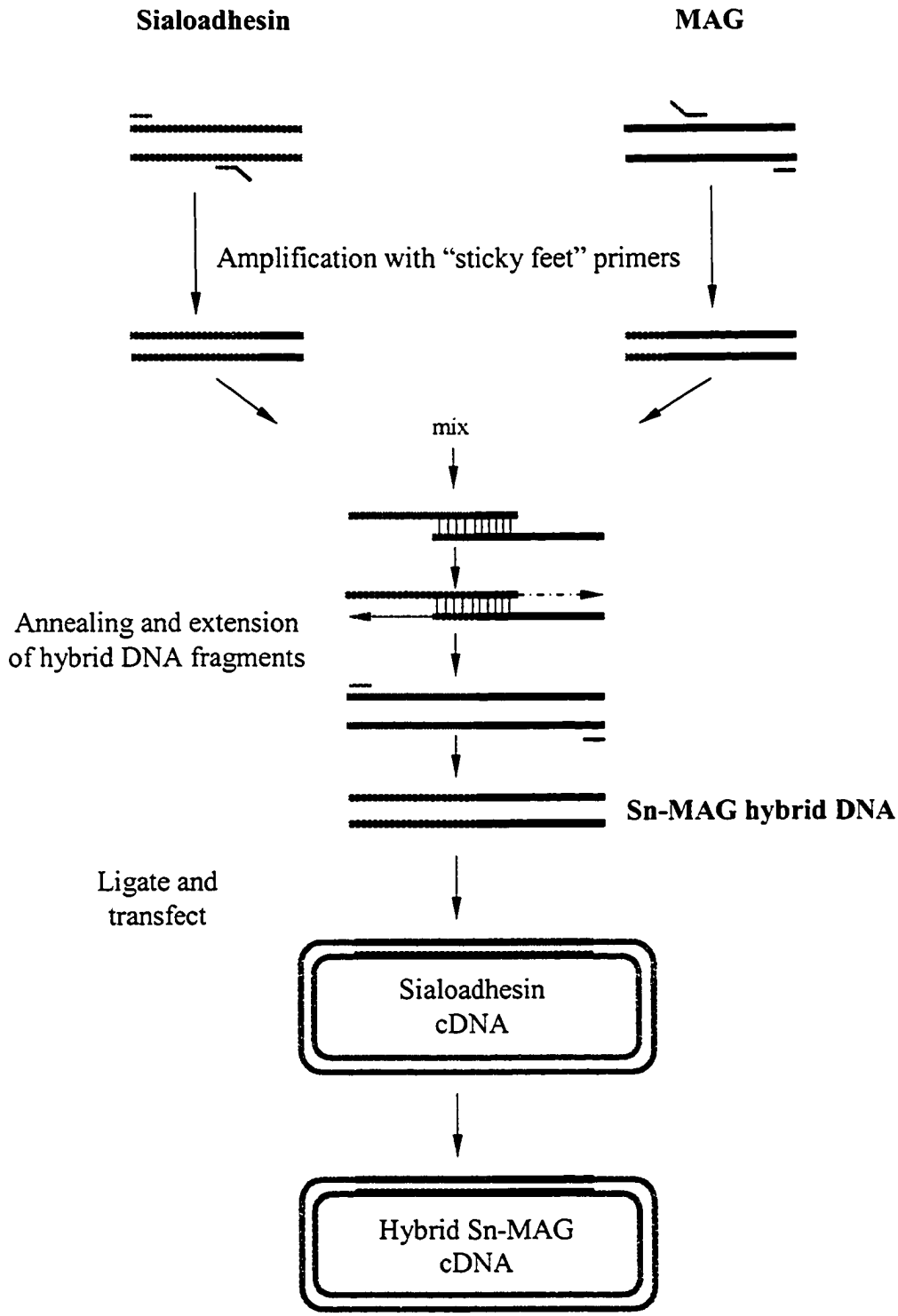


Figure 4.1: Construction of Sn-MAG chimeric proteins by "overlap extension PCR".

Once constructed, these chimeric forms of Sn-MAG fusion cDNA were transfected into CHO cells and expressed as integral membrane proteins. The expression of these chimeric Sn-MAG constructs were then analyzed by Western blotting and the cell surface localization of the proteins has visualized via immunofluorescent staining. At last, the sialic acid-dependent binding and neurite outgrowth inhibition abilities of these chimeric Sn-MAG proteins were assessed by our *in vitro* binding and neurite outgrowth assays as described in the previous chapter.

Furthermore, with the collection of evidence demonstrating that the three major myelin-associated inhibitors, MAG, Nogo-66 and OMgp, share the same neuronal receptor signaling complex and contribute predominantly to the inhibitory effect of CNS myelin in axonal regeneration, we wanted to determine if blocking the interaction between the MAG inhibition site and the neuronal receptor would be able to block the inhibition by MAG and by myelin in general. To test this, a series of peptides with amino acid sequences derived from various regions in the MAG Ig domains 4 or 5 were synthesized by the company SynPep, and their functions as antagonists to MAG in blocking MAG-mediated inhibition of neurite outgrowth was addressed in our neurite outgrowth assays.

Finally, in addition to mapping the entire Ig domain that is indispensable for the inhibitory function of MAG on neurite extension, we also sought to narrow down this critical "inhibitory site" into specific amino acid(s). To do this, we introduced single-amino-acid mutations into certain target regions within the MAG gene. Since there was evidence from our previous data indicating that the last two extracellular Ig domains of MAG are the most likely to carry this inhibition site, we therefore focused our search on

Ig domain 4 and 5. After the analysis of the predicted 3-D structure of the MAG protein, we selected eight amino acid residues located in Ig domains 4 or 5 and individually mutated each. These mutant MAG proteins were expressed in soluble form by transfected COS cells, followed by a series of binding and neurite outgrowth assays to characterize their functional activity.

4.2 Results

4.2.1 Construction of chimeric Sn-MAG fusion cDNAs

Based on the principle of previously described "overlap extension PCR" strategy, the first step of our approach to generate chimeric Sn-MAG fusion constructs was to design primers with about 15-20 nucleotides containing each individual fusion site. Once designed, these primers were produced by Gibco GRL. Then, hybrid DNA fragments were synthesized via PCR amplification followed by insertion into a pPCR-Script Amp SK(+) cloning vector using the PCR-Script Amp Cloning Kit (Stratagene). Afterward, Amp cloning vectors carrying the constructed DNA fragment were transformed into XL-10 Gold Kan ultracompetent E.Coli cells and positive clones were selected via Ampicillin resistance (50ug/ml). Each construct was verified for its nucleotide sequence accuracy by restrictive endonuclease digestion and DNA sequencing before being further sub-cloned into the expression plasmid pcDNA3.1. Figure 4.2 illustrates three chimeric Sn-MAG fusion constructs that have been generated, together with the wild type MAG gene as a control.

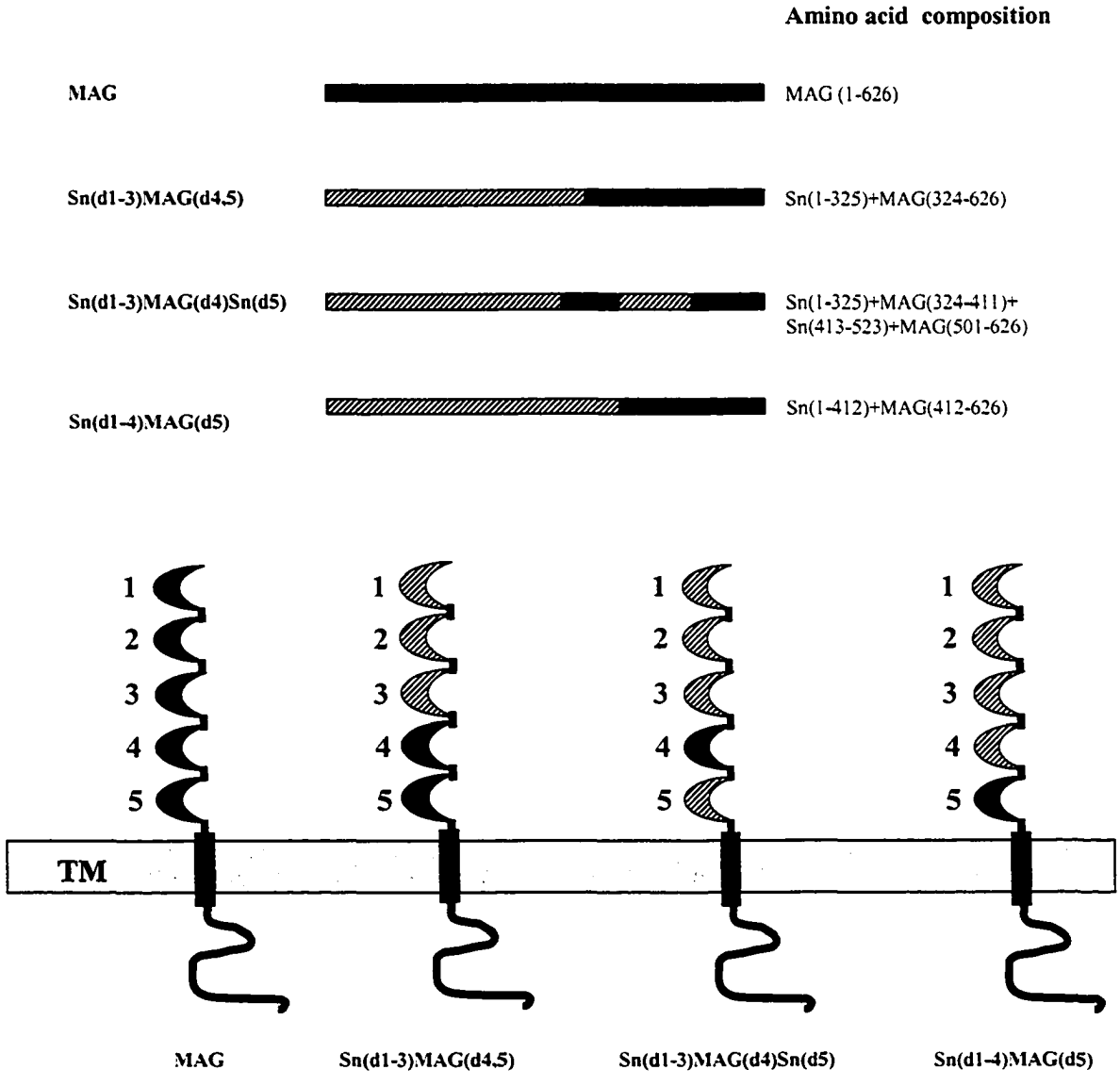


Figure 4.2: Schematic diagram of construction of chimeric Sn-MAG fusion proteins. Three chimeric Sn-MAG constructs, Sn(d1-3)MAG(d4,5), Sn(d1-3)MAG(d4)Sn(d5) and Sn(d1-4)MAG(d5) were generated by swapping Ig domains between Sialoadhesin and MAG using "overlap extension PCR". All the constructs contain intact transmembrane and cytoplasmic domains from MAG and are expressed as integral membrane proteins in transfected CHO cells. As a control, the wild type MAG gene is also transfected.

4.2.2 Expression of chimeric Sn-MAG fusion proteins by transfected CHO cells

Both the chimeric constructs and the wild type MAG cDNA were transfected into CHO cells using the SuperFect transfection reagent (Qiagen) and selected via Geneticin (G418 sulfate) resistance. In order to obtain cell lines with stable gene expression, the heterogeneous population of transfected CHO cells were immunostained with anti-Sialoadhesin or anti-MAG antibodies to indicate the expression of chimeric Sn-MAG or wild type MAG proteins, respectively, followed by Fluorescent Activated Cell Sorting. Cells with highest protein expression were isolated and cultured as single-cell clones. After 2-3 passages in culture, the expression of the transfected gene by each clone was analyzed by Western blotting (Figure 4.3). 10μg of total protein was extracted from non-transfected CHO cells (C), previously established MAG-expressing cells (MAG21), three clones of control full length MAG transfected cells (MAG(d1-5)), four clones of Sn(d1-3)MAG(d4,5) transfected cells, two clones of Sn(d1-4)MAG(d5) transfected cells and one clone of Sn(d1-3)MAG(d4)Sn(d5) transfected cells were loaded in each lane of a 10% SDS-PAGE gel. In blot A, the mouse anti-MAG monoclonal antibody B11F7, which recognizes denatured MAG Ig domain 4, was used and followed by HRP-conjugated secondary antibody. We were able to detect a strong band with a molecular weight of about 100kDa from each sample except control CHO cells, representing the expression of full length MAG or Sn(d1-3)MAG(d4,5) chimeric MAG by transfected cells. In blot B, a combination of two types of mouse anti-Sialoadhesin antibodies, SD6 and SER4, which recognize Sialoadhesin Ig domain 1 and 2/3, respectively, was used and followed by HRP-conjugated secondary antibody. One single band with the size of 100kDa was recognized from samples of Sn(d1-3)MAG(d4,5), Sn(d1-4)MAG(d5) and

Sn(d1-3)MAG(d4)Sn(d5) transfected cells, indicating the expression of the corresponding proteins.

The localization of expressed proteins was assessed by cell surface immunofluorescent staining (Figure 4.4). Based on the Western blot results, only one clone was selected from each construct-expressing cell line, each with approximately equivalent protein expression levels, to undergo immunostaining and further functional assessment. For staining, living cells were incubated with either anti-MAG or anti-Sialoadhesin antibody to detect the surface expression of wild type or chimeric forms of MAG proteins. Results showed strong expression of all three chimeric forms of Sn-MAG as integral membrane proteins by transfected CHO cells, and the expression level was similar to that of the full-length MAG.

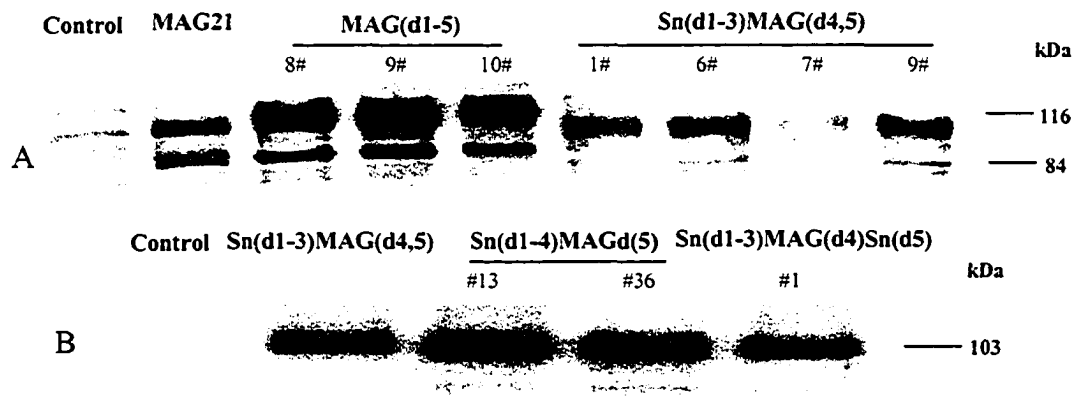


Figure 4.3: Western blot analysis for expression of transfected wild type MAG, Sn(d1-3)MAG(d4,5) (panel A), Sn(d1-3)MAG(d4)Sn(d5) and Sn(d1-4)MAG(d5) (panel B) by CHO single-cell clones. 10 ug total protein extracted from wild type MAG (MAG21 and MAG(d1-5)), chimeric Sn-MAG or control transfected CHO cells were loaded per lane. In panel A, mouse anti-MAG monoclonal antibody B11F7 was used as the primary antibody; In panel B, a combination of two mouse anti-Sialoadhesin monoclonal antibodies, 3D6 and SER4 were used. Samples were prepared under denaturing conditions and HRP-conjugated goat anti-mouse IgG was used as the secondary antibody in both of the experiments

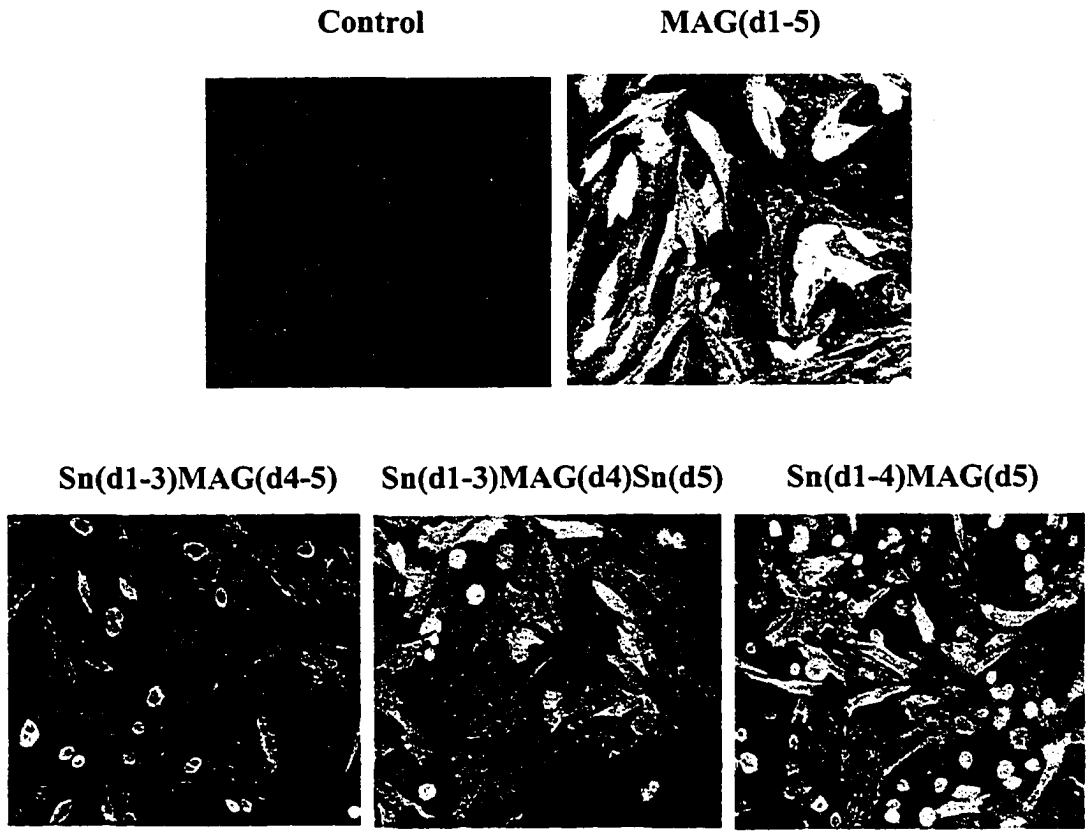


Figure 4.4: Live cells were surface immunostained with mouse anti-MAG monoclonal antibody 513 for MAG(d1-5) expression; or with mouse anti-Sialoadhesin monoclonal antibodies 3D6 and SER4 for Sn(d1-3)MAG(d4-5), Sn(d1-3)MAG(d4)Sn(d5) and Sn(d1-4)MAG(d5) expression. Oregon green-conjugated goat anti-mouse IgG was then used as the secondary antibody. Results show that all the three types of chimeric Sn-MAG proteins and wild type, full length MAG were expressed and located on the surface of transfected CHO cell membranes.

4.2.3 Sialic-acid-dependent binding and neurite outgrowth inhibition activities of chimeric Sn-MAG proteins

Before we could study how much of the neurite outgrowth inhibitory activity was retained by each of the chimeric Sn-MAG fusion proteins, we needed to determine that the sialic-acid-dependent binding activity was not abolished in these proteins, since losing the sialic-acid binding activity may attenuate the effect on inhibiting neurite extension. Thus, we performed the same sialic-acid-dependent binding assay, using human erythrocytes, as was described in the previous chapter (Figure 4.5). Results show that compared to control transfected CHO cells, cells that express each single type of chimeric Sn-MAG protein were able to bind and cluster human erythrocytes on their membrane surface, with similar affinity as that observed for wild type MAG-expressing cells. However, none of the chimeric Sn-MAG proteins were able to bind to human erythrocytes pre-treated with neuraminidase, indicating that the binding is sialic-acid-dependent (Figure 4.6). Therefore, the three chimeric Sn-MAG fusion proteins we constructed maintain the same sialic-acid-dependent binding characteristics as wild type MAG.

Next, we assessed the effect of each chimeric Sn-MAG fusion protein on neurite extension, using our established neurite outgrowth assay (Figure 4.7). Here, dissociated cerebellar granular neurons from postnatal rats were grown on monolayers expressing control, wild type MAG, truncated MAG or chimeric Sn-MAG proteins for 18 hours before fixation and staining with anti-GAP43 antibody. Consistent with our previous observations, both wild type MAG and MAG(d3-5) were able to inhibit neurite extension whereas control cells and MAG(d1-3) had no effect. Surprisingly, differences were

shown by the three types of chimeric Sn-MAG fusion proteins on their abilities to inhibit neurite outgrowth. Both Sn(d1-3)MAG(d4,5) and Sn(d1-4)MAG(d5) exhibited a significant inhibitory effect on neurite extension, to the same extent as wild type MAG; however, cells expressing Sn(d1-3)MAG(d4)Sn(d5) are very permissive to neurite extension. Since Ig domains from Sialoadhesin have no effect on neurite outgrowth, these data suggest that the neurite outgrowth inhibition site on MAG is carried by its Ig domain 5, which alone is able to inhibit neurite extension *in vitro*.

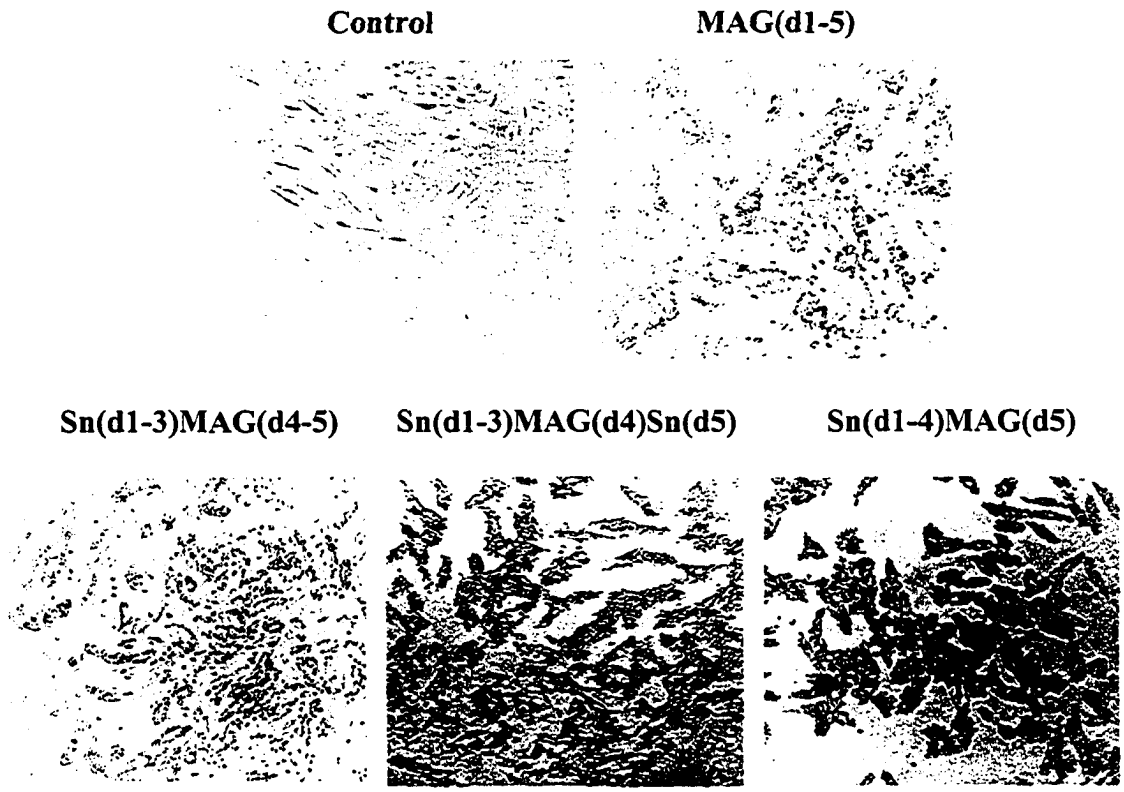


Figure 4.5 Binding of wild type MAG and chimeric Sn-MAG proteins to sialic acid-bearing human erythrocytes. Confluent monolayers of control CHO, wild type MAG(d1-5) or chimeric Sn-MAG expressing CHO cells were first treated with 100mU/ml neuraminidase for removal of *cis*-sialic acid residues. Then, incubated with native human erythrocytes at 37°C for 1 hour. Binding of cell surface proteins to sialic acid-containing glycoconjugates is visualized by the formation of "rosettes"-like erythrocyte clusters on top of the monolayer. Results reveal a significant binding of full-length MAG and all the three types of chimeric Sn-MAG proteins to erythrocytes, while no binding was observed with control transfected CHO cells.

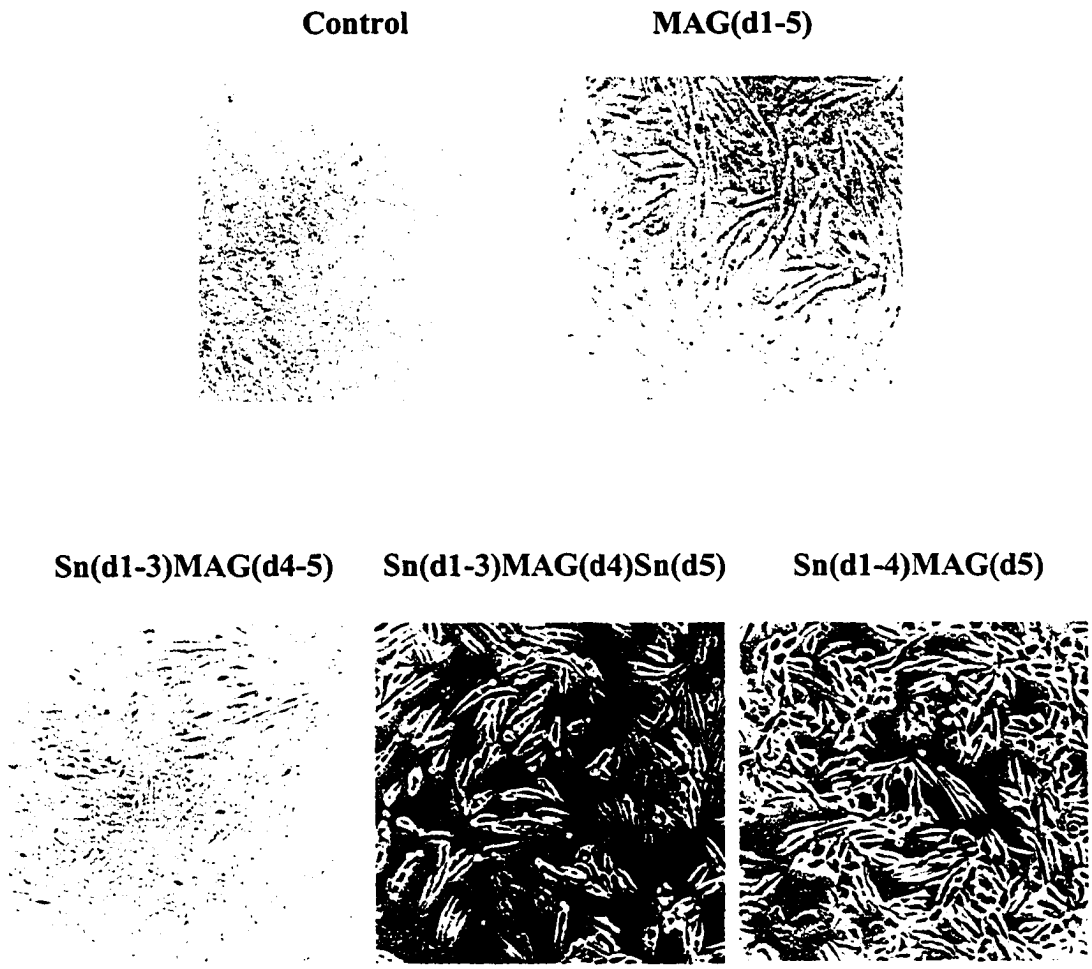


Figure 4.6 Binding of wild type MAG and chimeric Sn-MAG proteins to human erythrocytes are sialic acid-dependent. Here, human erythrocytes were pretreated with 100mU/ml neuraminidase to remove all the surface sialic acid residues before being applied to the CHO monolayers. Results show that all the previously observed erythrocyte bindings exhibited by either wild-type MAG or chimeric Sn-MAG proteins were completely abolished, which indicates its sialic acid-dependency.

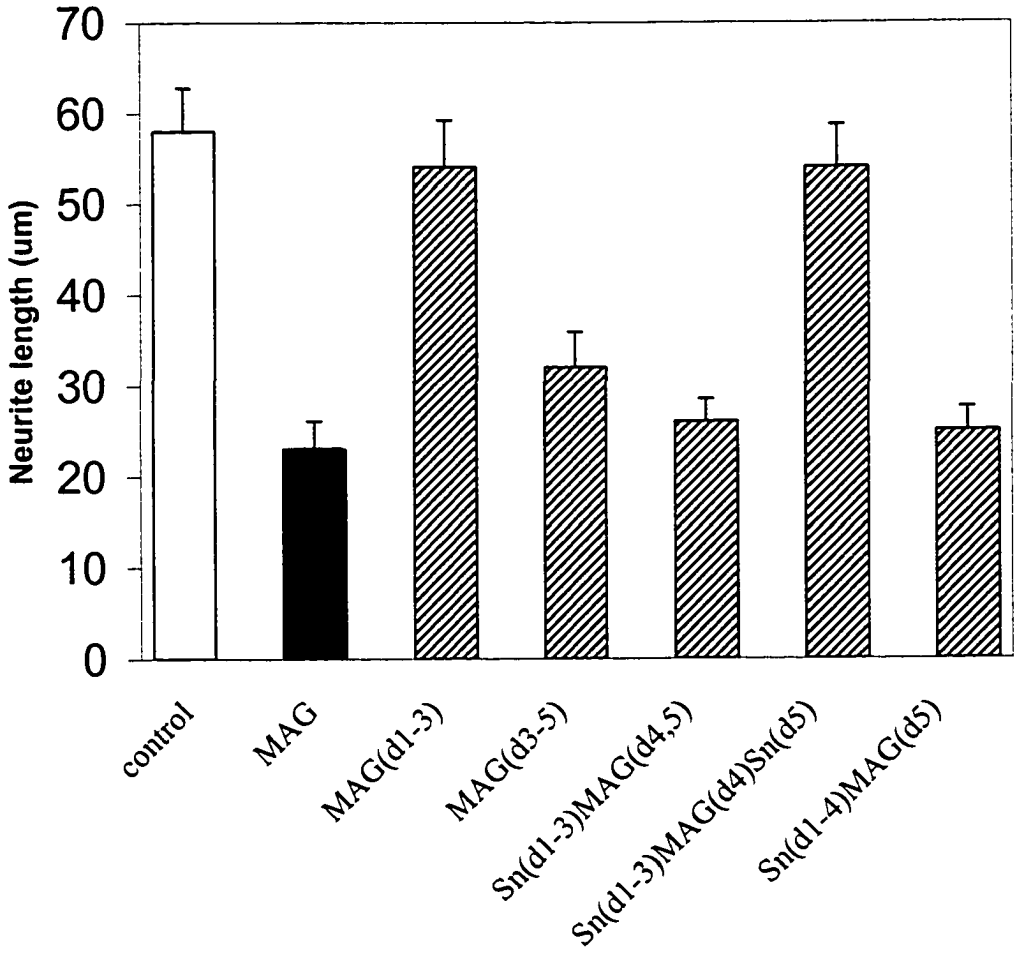
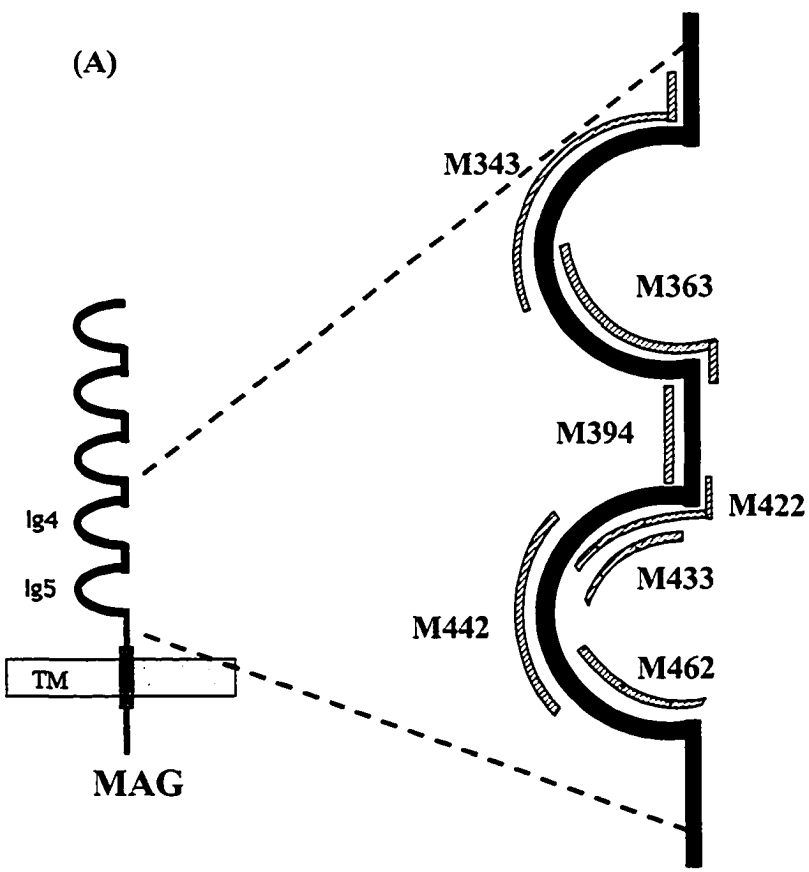


Figure 4.7: Effect of chimeric Sn-MAG proteins on neurite outgrowth. Dissociated cerebellar granular neurons from PND3 rats were cultured for 18 hours on top of monolayers of control CHO cells (control), wild-type MAG-expressing cells (MAG), two truncated forms of MAG-expressing cells (MAG(d1-3) and MAG(d3-5), and three chimeric forms of Sn-MAG-expressing cells (Sn(d1-3)MAG(d4,5), Sn(d1-3)MAG(d4)Sn(d5) and Sn(d1-4)MAG(d5)). Neuron-CHO co-cultures were then fixed with 4% paraformaldehyde and stained for GAP-43. The mean length of the longest neurite per cell for 100-200 randomly chosen individual neurons was quantified.

4.2.4 MAG Ig domain 5 antagonist peptides promote neurite outgrowth

With results from chimeric Sn-MAG proteins showing that the inhibition site of MAG is carried on its Ig domain 5, the next question we sought to answer was which region within this domain is responsible for interaction with the MAG neuronal receptor, NgR, that leads to inhibition of axon outgrowth. In order to address this question, four 25 to 30-residue peptides, consisting of overlapping segments of the MAG Ig domain 5 sequence were designed and synthesized by SynPep. We also synthesized three 30 to 35-residue peptides derived from the sequence of MAG Ig domain 4 and one 30-residue peptide from Sialoadhesin Ig domain 5 as control peptides (Figure 4.8).



(B)

M343: VSILCSTQSNPDPILTIFKEKQILATVIYE
M363: KQILATVIYESQLQLELPAVTPEDDGEYWCVAENQ
M394: AENQYGQRATAFNLSVEFAPIILLESHCAAARDTV
M422: AAARDTVQCLCVVKSNPEPSVAFELPSRNV
M433: VVKSNPEPSVAFELPSRNVTVNETE
M442: VAFELPSRNVTVNETEREFVYSERSGLLLT
M462: YSERSGLLLTSILTLRGQAQAPPRV
Sn447: SHGGLTLASNSGENDFNPRFISSAPNSLR

Figure 4.8: Schematic diagram illustrating seven synthetic peptides, consisting of overlapping segments of MAG Ig domains 4 and 5 (A), and the amino acid sequence of each peptide (B).

These synthetic peptides were purified to about 75-80% purity via HPLC purification, and were then diluted with ddH_2O to a concentration of $2\mu g/\mu l$ in stoke. To test the effect of these peptides on the inhibition of neurite outgrowth by MAG-expressing cells, dissociated primary cerebellar granular neurons from PND3 rats were grown on top of MAG-expressing or control CHO cell monolayers, in the presence of $50\mu g/ml$ MAG-derived peptides or control, Sialoadhesin-derived peptide. Neuron-CHO co-cultures were incubated at 37°C for 18 hours before fixation and staining for GAP-43. After quantification of average neurite length, results showed a significant improvement in neurite length on MAG-expressing cells in the presence of peptide M433 and M462, a slight improvement with peptides M422 and M442, while no improvement at all was observed with neurons grown on MAG-expressing cells in the presence of control Sialoadhesin or MAG Ig domain 4-derived peptides (Figure 4.9). This finding implies that each of the four MAG Ig domain 5 derived peptides, M422, M433, M442 and M462, contains part of the region that is essential for MAG to interact with the NgR-p75 receptor complex and consequently inhibit neurite outgrowth. In addition, since the

summation of these four peptides covers the entire MAG Ig domain 5 with overlapping regions, it appears that it may require at least the majority, if not all, of Ig domain 5 to exhibit its inhibitory effect toward neurite extension. Each of the four peptides derived from this domain comprises only 25-30 amino acids, which is adequate to interfere with full length MAG for binding to NgR, but is insufficient to bind to NgR alone with affinity high enough to trigger downstream signaling and mimic the effect of full length MAG. In this case, the four Ig domain 5-derived peptides function only as antagonists, not agonists, of MAG's inhibition of neurite outgrowth.

Moreover, with the recent elucidation of the shared neuronal receptor complex, NgR-p75, by all three major myelin-associated inhibitory molecules, Nogo66, OMgp and MAG, and their redundant effect in preventing axonal regeneration, it seems possible that blocking the binding of NgR to any one of the ligands would result in a more dramatic declination of overall inhibition by myelin. To test this possibility, the same neurite outgrowth experiment was carried out on an adult CNS myelin substrate (Figure 4.10). Results show that compared to poly-L-lysine, CNS myelin is indeed an inhibitory substrate for neurite extension, as neurons grown on it only protrude neurites half as long as the ones from neurons grown on top of poly-L-lysine. However, with the addition of individual MAG Ig domain 5-derived peptides into neuron-myelin co-cultures, the inhibitory effect of myelin on neurite outgrowth was blocked. On the other hand, addition of control Sialoadhesin-derived peptide has no effect on myelin's inhibition. These data not only confirm our finding that the neurite outgrowth inhibitory site of MAG is located on its Ig domain 5, the blocking of which can abolish MAG inhibitory function on neurite extension, but it also imply that interfering with the binding of MAG to its neuronal

receptor signaling complex using competitive antagonist peptides can result in an abrogation of inhibition by CNS myelin in general.

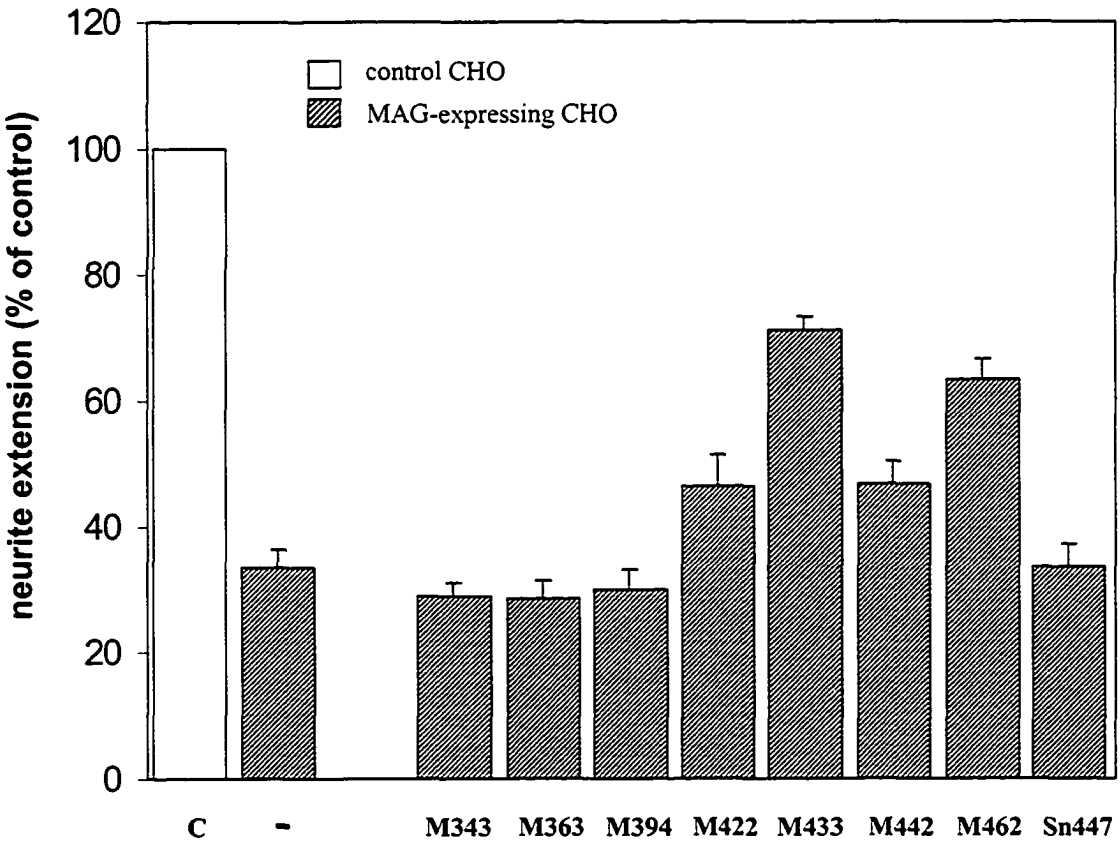


Figure 4.9: Blocking of MAG inhibition on neurite outgrowth by antagonist peptides. Dissociated cerebellar granular neurons from PND3 rats were cultured for 18 hours on top of monolayers of control CHO cells (white bar) or MAG-expressing CHO cells (stripe bars) with or without synthetic peptides. Neuron-CHO co-cultures were fixed with 4% paraformaldehyde and stained for GAP-43. The mean length of the longest neurite per cell for 100-200 randomly chosen individual neurons were quantified, and the extent of neurite outgrowth was represented as percentage of control.

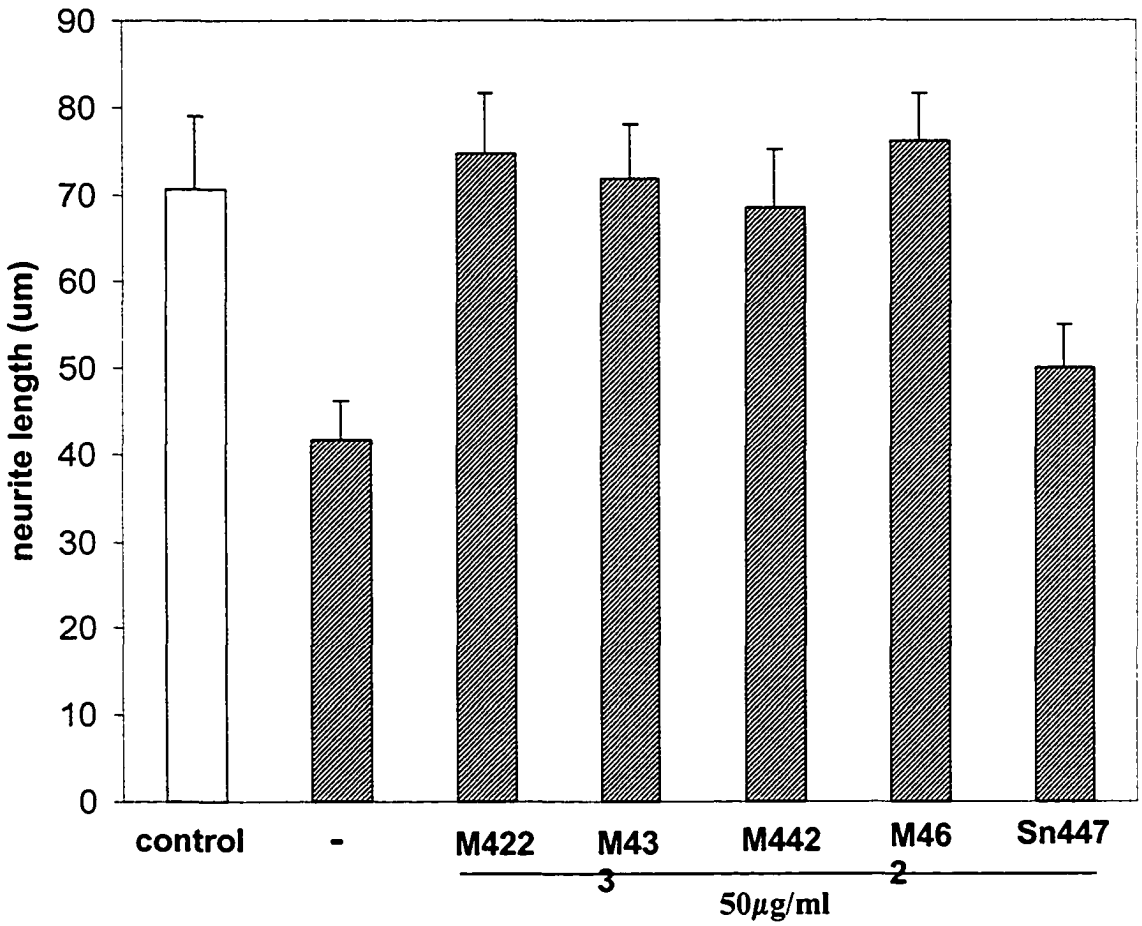


Figure 4.10: Blocking of CNS myelin inhibition on neurite outgrowth by antagonist peptides. Dissociated cerebellar granular neurons from PND1-3 rats were cultured for 18 hours on a substrate of poly-L-lysine (white bar), or CNS myelin (stripe bars) with or without different synthetic peptides. Neuron cultures were fixed with 4% paraformaldehyde and stained for GAP-43. The mean length of the longest neurite per cell for 100-200 randomly chosen individual neurons was measured.

4.2.5 Identification of amino acid residues crucial for the neurite outgrowth inhibitory activity of MAG

Previous domain deletion experiments with different truncated and chimeric forms of MAG have demonstrated that the active site on MAG, responsible for the inhibition to neurite outgrowth, is localized on its Ig domain 5. In order to further narrow down the location of this inhibition site to specific amino acid(s), we introduced single amino acid mutations into this region. As we know, except for Ig domain 1, each of the extracellular Ig domains of MAG adopts a C-2 type Immunoglobulin superfamily fold, which contains primary amino acid sequences more closely resembling those in V regions of immunoglobulin but with the cysteine spacing characteristic of C regions (Williams and Barclay 1988). Each of these C-2 type domains of MAG consists of seven ß-strands and at least two cysteines spaced approximately 50 amino acids apart. In order to identify the amino acid(s) in Ig domain 5 that carry the inhibitory site for MAG, our original thought was to focus on those residues which are predicted to be on the surface of the ß-strands and whose side chain(s) would be able to form a charge-gated pocket or interact directly with receptor molecules. Here, a careful study of the 3-D structure of the MAG protein was made by Dr. Nathan Zaccai. The basis upon which the predictions were made are, first, since the 3-D structure of MAG has yet to be resolved, alignment with other solved 3-D structures of C-2 domains helped to evaluate the likelihood of residues pointing outward in MAG Ig domain 5. Second, the location of disulfide bonds and carbohydrate binding sites in Ig domain 5 can serve as a basis for the elucidation of the orientation of the ß-strands.

Based on these studies, residues that could be on the protein surface were classified and five residues on Ig domain 5 were isolated with strong indication that they may play roles in MAG inhibitory activity. At the same time, three residues from the surface of Ig domain 4 were also chosen to evaluate their function in neurite outgrowth inhibition. As summarized in Figure 4.11, the five amino acid residues from MAG Ig domain 5— Leucine 431, Lysine 435, Glutamic acid 445, Arginine 485 and Argnine 491— and three residues from Ig domain 4— Leucine 346, Alanine 404 and Glutamic acid 410— were subjected individually to drastic mutation. The single-site mutations were introduced into molecular engineered cDNA encoding wild type MAG fused to the Fc region of human IgG, carried in the pIG plasmid. The accuracy of each mutant was verified by DNA sequencing. Then, mutant MAG-Fc constructs were transiently transfected into COS-1 cells and the expressed proteins were collected from culture media via Protein A sepharose chromotography. The purified mutant MAG-Fc proteins were verified by Western blot under non-reducing condition using mouse anti-MAG monoclonal antibody 513 (Figure 4.12, A). The result not only confirms a good yield of soluble MAG-Fc proteins through our transfection and purification processes, but also indicates that the mutant MAG-Fc proteins still possess the native conformation of wild type protein, for the recognition by antibody 513 is conformation-dependent. Next, we sought to assess the ability of these mutant MAG-Fc proteins to bind primary neurons using a solid-phase binding assay (Crocker and Kelm, 1998). In this assay, MAG-Fc molecules were immobilized on a 96-well plate coated with anti-human Fc antibody. Then, isolated postnatal cerebellar neurons were vitally labeled with the fluorescent dye calcein AM and incubated with the immobilized MAG-Fc. Unbound neurons were

washed off and bound neurons were counted with the FluorImager (Figure 4.12, B). Results demonstrate that all the mutant MAG-Fc proteins can bind to primary cerebellar neurons as effectively as wild type MAG-Fc.

Given the observation that these mutant MAG-Fc proteins are unaffected in their ability to bind neurons, we carried out a series of neurite outgrowth assays on immobilized substrates to investigate the effect of these mutant proteins on neurite extension. In order to give dissociated primary neurons a substantial control growth, L1-Fc protein, a cell adhesion molecule that has been shown to promote neurite outgrowth from a variety of neurons (Doherty, Williams et al. 1995), was immobilized on a 8-chamber slide pre-coated with anti-Fc antibody. Then isolated cerebellar neurons from PND2 to PND5 rats were plated onto immobilized L1-Fc with wild type or mutant MAG-Fc proteins added into the neuronal culture (Figure 4.13). The results show that when proteins with single amino acid mutations on Ig domain 4, namely L346D, A404D, E410K, were added to neurons grown on top of immobilized L1-Fc, the extension of neurites was inhibited to the same extent as was seen with wild type MAG-Fc. However, the other five mutant MAG-Fc proteins with mutation site on Ig domain 5, L431D, K435E, E445K, R485D and R491E had partially lost their ability to inhibit neurite outgrowth. Therefore, all the five amino acids we have chosen from Ig domain 5 appera to be required for MAG to exert its inhibitory effect on neurite outgrowth. Mutation of each of them can attenuate MAG's inhibition. Conversely, Ig domain 4 is suggested to be dispensable in this case, for drastic mutation in each of the three residues located on the surface of this domain has no effect on the inhibition by MAG.

Mutants	Change in nucleotides	Ig domain
L346D	CTG/GAC (1186-1188)	4
A404D	GCC/GAC (1360-1362)	4
E410K	GAG/AAG (1378-1380)	4
L431D	CTA/GAC (1441-1443)	5
K435E	AAA/GAA (1453-1455)	5
E445K	GAG/AAG (1483-1485)	5
R485D	CGC/GAC (1603-1605)	5
R491E	AGG/GAG (1621-1623)	5

/translation="MIFLATLPLFWIMISASRGGHWGAWMPSTISAFEGTCVSIPCRF
DFPDELRPAVVHGVWYFNSPYPKNYPPVVFKSRTQVVHESFQGRSRLLGDLGLRNCTL
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VPDNCPELRPELSWLGHEGLGEPTVLGRLREDEGTWVQVSLLHFVPTREANGHRLGCQ
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VVAVEGETVSIRCSTQSNPDPILTIFKEKQILATVIYESQLQLELPAVTPEDDGEYWC
VAENQYGQRATRFNLSVRFAPIILLESHCAAARDTVQCRCVVRSNPEPSVAFRLPSRN
VTVNETEREFVYSERSGLLLTSILTIRGQAQAPPRVICTSRNLYGTQSLELPFQGAHR
LMWAKIGPVGAVVAFAILIAIVCYITQTRRKKNVTESSSFSGGDNPHVLYSPEFRISG
APDKYESREVSTRDCH"

Figure 4.11: Illustration of the single amino acid mutations within MAG Ig domain 4 and 5. Table shows the introduced nucleotide changes and domain localization of each of the corresponding mutations. Below is the sequence of wild type rat MAG protein, with the regions of extracellular domains underlined and the sites of single-amino-acid mutations bold and highlighted.

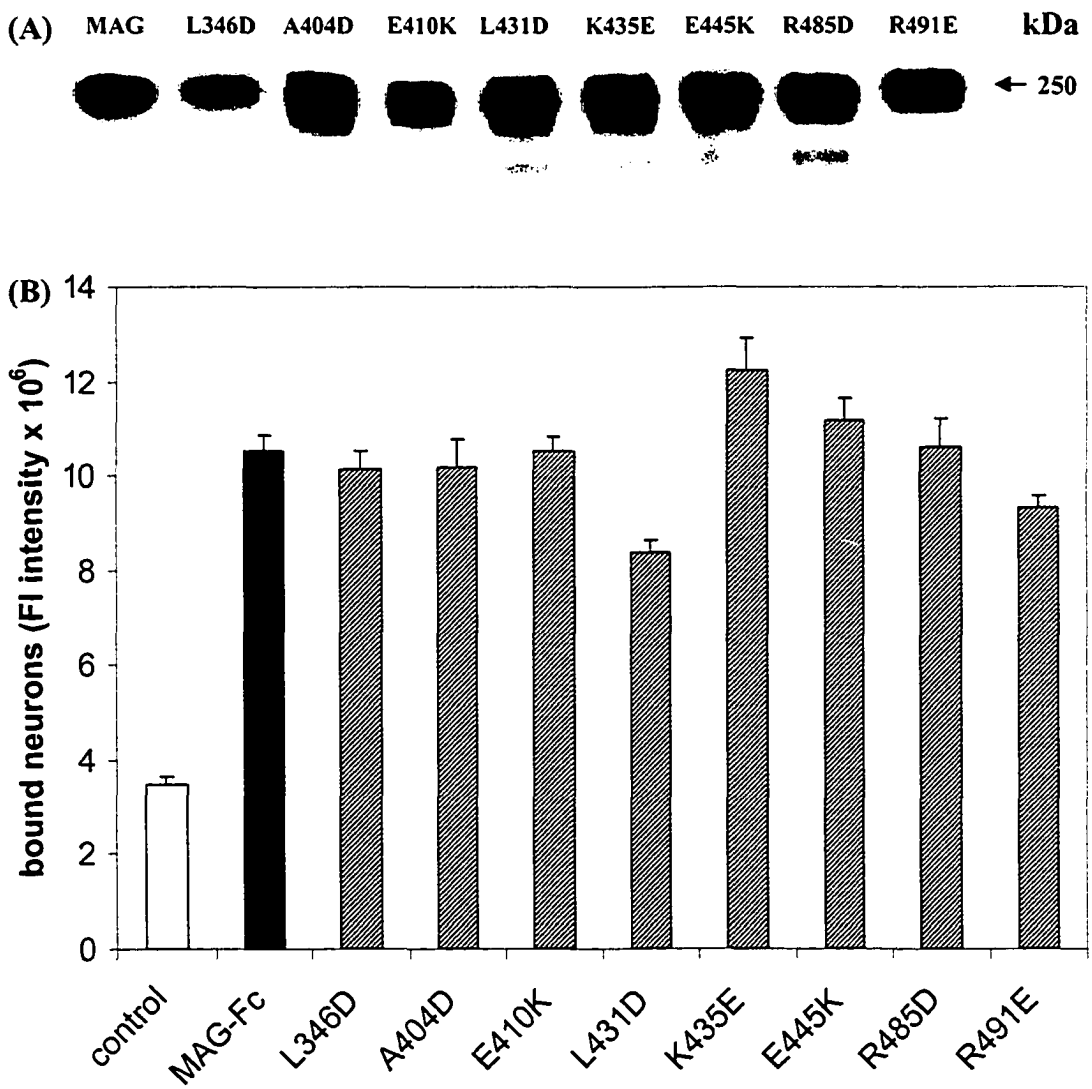


Figure 4.12: (A) Western blot of purified wild type and mutant MAG-Fc proteins with anti-MAG monoclonal antibody 513. (B) Binding of wild type (black bar) and mutant MAG-Fc proteins (stripe bars) to dissociated primary cerebellar neurons. Compared to the control PLL-coated well, wells coated with 25μg/ml of wild type or mutant MAG-Fc bound substantial and relatively equal amount of neurons.

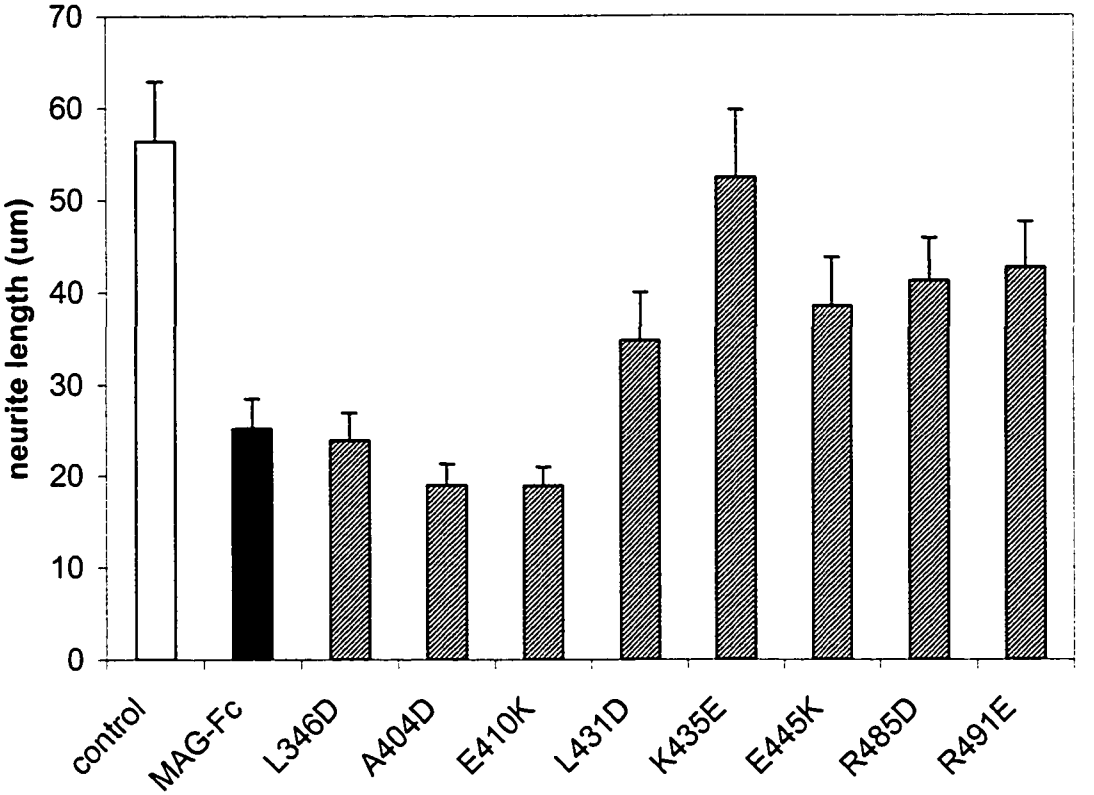


Figure 4.13: Neurite outgrowth of cerebellar neurons on immobilized L1-Fc substrate. Cerebellar granular neurons were isolated from PND 2-5 rats and grown on top of an immobilized L1-Fc substrate. 25μg/ml of wild type (black bar) or mutant MAG-Fc proteins (stripe bars) were added into the neuronal culture and incubated at 37°C for 18 hours before fixation and staining with anti-GAP43 antibody. The average neurite length was quantified by measuring the longest neurite from each of the randomly chosen 100 neurons.

4.3 Discussion

The results presented here suggest that the neurite outgrowth inhibitory function of MAG is predominantly mediated by Ig domain 5. First, a chimeric form of a mutant MAG protein, Sn(d1-4)MAG(d5), which contains only MAG Ig domain 5 combined with the extracellular domain counterparts from Sialoadhesin, exhibits significant potency in inhibiting neurite outgrowth comparable to wild type MAG. Secondly, the inhibition of neurite outgrowth by MAG can be partially reversed using peptides derived from various regions of MAG Ig domain 5, whereas peptides with the sequence of MAG Ig domain 4 or Sialoadhesin Ig domain 5 showed no antagonistic effects. Third, introducing single-site-mutations to several potential protein-surface amino acids located in Ig domain 5 results in improved neurite outgrowth in the presence of MAG. Taken together, these results reveal that there are indeed two distinct functional sites in MAG, a sialic-acid-dependent binding site carried by amino acid Arg118 in MAG Ig domain 1, and a neurite outgrowth inhibition site located on MAG Ig domain 5. The inhibition site is both sufficient and necessary for MAG-mediated axonal regeneration. On one hand, blocking or mutating of this site results in the abrogation of inhibition by MAG. On the other hand, when presented of the surface of living cells, this site alone can inhibit neurite outgrowth regardless the absence of the rest domains.

It was established by both our lab (Domeniconi, Cao et al. 2002) and Strittmatter's group (Liu, Fournier et al. 2002) that NgR, a GPI-linked neuronal membrane protein that was first identified as the Nogo66 receptor, is also the ligand-binding receptor for MAG. The direct interaction of MAG and NgR leads to the activation of the neurotrophin receptor p75, which associates with NgR and transduces

the inhibitory signals downstream into the neuron resulting in the inhibition of axonal regeneration (Wang, Kim et al. 2002; Wong, Henley et al. 2002). In both Strittmatter's and our studies, the binding of MAG to NgR was found to be sialic-acid-independent, which indicates that the NgR-recognition site on MAG is distinct from the sialic-acid-dependent binding site. This is further supported by the results presented here, that the Ig domain 5 of MAG, which does not possess the sialic-acid-recognition activity yet carries the inhibitory function of MAG, could possibly be the protein region responsible for binding to NgR. In the future, two other sets of experiments may be useful to address this postulation. First, whether mutation in this domain will interrupt the binding of MAG to NgR. Secondly, whether the antagonistic peptides that block MAG-mediated neurite outgrowth can also block the MAG-NgR interaction.

Even though from the results we presented here, the sialic-acid-dependent binding site of MAG seems to be non-essential for MAG-mediated inhibition, however it may play important roles in the facilitation of the MAG's effect when MAG is exposed to neurons in a soluble form or as an immobilized substrate. It has been previously shown that when MAG was presented to neurons in a soluble form, mutation of the sialic-acid-binding site abolishes the overall inhibitory effect (Tang, Shen et al. 1997). In recent years, a collection of evidence has implied that neuronal sialylated gangliosides, especially GT1b and GD1a, not only bind to MAG but also are essential for the neurite outgrowth inhibition induced by soluble MAG (Vinson, Strijbos et al. 2001; Vyas, Patel et al. 2002). This suggests the possibility that in order to exert inhibition, soluble MAG proteins may require the ganglioside binding, which is mediated by the sialic-acid-binding site on MAG Ig domain 1, to increase the avidity of the interactions with the

receptor complex and signal transducing molecules. Indeed, it was documented that gangliosides are enriched in lipid rafts on cell membranes, where many GPI-liked proteins, such as NgR, and signal transducing molecules are also localized (Simons and Ikonen 1997; Masserini, Palestini et al. 1999). Thus, it is likely that binding of MAG to these gangliosides potentiates and augments MAG's effect by inducing clustering of the receptor subunits and other active molecules.

Finally, it is promising to notice that the peptides derived from MAG Ig domain 5 can function as antagonists to block MAG-mediated neurite outgrowth inhibition *in vitro*. Since all the three major myelin-associated inhibitors exert their effects through binding to the neuronal receptor NgR (Domeniconi, Cao et al. 2002; Liu, Fournier et al. 2002; Wang, Koprivica et al. 2002), and considering the hypothesis that these inhibitors may also compete for the same binding site (Domeniconi, Cao et al. 2002; Wang, Koprivica et al. 2002), it may be possible that these MAG antagonist peptides are also able to interfere with the other two inhibitors for binding to NgR. Furthermore, it would be very important to investigate whether these peptides can also work as functional antagonists to block the overall inhibition of axonal regeneration by CNS myelin after injury *in vivo*.

CHAPTER V

INTERLEUKIN-6 IS A DOWNSTREAM EFFECTOR OF CYCLIC AMP AND CAN PROMOTE AXONAL REGENERATION

5.1 Introduction

The regenerative capacities of injured CNS neurons are not only influenced by the inhibitory environment they encounter, but also determined by their intrinsic growth states. One indication of this change in intrinsic growth state is an improved regeneration of injured DRG neurons can be achieved by delivering a conditioning, peripheral nerve axotomy prior to the CNS injury (Richardson and Issa 1984; Richardson and Verge 1986; Oudega, Varon et al. 1994). This conditioning lesion significantly increases the ability of injured central branch adult DRG neurons to regenerate along spinal tracts (Richardson and Verge 1986; Neumann and Woolf 1999). Meanwhile, this increased intrinsic growth state can also be illustrated *in vitro* as an enhanced rate of neurite elongation in dissociated adult DRG neurons after a prior conditioning injury *in vivo* (Hu-Tsai, Winter et al. 1994; Edstrom, Ekstrom et al. 1996; Smith and Skene 1997). Recently, we demonstrated that neuronal cAMP levels might serve as an indicator of the intrinsic growth state of neurons and play an important role in determining neuronal response to myelin inhibitors and the extent of regeneration. First, we showed that elevating neuronal cAMP, either with a cAMP analog or by pre-incubating with various neurotrophins, overcomes inhibition by MAG and myelin *in vitro* (Cai, Shen et al. 1999). Second, elevated cAMP levels not only are responsible for the spontaneous regeneration of neonatal spinal cord axons after injury (Cai, Qiu et al. 2001), but also explain the mechanism underlying the switch in neuronal intrinsic growth state induced by a conditioning lesion as well. Results show that a conditioning peripheral nerve lesion is accompanied by an increase in neuronal cAMP levels, blocking of which abrogates the regeneration-promoting effect of the conditioning lesion. More importantly, elevated

cAMP levels not only mimic the effect of a conditioning lesion *in vitro* without peripheral nerve injury (Cai, Qiu et al. 2001), but also result in regeneration of mature spinal axons *in vivo* (Qiu, Cai et al. 2002).

Since then, we sought to identify the downstream consequences of elevated cAMP that lead to enhanced axonal regeneration. One gene that has been found to participate in this cAMP-induced regeneration is Arginase I (Arg I), an enzyme that plays an important role in the synthesis of polyamines. Many studies have shown that polyamines influence growth and development of the nervous system, as well as axonal regeneration (Ingoglia, Sharma et al. 1982; Gilad and Gilad 1988; Gilad, Tetzlaff et al. 1996); (Slotkin and Bartolome 1986). Our lab has shown, for the first time, that Arg I is up-regulated by cAMP in the nervous system in a transcription-dependent manner in response to cAMP, and the synthesis of polyamines catalyzed by Arg I is able to block the inhibition of neurite outgrowth by MAG and myelin (Cai, Deng et al. 2002).

In order to identify other genes that are associated with the cAMP-induced improvement of regenerative capacity in injured CNS neurons, we used microarray system to screen for those genes whose expression is up-regulated after treatment with dibutyryl-cAMP (dbcAMP), a cAMP analog. To our surprise, the gene whose expression is the most robustly elevated is the one encoded for Interleukin-6 (IL-6). IL-6 is a small cytokine molecule produced by various types of cells and is involved in multiple forms of cell-cell communication regulating cell survival, proliferation and differentiation (Heinrich, Horn et al. 1998). In the nervous system, IL-6 is expressed by neurons, glial cells and macrophages (Schobitz, Voorhuis et al. 1992; Gadient and Otten 1995). The levels of IL-6 expression in the CNS remain very low under normal conditions. However,

they are significantly up-regulated during brain injury, infection, inflammation, and neuronal diseases (Strauss, Bauer et al. 1992; Tarkowski, Rosengren et al. 1995; Muller and Ackenheil 1998; Hans, Kossmann et al. 1999; Murphy, Borthwick et al. 1999; Suzuki, Tanaka et al. 1999). The function of IL-6 in the CNS can be both beneficial and detrimental. On one hand, endogenous IL-6 improves the survival and differentiation of neuronal cell lines and primary neurons, protects neurons against glutamate excitotoxicity, reduces susceptibility towards seizures and ischemia, and modulates the expression of a number of neurotrophic factors and other members of IL-6-type cytokines which result in positive feed-back signals for neuronal survival and differentiation. On the other hand, as a proinflammatory factor, IL-6 regulates the expression of many inflammatory and immune-related molecules, such as IL-1β and TNF-α, both of which play important roles in the inflammatory and acute-phase responses after CNS injury. In glial cells, IL-6 promotes astrocyte proliferation, maturation, macrophage activation and astrogliosis. Dysregulation and overexpression of IL-6 in vivo are associated with many neuropathological disorders, such as neurodegeneration, breakdown of the blood-brain-barrier (BBB), angiogenesis and impaired learning (Van Wagoner and Benveniste 1999; Juttler, Tarabin et al. 2002). Taken together, the functions of IL-6 in the CNS are very complicated and can result in both beneficial and destructive effects.

In recent years, another member of the IL-6-type cytokine family, Leukemia inhibitory factor (LIF), has been shown to augment neurotrophin-3 expression and corticospinal axon regeneration after adult CNS injury (Blesch, Uy et al. 1999). Results from LIF knockout mice also suggest that LIF is one of the factors involved in the mechanism which results in improved spinal cord regeneration after a conditioning

lesion, as the increase in the intrinsic growth capacity of injured DRG axons after a conditioning lesion is abrogated in LIF-/- mice (Cafferty, Gardiner et al. 2001). In contrast, although results have shown that IL-6 may be important for the regeneration of axotomized sensory neurons in the PNS (Zhong, Dietzel et al. 1999), little is known regarding its role in axonal regeneration in the CNS. Here, we demonstrate that an increased expression of IL-6 is one of the most prominent consequences after the elevation of cAMP levels in the CNS. More importantly, enhancement of IL-6 signaling by *in vitro* application or *in vivo* delivery of recombinant IL-6 can overcome the inhibitory effects of MAG and myelin on cultured CNS neurons.

5.2 Results

5.2.1 Elevation of IL-6 expression in cultured DRG and Hippocampal neurons after dbcAMP treatment

In our previous studies, we showed that elevating neuronal cAMP levels with a cAMP analog, dbcAMP, overcomes inhibition by MAG and myelin (Cai, Shen et al. 1999). In order to identify the genes whose expressions are consequently changed by the elevated cAMP levels, and thus may be associated with the cAMP-induced improvement of axonal regeneration, we used a microarray analysis system for a large scale of screening of RNA that is increased. First, about 1x 10^6 dissociated PND5 DRG neurons were plated in each well of a poly-L-Lysine-coated 24-well plate. Then neurons were cultured in SATO media with 1mM dbcAMP for 18 hours at 37°C. After that, total mRNA was quickly extracted from both dbcAMP treated and control samples. In collaboration with Dr. Ronald Hart and Mr. Jason Carmel at Rutgers University, cDNA

were systhesized and labeled with fluorescent dyes before hybridization onto a microarray gene chip containing oligonucleotides of 5000 different genes. Analyzed data show that after 18 hour treatment with dbcAMP, the levels of IL-6 mRNA in dissociated DRG cultures are increased by more than 14 folds. Consistent with this result, quantification of mRNA levels using RT-PCR also shows a robust up-regulation of IL-6 mRNA levels in DRG neurons treated with dbcAMP, although the extent of increase is slightly different from the microarray analysis (Figure 5.1). Meanwhile, a two-fold increase in Arg I mRNA levels is also detected by both methods, which is similar to our previous findings (Cai, Deng et al. 2002) and confirms the reliability of this analyzation.

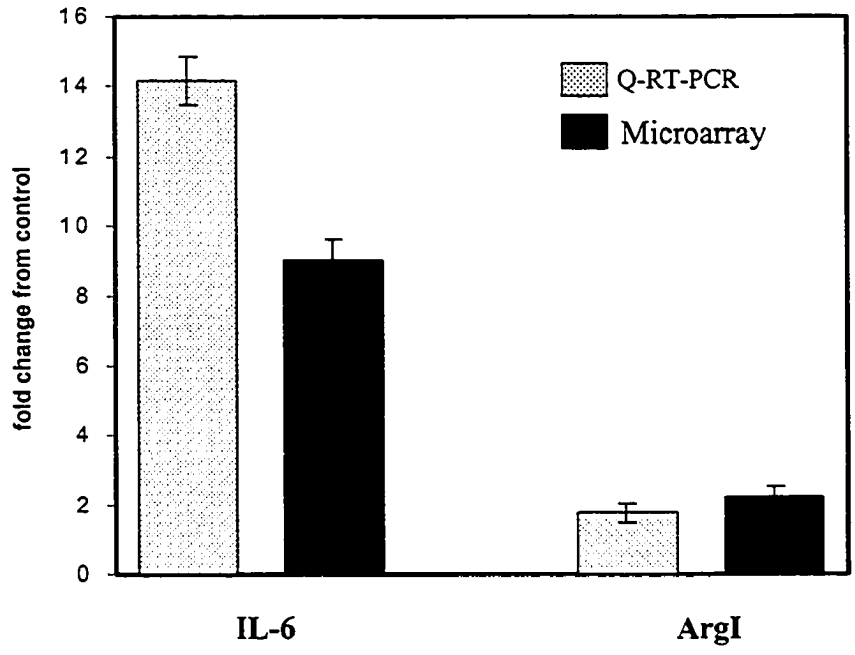


Figure 5.1: Quantification of IL-6 mRNA levels in DRG neurons treated with dbcAMP. DRG neurons were dissociated from PND 5 rats and plated in PLL-coated 24-well plate at a concentration of $1x10^6$ neurons per well. Cultured neurons were incubated with 1mM dbcAMP for 18 hours at 37°C. Total mRNA was isolated and subjected to Microarray or quantitative RT-PCR (Q-RT-PCR) analysis and the results are compared to that of untreatment control cultures. Results show a robust increase in IL-6 mRNA levels after dbcAMP treatment in both Microarray and Q-RT-PCR analysis. As a positive control, a two-fold elevation of Arg I mRNA levels was also detected by either method.

The expression and secretion of IL-6 from cultured neurons was investigated with via Western blot analysis and enzyme-linked immunosorbent assay (ELISA). In Western blot analysis (Figure 5.2 A), dissociated PND 5 DRG neurons were cultured at a concentration of $1x10^6$ cells/ml in SATO media with 1mM dbcAMP. At each indicated time point, supernatants from cultured neurons were collected and 50μg of total protein from each sample was loaded and separated on a 4-20% gradient SDS-PAGE gel. As a control, 5ng of recombinant rat IL-6 was also loaded. The secretion of IL-6 from cultures was detected with an anti-IL-6 antibody. Here, results indicate a dramatic increase in IL-6 protein expression by neurons treated with dbcAMP for 18 hours, and this elevation persists for at least 44 hours after treatment. In ELISA analysis (Figure 5.2 B), dissociated PND5 DRG or PND1 hippocampal neurons were plated onto poly-L-lysine-coated 96-well tissue culture plates and incubated with 1mM dbcAMP for 24 hours at 37°C. The supernatant was collected and IL-6 protein levels were measured immediately via a competitive immunoassay kit. Results reveal a 2- and 3-fold increase in IL-6 protein expression in dbcAMP treated hippocampal and DRG neurons, respectively. Taken together, these data demonstrate that up-regulation of IL-6 expression is one of the downstream events that are induced by the elevation of cAMP levels in cultured neuronal cells. This brings us to the question of whether this elevation of IL-6 expression is associated with enhanced axonal regeneration.

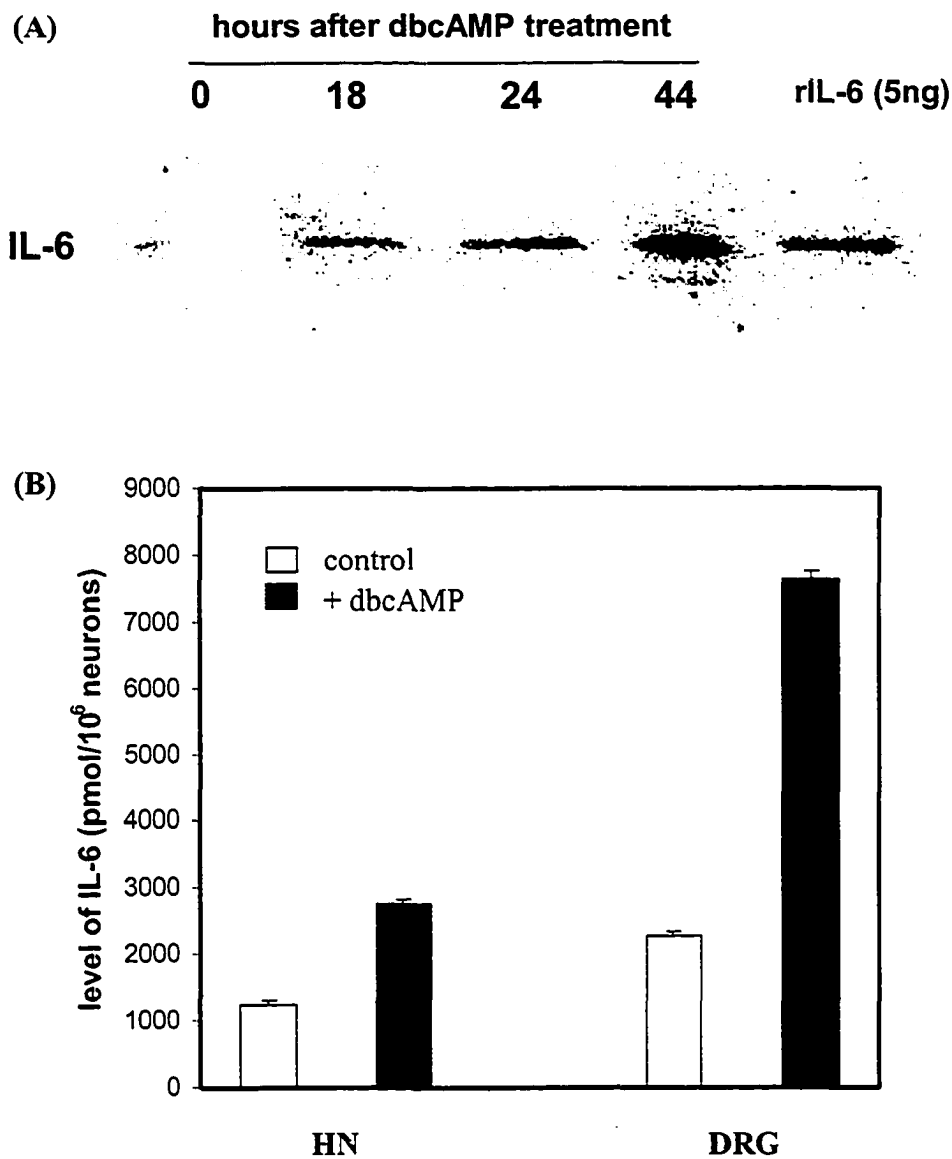


Figure 5.2: Analysis of the regulation of IL-6 protein expression after dbcAMP treatment. (A) Western blot analysis. Dissociated PND5 DRG neurons were cultured with 1mM dbcAMP. Where indicated, total proteins from supernatant of neuron culture were collected at different time points and separated in a gradient SDS-PAGE gel. Using anti-IL-6 antibody, a significant increase of IL-6 expression was detected after 18 hours of dbcAMP treatment, which persists till 44 hours after. (B) ELISA. Dissociated PND5 DRG and PND1 hippocampal (HN) neurons were incubated with 1mM dbcAMP for 24 hours. Then supernatant was collected and IL-6 protein levels were measured immediately with a competitive immunoassay kit (R&D Systems). Results indicated that with dbcAMP treatment, a dramatic elevation of IL-6 expression occurs in both neuronal cultures.

5.2.2 *In vitro* application of IL-6 alone is sufficient to overcome the inhibition by MAG and myelin in cultured DRG and hippocampal neurons but not in cerebellar granule cells

We have shown that elevation of neuronal cAMP levels, either by direct addition of the cAMP analog, dbcAMP, to neurons cultured on myelin inhibitors, or by priming neurons with various types of neurotrophins prior to their encountering the inhibitors, overcomes the inhibitory effects of MAG and myelin on neurite extension (Cai, Shen et al. 1999). To determine whether the up-regulation of IL-6 expression in neuronal cultures stimulated by dbcAMP plays any role in the reversal effect of cAMP on MAG/myelin inhibition, we assessed the ability of exogenous IL-6 to block MAG/myelin inhibition of neurite outgrowth. Initially, we analyzed neurite growth on MAG-expressing CHO monolayers to study the effect of IL-6 in overcoming the inhibition by MAG (Figure 5.3). Here, DRG and hippocampal neurons were isolated from PND5 and PND1 rats, respectively, and plated onto MAG-expressing CHO cell monolayers. Different doses of recombinant rat IL-6 were added directly to the culture media. The neuron-CHO co-cultures were incubated at 37°C for 18 hours before fixation and staining. A quantification of average neurite length revealed that, compared to control neurons, both DRG and hippocampal neurons treated with IL-6 extend significantly longer neurites in the presence of MAG. This block of MAG-mediated inhibition by IL-6 is dose-dependent, with the maximum reversal achieved at an IL-6 concentration of 200ng/ml in both types of neuronal cultures. The same results were also obtained for neurons grown on a CNS myelin as a substrate (Figure 5.4). When IL-6 was applied to PND5 DRG or PND1 hippocampal neurons grown on a CNS myelin, a dose-dependent block of

myelin's inhibition was observed in both neuronal populations, with a maximum reversal effect observed in cultures containing 100ng/ml of IL-6. In both neurite outgrowth assays, excessive IL-6 has no effect on neurite outgrowth from neurons grown on control cells or on PLL. The block of MAG or myelin's inhibition by IL-6 was nearly complete, as growth of neurons on MAG/myelin substrate reached about 90% of control growth.

These results provide strong evidence that elevation of IL-6 alone, in DRG and hippocampal neurons, is sufficient to block the inhibition of neurite outgrowth by MAG and myelin *in vitro*. But, different results were observed with cultured cerebellar granule cells. When dissociated postnatal cerebellar granule neurons were grown on an inhibitory substrates, either MAG-expressing CHO cell monolayers or CNS myelin, neurite extension was strongly inhibited. This inhibition by MAG or myelin can be efficiently blocked by including 1mM of dbcAMP into the culture. However, unlike what was observed for DRG and hippocampal neurons, addition of IL-6 alone in the neuronal culture was insufficient to induce significant reversal of inhibition in cerebellar granule neurons (Figure 5.5 A). In order to identify whether this unresponsiveness of cerebellar neurons to IL-6 is due to lack of binding receptors, we included soluble IL-6 binding receptors (sIL-6R) into the neurite outgrowth assays. It has been reported that sIL-6R is endogenously generated *in vivo* via shedding of the membrane-bound receptor or by alternative mRNA splicing (Lust, Donovan et al. 1992; Mullberg, Schooltink et al. 1993). It can bind to IL-6 and recruit signal transducer receptor gp130 as efficiently as the membrane-bound form. Based on these findings, we tried to increase the activation of the IL-6-receptor signaling complex by adding sIL-6R together with IL-6 directly into the cerebellar neuron-MAG-expressing CHO cell co-culture (Figure 5.5 B), or via overnight

incubation with neurons prior to transfer to the monolayers (Data not shown). To our surprise, both of these treatments show mild improvement of growth against MAG's inhibition, but neither of them have a significant effect. These results indicate that the lack of responsiveness of cerebellar granular neurons to IL-6 in terms of neurite outgrowth is not due to the missing of binding receptors, but may attribute to the inactivity of downstream signaling pathways.

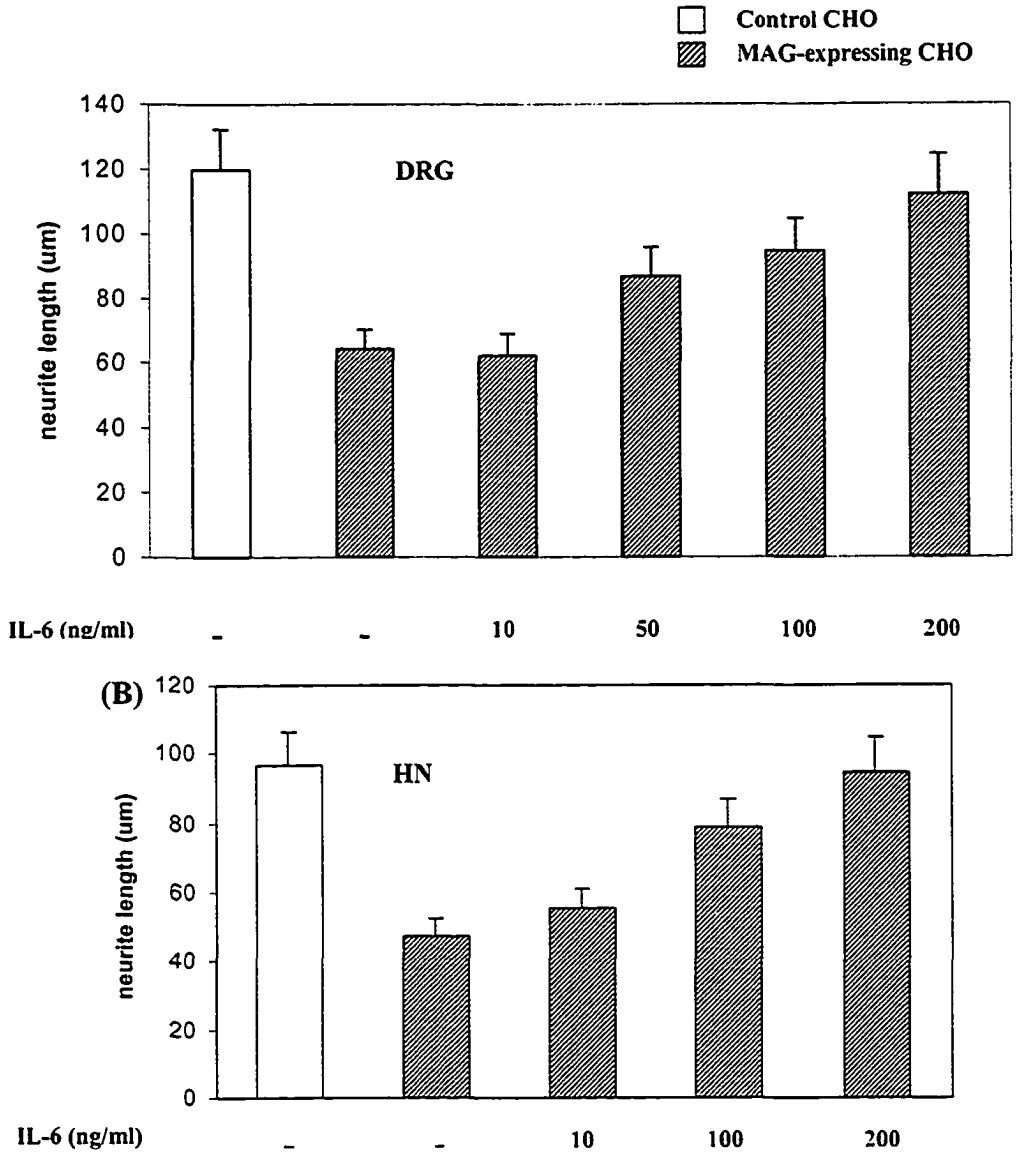


Figure 5.3: Blocking of MAG inhibition of neurite outgrowth by IL-6. DRG neurons from PND5 rats (A) or hippocampal neurons (HN) from PND1 rats (B) were isolated and cultured on top of control or MAG-expressing CHO cell monolayers. Various concentrations of recombinant rat IL-6 were added directly to the cultures. Neurons were cultured at 37°C for 18 hours before fixation and staining with anti-GAP43 antibody. In each experiment, the mean length of the longest neurite for 180-200 neurons was measured for at least three experiments.

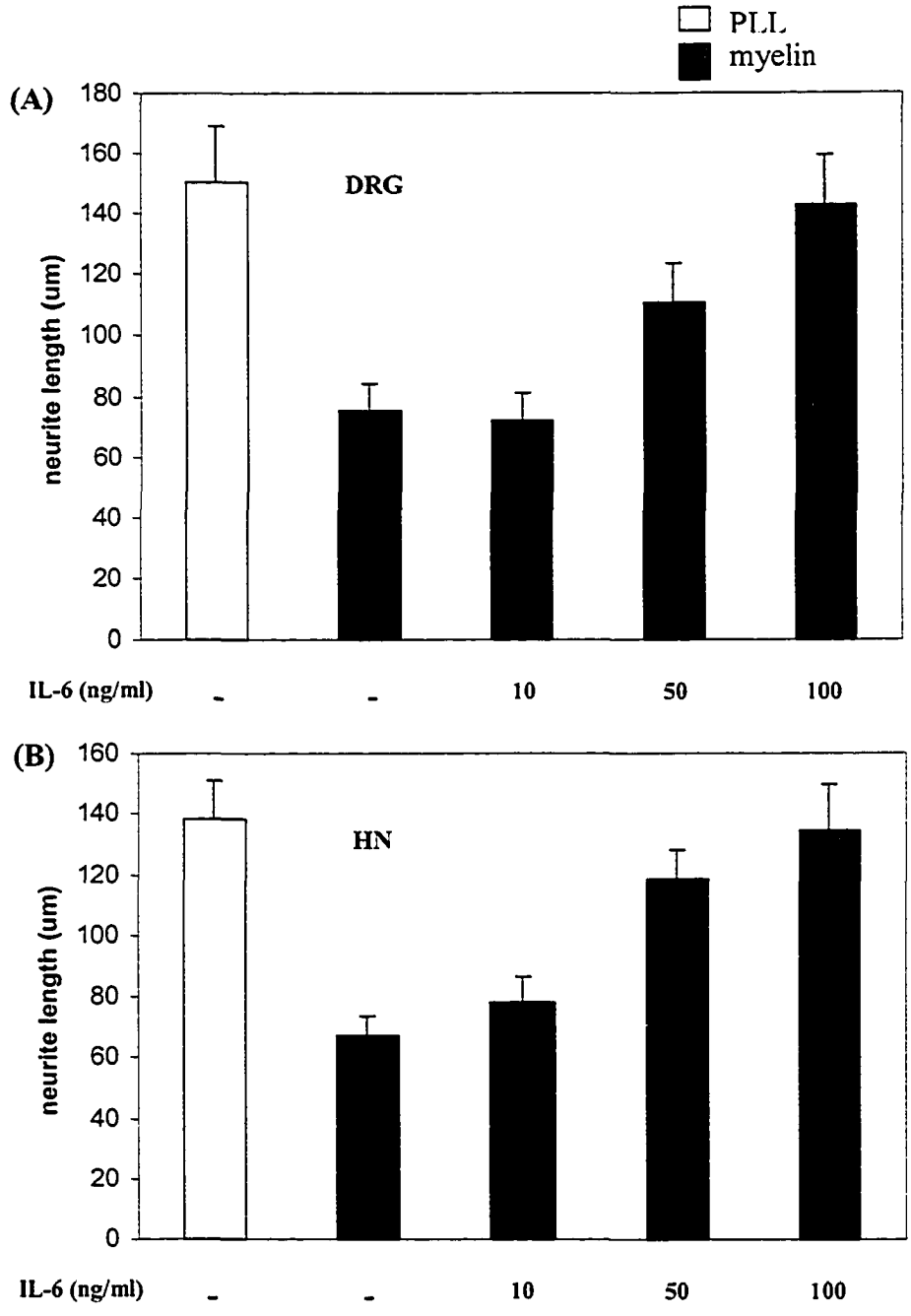


Figure 5.4: Blocking of myelin inhibition of neurite outgrowth by IL-6. DRG neurons from PND5 rats (A) or hippocampal neurons (HN) from PND1 rats (B) were isolated and cultured on poly-L-lysine (PLL) or a CNS myelin substrate. Various concentrations of recombinant rat IL-6 were added directly to the cultures. Neurons were cultured at 37°C for 24 hours before fixation and staining. In each experiment, the mean length of the longest GAP-43-positive neurite for 180-200 neurons was measured for at least three experiments.

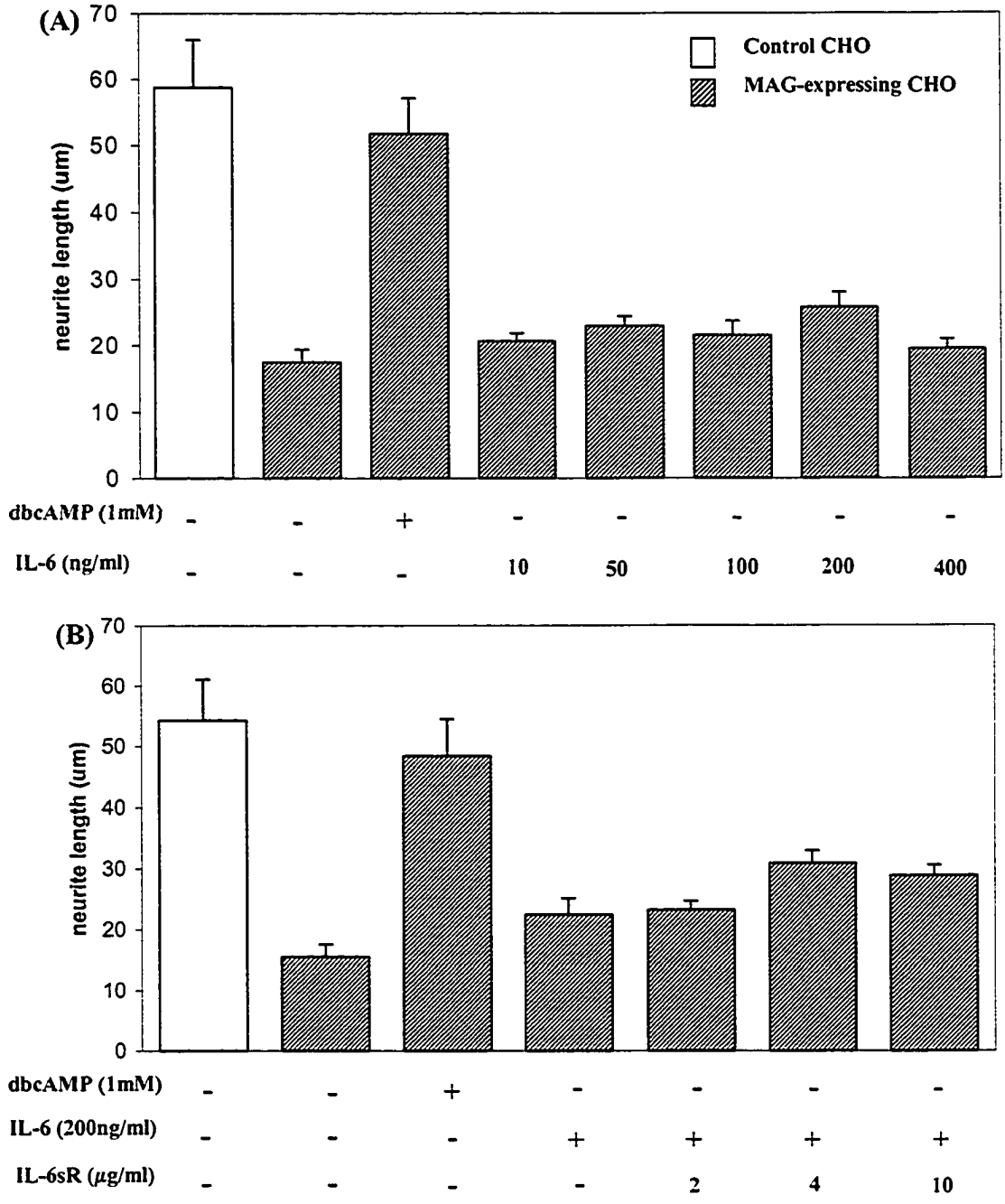


Figure 5.5: Addition of IL-6 alone or together with soluble IL-6 receptor is insufficient to overcome MAG-mediated neurite outgrowth inhibition in cerebellar granule neurons. Cerebellar granule neurons from PND3-5 rats were isolated and cultured on top of control or MAG-expressing CHO cell monolayers. Various concentrations of recombinant rat IL-6 (A), or 200ng/ml of IL-6 together with indicated concentrations of soluble IL-6 receptor (IL-6sR) (B) were added directly to the cultures. Neurons were cultured at 37°C for 18 hours before fixation and staining. In each experiment, the mean length of the longest neurite for 180-200 neurons was measured.

5.2.3 *In vivo* administration of IL-6 improves the growth of adult DRG neurons and overcomes inhibition by MAG and myelin *in vitro*

Previous studies concerning IL-6 in the nervous system were concentrated mostly on its role in neuronal survival, protection, differentiation and the immune-response in various diseases. Little is known about the role that IL-6 may play in axonal regeneration. Hirota and colleagues have shown an accelerated regeneration of axotomized hypoglossal nerve in transgenic mouse models in which IL-6 and the soluble IL-6 receptors are both overexpressed (Hirota, Kiyama et al. 1996). Similarly, in IL-6 deficient mice, delayed regeneration of sensory axons are observed (Zhong, Dietzel et al. 1999). These results implicate IL-6 involvement in the regeneration of the PNS. However, results indicating that IL-6 can improve axonal regeneration in the injured CNS are still lacking. With our findings that IL-6 expression is one of the downstream events induced by elevated cAMP levels in neuron cultures, and with evidence showing the block of neurite outgrowth inhibition by MAG and myelin with exogenous IL-6 applied directly to cultured neurons, we are encouraged to postulate that up-regulation of IL-6 levels in the CNS *in vivo* may have some function in improving axonal regeneration after injury.

It has been demonstrated in our lab that the growth of dissociated adult DRG neurons on substrates of CNS myelin or MAG-expressing CHO cells parallels very well the regenerative capacity of dorsal column fibers in the injured adult spinal cord. Therefore, the growth of adult DRG neurons *in vitro* may represent the regeneration of corresponding spinal cord axons *in vivo*. Based on this, we assessed the potential role for IL-6 in the regeneration of adult DRG neurons *in vitro*. First, we intrathecally delivered recombinant IL-6 cytokine into adult rats to acquire high circulating levels of IL-6 in the

cerebrospinal fluid (CSF). To do this, each postnatal day 21-23 rat was implanted with an osmotic mini-pump filled with various concentrations of IL-6 or saline. The pumps were embedded underneath the muscles on top of the spinal cord, with an attached cannula inserted beneath the dura membrane between L4 and L5, which delivers IL-6 directly into the CSF. Animals received this intrathecal administration of IL-6 for 24 hours, after which DRG neurons located at L4-L6 were isolated bilaterally from each animal. Dissociated neurons were cultured with MAG-expressing CHO cell monolayers for 18 hours at 37°C before fixation and staining. Results demonstrate an extensive neurite outgrowth in DRG neurons taken from IL-6 treated animals, overcoming the inhibition by MAG-expressing CHO cells. This was illustrated by both anti-GAP43 immunofluorescent staining (Figure 5.6 A) and neurite length quantification (Figure 5.6 B). The regeneration-promoting effect of intrathecal delivery of IL-6 is also dose-dependent, with the plateau of reversal occurring at a dose of 2pmol/kg/hr.

Since it has been shown that excess IL-6 expression in the CNS leads to the breakdown of blood-brain-barrier (BBB) (Brett, Mizisin et al. 1995), we sought to determine if subcutaneous delivery of IL-6 could also exhibit similar function in CNS neuronal regeneration. For this approach, the same osmotic mini-pumps filled with saline or different concentrations of IL-6 were inserted underneath the skin on the back of each animal. Operated animals were kept for 24 hours before sacrificing and L4-L6 DRG neurons from each animal were isolated and subjected to the same neurite outgrowth experiments as described above. Results show that subcutaneous delivery of IL-6 has the same effect on the block of MAG-mediated inhibition of neurite outgrowth in adult DRG neurons (Figure 5.7).

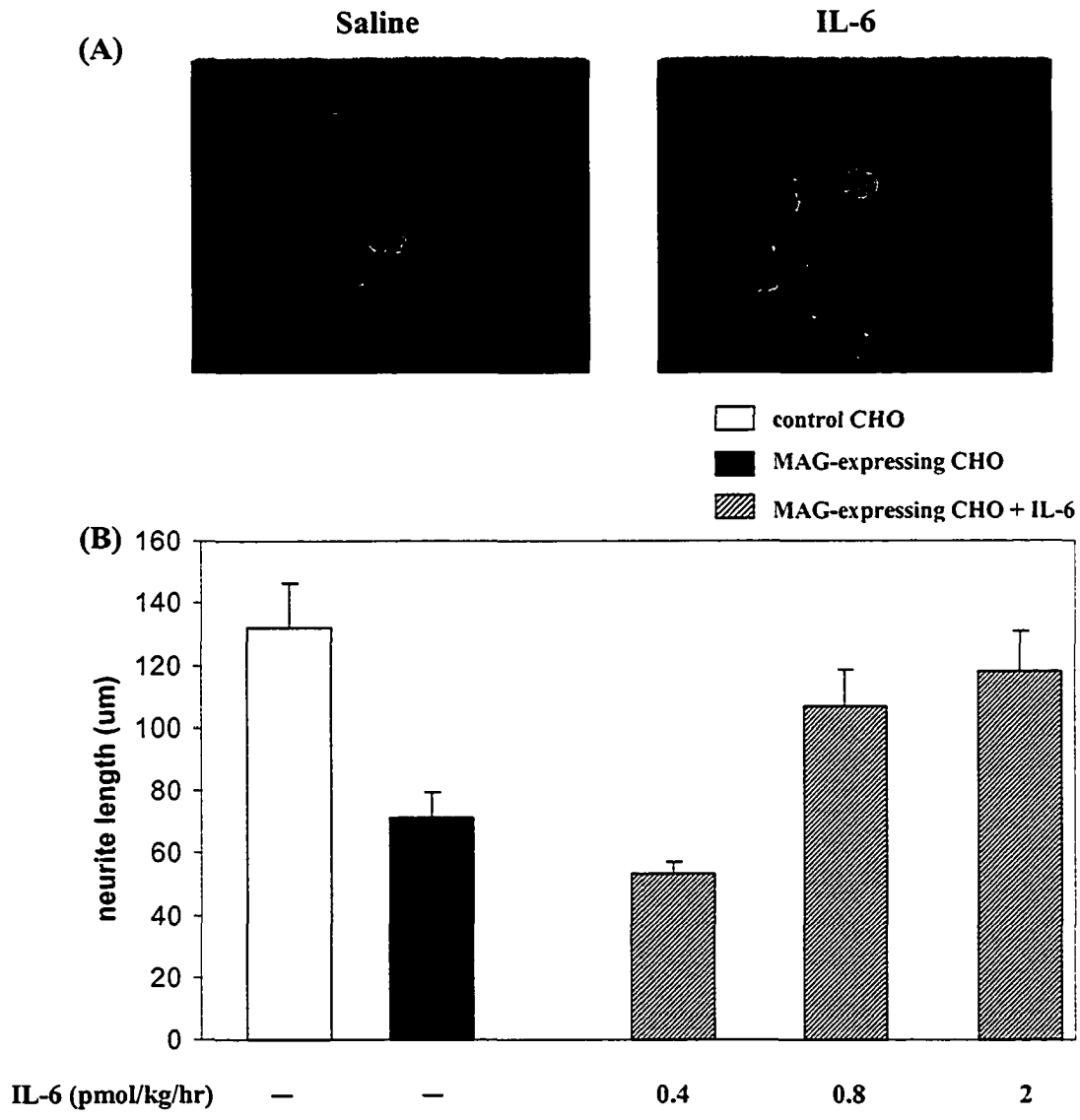


Figure 5.6: Intrathecal delivery of IL-6 overcomes inhibition by MAG in adult DRG neurons. (A), anti-GAP43 immunostaining of control or IL-6 administered adult DRG neurons grown on MAG-expressing CHO cell monolayer. (B), quantification of neurite extension of saline or IL-6 administered adult DRG neurons.

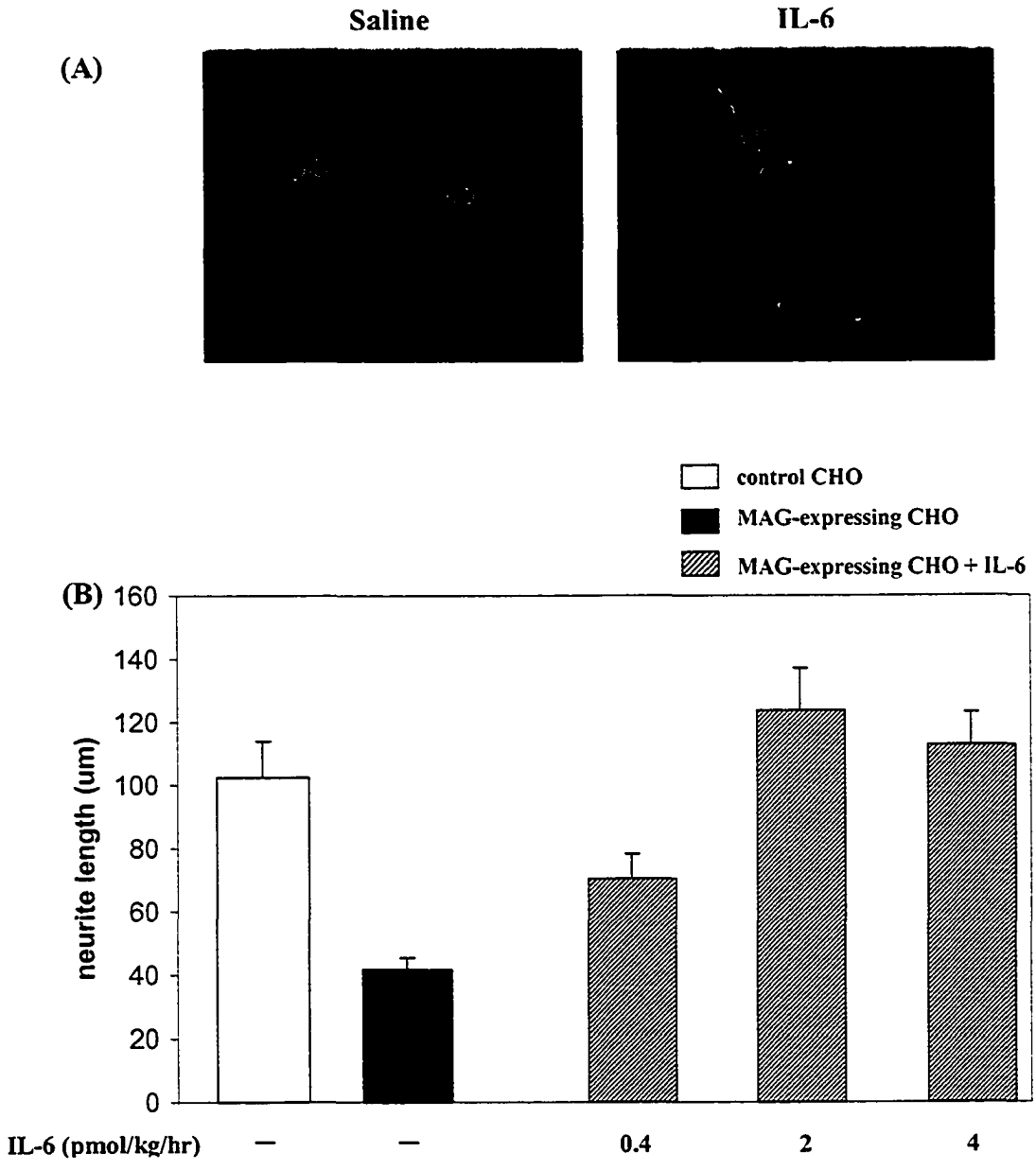


Figure 5.7: Subcutaneous delivery of IL-6 overcomes inhibition by MAG in adult DRG neurons. (A), anti-GAP43 immunostaining of control or IL-6 administered adult DRG neurons grown on MAG-expressing CHO cell monolayer. (B), quantification of neurite extension of saline or IL-6 administered adult DRG neurons grown on MAG-expressing CHO cells.

5.3 Discussion

The theory that neuronal cAMP levels are an indicator of the intrinsic growth state of neurons, which in turn dictates the neuronal response to the CNS environment and consequently regulates the ability of injured CNS neurons to regenerate, has been well established (Cai, Shen et al. 1999; Cai, Qiu et al. 2001; Qiu, Cai et al. 2002). Many studies ongoing have tried to identify the cellular and molecular mechanisms underlining this regeneration-promoting effect of elevated cAMP levels (Cai, Deng et al. 2002). However, the majority of the components involved in this mechanism still remain elusive. Here, we report that one of the downstream consequences of elevated neuronal cAMP levels is the up-regulation of IL-6 expression. Importantly, exogenous elevation of neuronal IL-6 levels alone, either through *in vitro* application or *in vivo* administration, can block the inhibition of neurite outgrowth by MAG and CNS myelin in DRG and hippocampal neurons *in vitro*. Thus, manipulation of the neuronal IL-6 levels is likely to have significant therapeutic implications in improving nerve regeneration in the adult CNS after injury.

In the mammalian CNS, IL-6 is expressed at low levels by many cell types, including epidermal cells, endothelial cells, fibroblasts, lymphocytes, microglia and astrocytes, as well as neurons. Several transcription factor-binding elements have been found in the IL-6 promoter, including the cAMP response element (CRE), which indicates that cAMP levels may be one of the IL-6 expression regulators. Indeed, many reports have demonstrated the regulation of IL-6 production in astrocytes by modulating cAMP levels (Benveniste, Huneycutt et al. 1995; Kiriyama, Murayama et al. 1997), and it is very likely that cAMP levels may also contribute to the regulation of IL-6 expression

in other cell types as well. In the experiments presented here, the source of IL-6 induced by dbcAMP treatment is still unknown. It is very possible that the observed elevation of IL-6 levels in neuronal cultures is the synergistic outcome due to expression by multiple types of cells in response to cAMP.

It is of note that the neuronal response to elevated IL-6 levels with regard to the changes in growth ability and overcoming myelin inhibitors varies among different types of neurons. In DRG and hippocampal neurons, treatment with dbcAMP results in a robust increase in the expression of IL-6, and elevation of IL-6 alone is sufficient for these neurons to overcome the inhibition by MAG/myelin and extend long processes when growing. Conversely, although cerebellar neurons also respond to dbcAMP in up-regulating IL-6 expression, with a much lower basal level that can hardly be detected (data not shown), these neurons failed to overcome the neurite outgrowth inhibition by MAG or myelin in the presence of IL-6. This unresponsiveness of cerebellar neurons to IL-6 may not be attributed to the lack of IL-6 binding receptors. It has been shown that cerebellar Purkinje neurons exhibit intense immunostaining for the IL-6R, the ligand-binding receptor subunit for IL-6, while granule neurons also show expression but at a much lower level (Nelson, Campbell et al. 1999). In addition, many reports have suggested that the soluble form of IL-6R, sIL-6R, plays an important role in determining the expression and biological function of IL-6 *in vitro* and *in vivo*. The CNS cells that are normally slightly responsive or unresponsive to IL-6 can become responsive on the addition of sIL-6R (Kordula, Rydel et al. 1998; Marz, Cheng et al. 1998; Oh, Van Wagoner et al. 1998; Marz, Otten et al. 1999; Van Wagoner, Oh et al. 1999; Vyas, Patel et al. 2002). However, in our experiments, treating cerebellar neuronal cultures with IL-

6/sIL-6R, by which the activation of IL-6-receptor complex will be enhanced, is unable to rescue neurons from being inhibited by MAG in neurite outgrowth. This indicates that the failure of IL-6 to reverse MAG inhibition in cerebellar neurons is not due to insufficient binding or activation of its receptor complex, but is probably attributive to the inactivation of signal transducing pathways.

Here, we show that elevation of IL-6 levels by adding directly to the neuronal cultures induces the neurite outgrowth of postnatal hippocampal and older DRG neurons and overcomes the inhibition by MAG and myelin in a dose-dependent manner. The optimal reversal of MAG or myelin inhibition is achieved via an IL-6 concentrations of 200ng/ml and 100ng/ml, respectively, but the effect declines at higher dose (data not shown). This is consistent with the bi-directional biological functions of IL-6 *in vivo*. In the CNS, IL-6 is characterized for its multi-potency, either beneficial or detrimental. It can exert completely opposite actions on neurons, triggering either neuronal survival after injury or causing neuronal degeneration or cell death (Gadient and Otten 1997). There are several indicators which show that the initial presence of IL-6 following nerve injury may be correlated with physiological benefits. For example, increased IL-6 concentrations have been shown to be associated with protection against cell death (Vyas, Patel et al. 2002), promotion of survival (Hama, Kushima et al. 1991; Kushima and Hatanaka 1992), and protection against axotomy caused by nerve injury (Hirota, Kiyama et al. 1996). However, sustained high levels of IL-6 in the CNS appear to be harmful (Steffensen, Campbell et al. 1994). Many reports have suggested that the overproduction of IL-6 is associated with the development of several diseases and various types of neuropathologis (Gadient and Otten 1997). Taken together, this indicates the need for tight regulation of

IL-6 levels in the CNS to orchestrate its biological functions in order to maintain its beneficial functions and prevent its destructive effects.

Finally, *in vivo* intrathecal or subcutaneous delivery of IL-6 promotes the regeneration of cultured adult DRG neurons to overcome MAG inhibition *in vitro*. This may also shed some light on the potential development of therapeutic strategies to encourage axonal regeneration after CNS injury *in vivo* by regulating IL-6 levels.

CHAPTER VI

SIGNALING COMPONENTS INVOLVED IN THE IL-6 INDUCED AXONAL REGENERATION

6.1 Introduction

IL-6 acts on target cells through a receptor complex composed of two subunits: the IL-6 receptor and gp130. IL-6 receptors exist both as membrane-bound and soluble forms *in vivo*. Binding of IL-6 to either form of the IL-6 receptor leads to association with gp130 and induces the formation of a gp130/gp130 homodimer (Hibi, Nakajima et al. 1996; Taga and Kishimoto 1997). This receptor complex formation induces phosphorylation and activation of gp130-associated tyrosine kinases (Jak kinase). In 1994, it was discovered that IL-6-type cytokines utilize tyrosine kinases of the Jak family and transcription factors of the STAT family as major mediators of signal transduction (Darnell, Kerr et al. 1994; Lutticken, Wegenka et al. 1994; Stahl, Boulton et al. 1994). Jaks are intracellular kinases that are widely expressed. There are four members of Jak family in mammalian cells: Jak1, Jak2, Jak3 and Tyk2. Although IL-6 has been shown to activate Jak1, Jak2, and Tyk2, Jak1 is the most important for signaling through gp130 (Guschin, Rogers et al. 1995; Rodig, Meraz et al. 1998). Upon ligand-induced receptor activation, dimerized gp130 bring the associated Jaks into close proximity, leading to their activation via inter- or intra-molecular phosphorylation (Ihle 1995; Pellegrini and Dusanter-Fourt 1997; Duhe and Farrar 1998). Jak activation further leads to the tyrosine phosphorylation of STATs (Signal Transducer and Activator of Transcription). STATs are a group of ubiquitously expressed transcription factors found in mammalian cells. Among them, STAT-1α and STAT-3 are the most responsive after treatment with various IL-6 family members (Taga and Kishimoto 1997). After Jaks activation, STAT1 and STAT3 proteins become tyrosine phosphorylated and dimerize, translocate to the nucleus and bind to elements in the promoters of IL-6 responsive genes (Wen, Zhong et al. 1995;

Wen and Darnell 1997). In addition to the Jak family of tyrosine kinases, the tyrosine phosphatase SHP2 is also found to bind to phosphorylated gp130 (Stahl, Farruggella et al. 1995). Therefore, the activation of IL-6 receptor complex also transduces signals through the Ras/MEK/MAPK signal transduction pathway (Nakajima, Kusafuka et al. 1993; Taga and Kishimoto 1997). Both the Jak/STAT and the Ras/MEK/MAPK pathways lead to the activation of a number of transcription factors (Hibi, Nakajima et al. 1996; Hirano, Ishihara et al. 2000), and in an integrative process, these transcription factors co-operatively regulate the expression of IL-6-responsive genes, thus mediating the biological effects of IL-6. Figure 6.1 is a schematic illustration of IL-6 induced signal transduction.

In our previous studies, we have demonstrated that artificial elevation of neuronal cAMP levels, either with dbcAMP or by priming the neurons with various neurotrophins, was sufficient to overcome inhibition by MAG/myelin and to encourage dorsal root spinal axons to regenerate *in vivo* (Cai, Shen et al. 1999; Cai, Qiu et al. 2001; Qiu, Cai et al. 2002). It was further shown that this regeneration-promoting effect of elevated cAMP requires not only the activation of protein kinase A (PKA) (Cai, Qiu et al. 2001; Qiu, Cai et al. 2002), but also new gene transcription and protein synthesis (Cai, Deng et al. 2002). Here, we have elucidated that one significant consequences of elevated cAMP is the up-regulation of IL-6, which alone can block the neurite outgrowth inhibition by MAG and myelin. However, it is unclear whether this cAMP-induced increment of IL-6 and the blocking of MAG/myelin inhibition by IL-6 is also PKA and transcription dependent, neither did we know what other signaling pathways might be involved.

Here, we sought to identify the influence of PKA and the involvement of the gp130/Jak signaling pathway in the IL-6-mediated axonal regeneration.

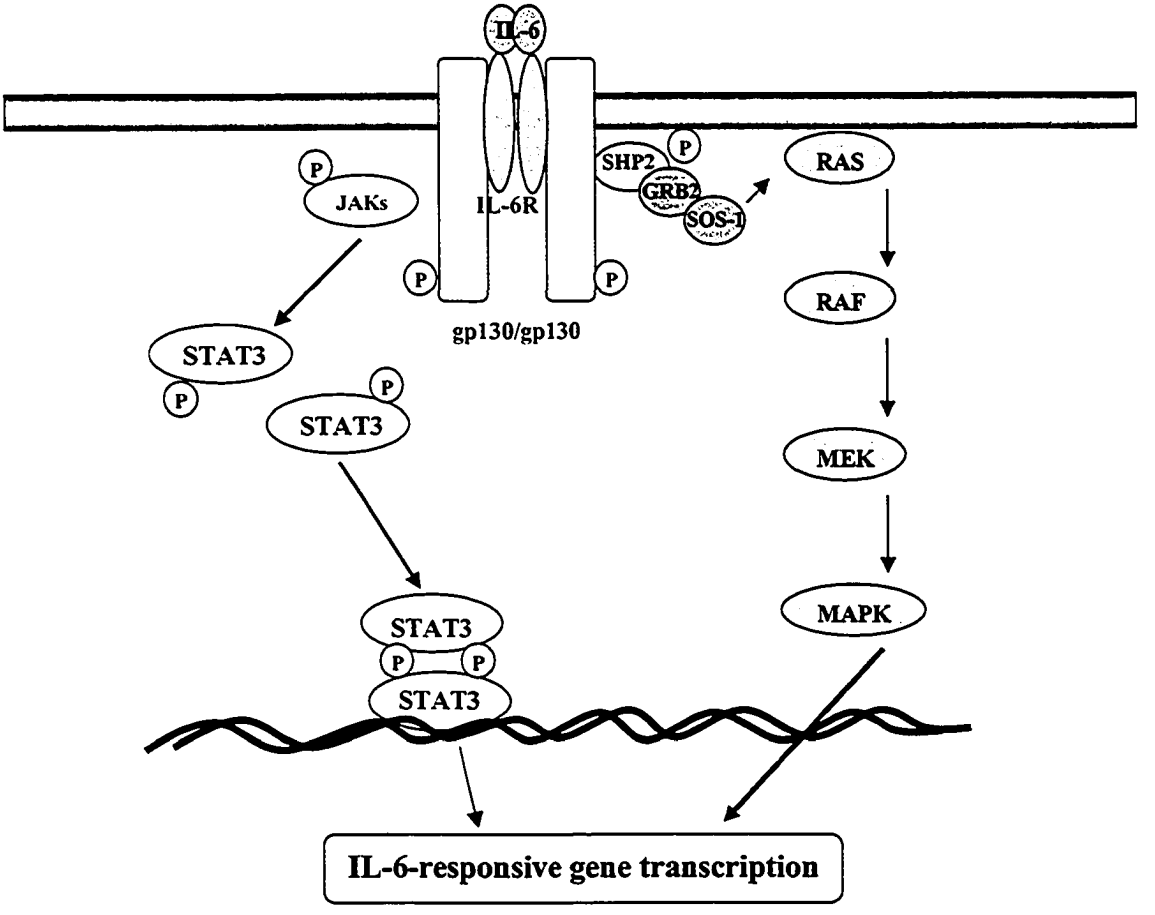


Figure 6.1: IL-6-induced signal transduction. IL-6 acts on target cells through a receptor complex composed of IL-6-binding receptor (IL-6R), the signal transducing component (gp130). Initiation of IL-6 signaling occurs when IL-6 binds to the IL-6 receptor, leading to the association and dimerization of gp130. This event leads to activation of gp130-associated tyrosine kinases (JAKs), which phosphorylate gp130, provide docking and activation sites for various signaling pathways (JAK/STAT and Ras/MEK/MAPK). The activation of these signaling pathways results in subsequent IL-6-responsive gene transcription.

6.2 Results

6.2.1 The axonal regeneration induced by IL-6 is mediated by gp130/Jak signaling pathway and requires gene transcription

With intensive studies in the past years, it was demonstrated that IL-6 proteins exert their biological functions mainly through activation of receptor gp130 and subsequent activation of Jak/STAT signaling pathway (Darnell, Kerr et al. 1994; Lutticken, Wegenka et al. 1994; Stahl, Boulton et al. 1994). In order to determine if this mechanism is also responsible for the ability of IL-6 to block the inhibition of MAG/myelin on neurite outgrowth, we first assessed the expression of gp130 and STAT3 transcription factors in cultured primary neurons (Figure 6.2). Here, total proteins were extracted from dissociated postnatal cerebellar (CN), DRG, and hippocampal (HN) neurons. Similar amount of protein were loaded and separated in a 4-20% gradient SDS-PAGE gel. The expression of IL-6 receptor gp130 and transcription factor STAT3 were illustrated using monoclonal anti-gp130 and anti-STAT3 antibodies, respectively. As a control for equivalent loading, the detection of actin was also performed. Results indicate that both DRG and hippocampal neurons exhibit high levels of gp130 and STAT3 expression, whereas the expression of both proteins in cerebellar neurons is significantly less. This correlates with the differential response of these neurons to IL-6 treatment, in which the neurite extension against the inhibitory effect by MAG and myelin from DRG and hippocampal neurons were greatly improved by addition of IL-6, yet cerebellar neurons show almost no response at all.

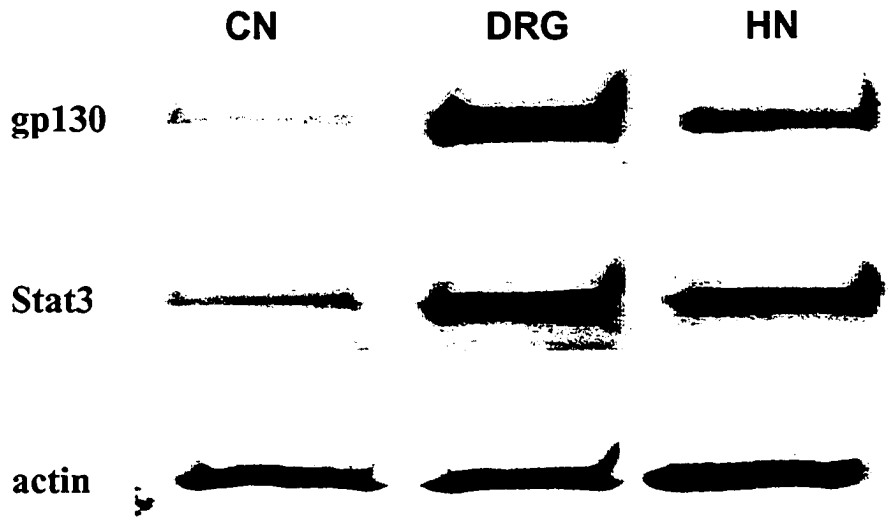
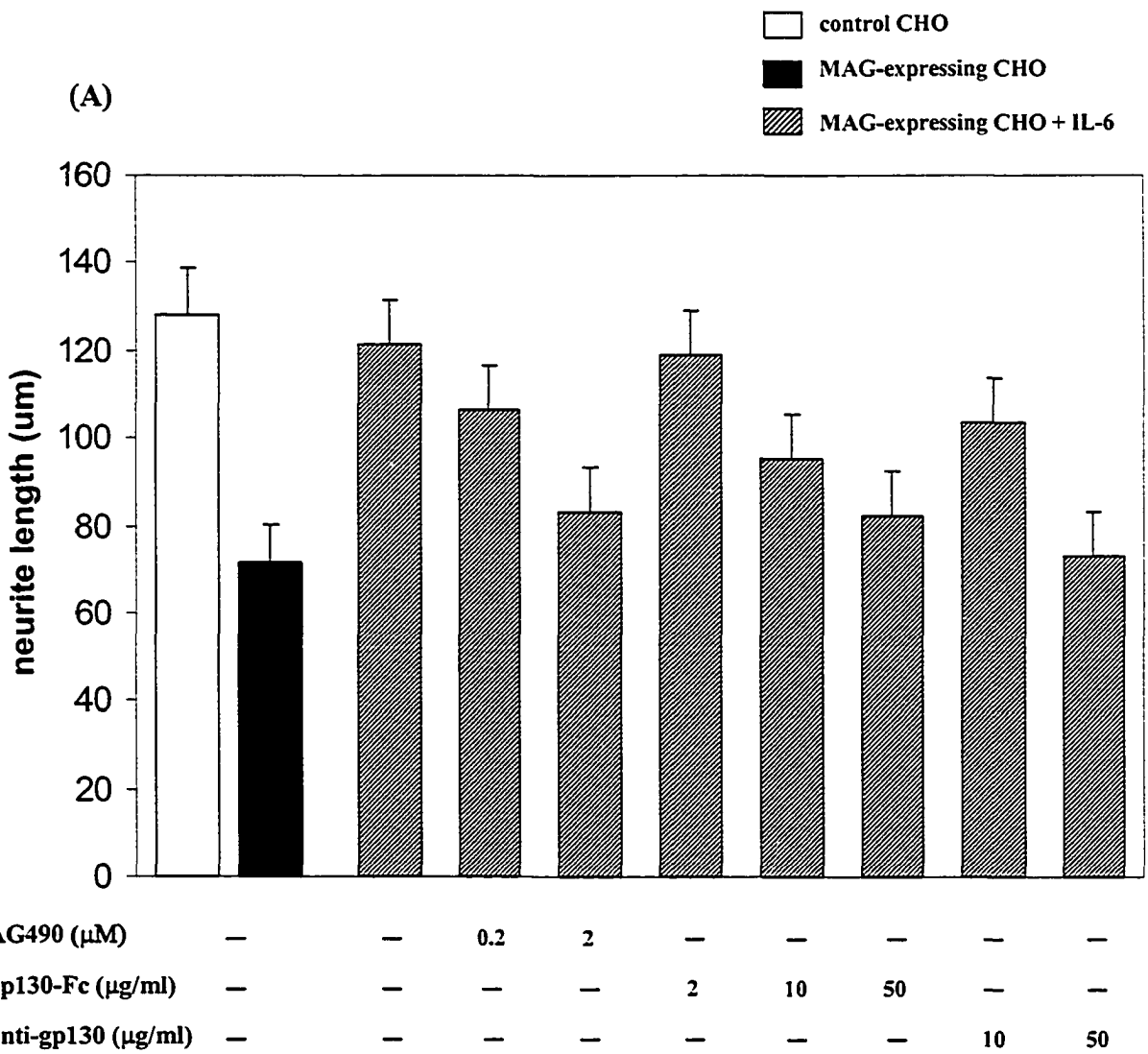


Figure 6.2: Western blot analysis of endogenous gp130 and STAT3 expression in primary neurons. Dissociated postnatal cerebellar (CN), DRG, and hippocampal (HN) neurons were cultured in SATO media overnight in 24-well culture plates with a concentration of $1x10^6$ cells/ml. Then total proteins were extracted from each sample and separated in a 4-20% gradient SDS-PAGE gel. The expression of receptor gp130 and transcription factor STAT3 was detected using monoclonal anti-gp130 and anti-STAT3 antibodies.

To determine the involvement of receptor gp130 and Jak1 tyrosine kinase in the ability of IL-6 to overcome the inhibition by MAG, we used three different types of molecules to block gp130/Jak signaling in our neurite outgrowth assay: AG490, a specific inhibitory molecule for Jak1 tyrosine kinase; gp130-Fc, a soluble form of IL-6 receptor gp130 antagonist; and functional-neutralizing anti-gp130 antibody (Figure 6.3). Primary DRG and hippocampal neurons were dissociated from PND5 and PND1 rats, respectively, and cultured on top of MAG-expressing CHO cell monolayers at 37°C for 18 hours. During incubation, neurons were exposed to 200ng/ml of recombinant rat IL-6 in the presence of each of the inhibitory molecules at various doses. After fixation and anti-GAP43 immunostaining, the mean length of the longest GAP-43-positive neurite for 180-

200 neurons from each sample was measured. Results show that as reported before, the inhibition of neurite outgrowth from both types of neurons by MAG is dramatically reversed by exogenous IL-6 treatment. However, this reversal effect is significantly abolished in a dose-dependent manner by each of the inhibitory molecules tested, which indicates the loss of IL-6-induced axonal regeneration by blocking of the gp130/Jak signaling pathway. All the inhibitory molecules we used here have no effect on the neurite growth on control cells.



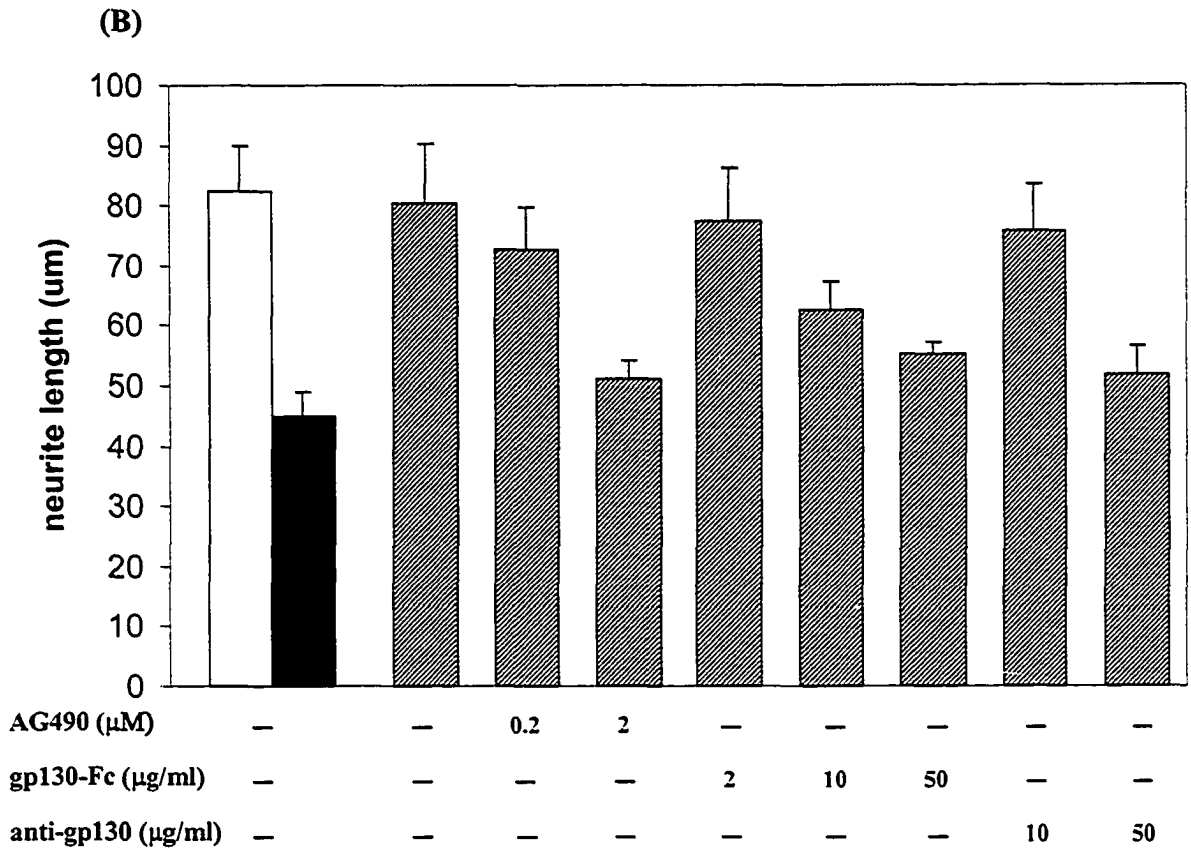


Figure 6.3: The effect of IL-6 in overcoming MAG inhibition of neurite outgrowth is mediated through receptor gp130 and Jak1 tyrosine kinase. Dissociated PND5 DRG neurons (A) and PND1 hippocampal neurons (B) were grown on top of MAG-expressing CHO cell monolayers and treated with 200ng/ml rat IL-6 in the presence of either Jak1 inhibitor, AG490, soluble receptor gp130 antagonist, gp130-Fc, or gp130 neutralizing antibody. After 18 hours of incubation, neurons were fixed and immunostained with anti-GAP43 antibody. In each graph, the mean length of the longest neurite for 100-180 neurons was measured for at least three experiments.

Here, we established that the abrogation of MAG-mediated neurite outgrowth inhibition by IL-6 requires the activation of IL-6 receptor gp130 and Jak1 tyrosine kinase. Next, we wanted to determine if the effect of IL-6 on axonal regeneration in the presence of MAG is dependent on gene transcription. To assess this, dissociated postnatal DRG and hippocampal neurons were cultured on top of control or MAG-expressing CHO cell monolayers. Consistent with previous observations, neurite outgrowth from both types of

neurons is strongly inhibited by MAG-expressing CHO cells, and this inhibition can be significantly reversed by addition of 200ng/ml IL-6 into the culture. However, when an inhibitor of transcription, 5,6-dichloro-1-b-D-ibo-furanosyl-benzimidazole (DRB), is added directly to the culture at a concentration of 20μM, the reversal effect of IL-6 is abolished (Figure 6.4). DRB has no effect on neurite outgrowth on control cells. That is to say, the block of MAG inhibition by IL-6 requires new gene expression.

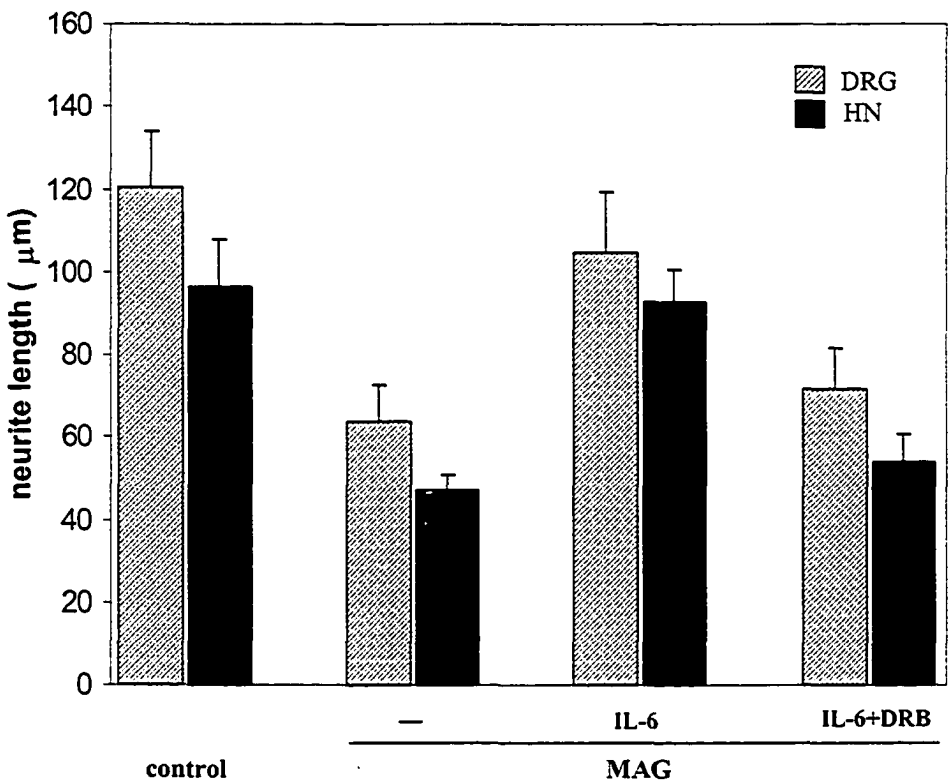


Figure 6.4: The ability of IL-6 to overcome inhibition by MAG is transcription dependent. PND5 DRG neurons (stripe bars) and PND1 hippocampal neurons (black bars) were isolated and grown on either control or MAG-expressing CHO cell monolayers. As indicated, some neurons were cultured with MAG-expressing CHO cells in the presence of 200ng/ml of IL-6 with or without DRB (20μM). After 18 hours of incubation, neurons were fixed and immunostained for GAP43. For each treatment, the mean length of the longest GAP-43-positive neurite for 180-200 neurons was measured for at least 3 separate experiments. Results indicate that in both neuronal types, blocking gene transcription by DRB abolishes the effect of IL-6 in overcoming the inhibition of neurite outgrowth by MAG.

6.2.2 The IL-6-induced signaling pathway is not necessary for cAMP-mediated axonal regeneration

In our previous studies, we have established that the inhibition of axonal regeneration by MAG and myelin can be blocked by elevating neuronal cAMP levels with dbcAMP (Cai, Qiu et al. 2001). Here, we have shown that up-regulation of IL-6 expression is one of the downstream consequences induced by dbcAMP treatment in neuronal culture. Furthermore, exogenous addition of IL-6 alone is able to overcome the inhibition of neurite outgrowth by MAG and myelin via activation of receptor gp130 and Jak1 tyrosine kinase, the blocking of which significantly abolishes the reversal effect of IL-6. Thus, the question that remains to be answered is whether this IL-6-induced activation of gp130 and Jak1 tyrosine kinase is also required for the cAMP-mediated blocking of MAG and myelin inhibition on axonal regeneration. To test this, primary DRG neurons were dissociated from PND5 rats and cultured on control or MAG-expressing CHO cell monolayers at 37°C for 18 hours. During incubation, neurons were treated with 1mM dbcAMP in the presence of the Jak1 inhibitor, AG490, or gp130 antagonist gp130-Fc, or neutralizing anti-gp130 antibodies. After fixation and anti-GAP43 immunostaining, the mean length of the longest GAP-43-positive neurite for 100-180 neurons from each sample was measured (Figure 6.5). In contrast to what we have observed in IL-6 treated neurons, none of the gp130/Jak signaling blocking molecules tested here was able to abrogate the ability of dbcAMP to overcome the inhibition of neurite outgrowth by MAG. This implies that, even though the elevation of IL-6 levels and the subsequent activation of gp130/Jak signaling by IL-6 are downstream events

induced by dbcAMP, they are not necessary for dbcAMP to overcome the inhibition by MAG.

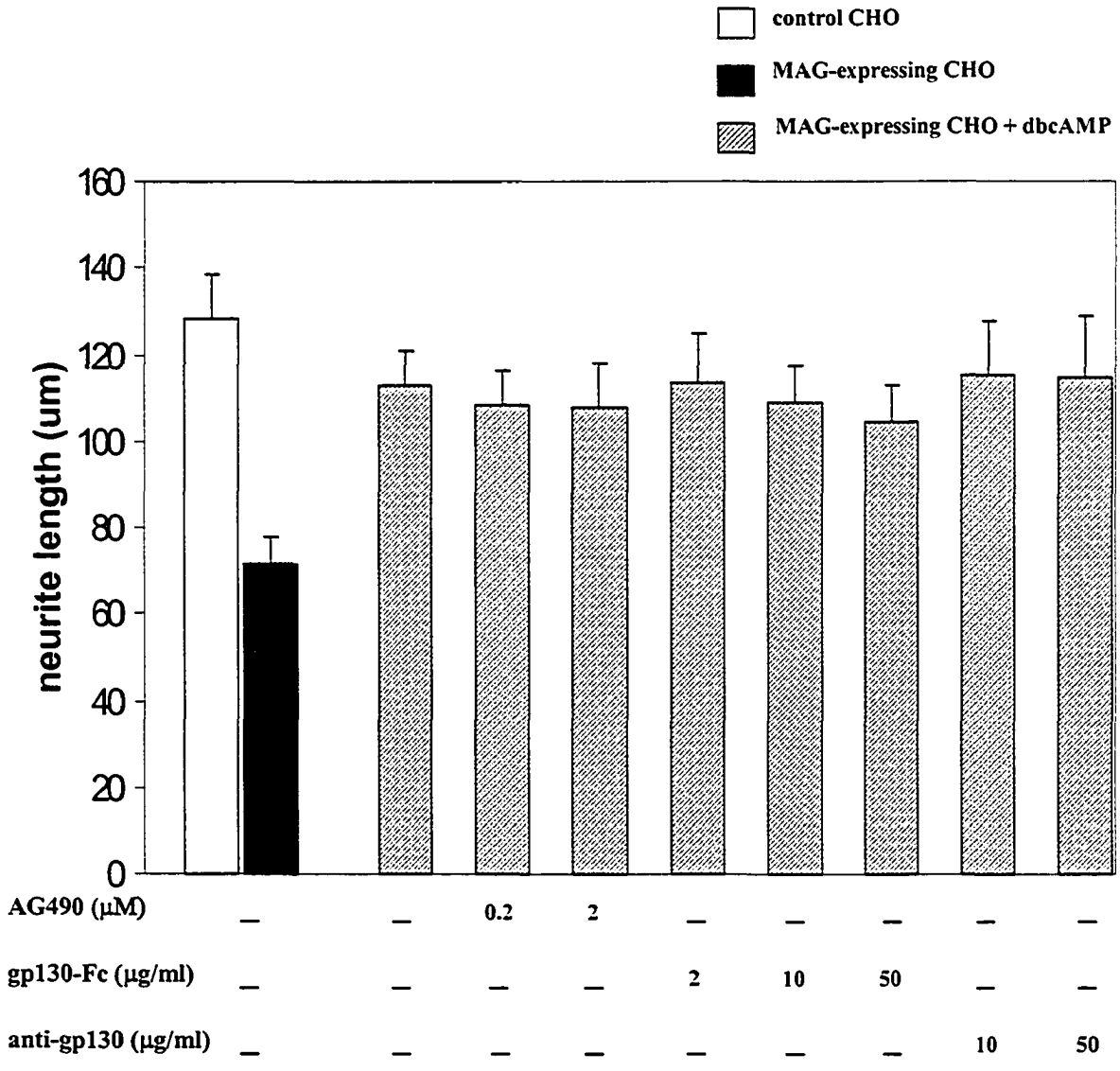


Figure 6.5: The role of dbcAMP in overcoming MAG inhibition on neurite outgrowth is independent of gp130 and Jak1 tyrosine kinase activities. Dissociated PND5 DRG neurons were grown on MAG-expressing CHO cell monolayers and treated with 1mM dbcAMP in the presence of either Jak1 inhibitor, AG490, IL-6 receptor gp130 antagonist, gp130-Fc, or gp130 neutralizing antibody. After 18 hours of incubation, neurons were fixed and immunostained with anti-GAP43 antibody. In each graph, the mean length of the longest neurite for 100-180 neurons was measured for at least three experiments.

6.3 Discussion

It was discovered that after ligand-induced activation of the receptor complex, which includes ligand-binding receptor IL-6R and signal transducing receptor gp130, IL-6 exerts its biological functions mainly through activation of the Jak family tyrosine kinases and the transcription factors of the STAT family, which is a common signal transduction pathway shared with many other cytokines and growth factors (Darnell, Kerr et al. 1994; Lutticken, Wegenka et al. 1994; Stahl, Boulton et al. 1994). Here, we not only illustrated the endogenous expression of the IL-6 receptor gp130 and transcription factor STAT3 in primary postnatal DRG and hippocampal neurons, but also demonstrated the requirement of gp130 and Jak1 tyrosine kinase for IL-6-induced axonal regeneration from these neurons, which indicates that the regeneration-promoting function of IL-6 in neurons is also mediated by the gp130/Jak/STAT pathway. This finding will be very useful for the future identification of axonal regeneration-associated genes. During the past years, many STAT-regulated, IL-6-responsive genes have been identified, which include many acute phase protein (APP) genes, transcription factors such as Jun B, c-Fos, interferon regulatory factor (IRF)-1, and a variety of other genes (Heinrich, Behrmann et al. 1998). Further studies of these STAT-regulated genes may shed some light on the mechanisms which overcome the inhibition of neurite outgrowth by MAG and CNS myelin. In addition to the Jak/STAT signaling pathway, the Ras/Raf/MAPK signaling cascade and transcription factor NF-κB were also shown to mediate certain neuronal functions of IL-6-type cytokines (Middleton, Hamanoue et al. 2000; Tancredi, D'Antuono et al. 2000). The roles that these alternative signaling

pathways and transcription factors play in the IL-6-induced axonal regeneration need to be further investigated.

Surprisingly, blocking the activity of gp130 or Jak1 tyrosine kinase had no effect on cAMP-induced block of MAG-mediated inhibition of neurite outgrowth. This suggests that, although up-regulation of IL-6 expression is one of the downstream events induced by cAMP elevation, the subsequent IL-6-induced signaling pathway is parallel and dispensable with respect to the reversal effect of dbcAMP. Both dbcAMP and IL-6 alone are able to overcome the inhibitory effect of MAG and myelin in neurite outgrowth, but the reversal effects of the two molecules may be redundant.

Based on these findings, we are revising our hypothesis on the mechanism of dbcAMP-induced IL-6 up-regulation and the action of IL-6 in promoting regeneration (Figure 6.6): When dbcAMP is added into the culture, the neuronal cAMP levels are immediately increased. This elevation of cAMP thus regulates gene expression through PKA (Cai, Qiu et al. 2001) and CREB (unpublished data) activation, as well as interactions with the Ca^{++} and Ras/Raf/MEK signaling pathways (unpublished data). The activation of multiple cAMP-induced signaling pathways results in the transcription of a host of regeneration-related genes in neurons, including Arg-1, an enzyme which catalyzes polyamine production and results in the block of MAG-mediated inhibition of neurite outgrowth (Cai, Deng et al. 2002), and IL-6. Moreover, application of dbcAMP not only induces neuronal production of IL-6, but also stimulates the expression of IL-6 from astrocytes and microglia cells (Van Wagoner and Benveniste 1999). These processes work synergeticly to increase the total IL-6 level in the culture media. Through binding to its neuronal receptors, IL-6 activates gp130 and the associated Jak/STATs

signaling cascade, which leads to the regulation of gene transcription and results in the block of neurite outgrowth inhibition by MAG and myelin. Other alternative IL-6-responsive pathways, including Ras/Raf/MEK and NF-κB, may also contribute to the ability of IL-6 to overcome regeneration inhibition by MAG and myelin, but this remains to be further investigated. The axonal regeneration-promotion pathways utilized by cAMP and IL-6 may be parallel but, via cross talk, they may exert their respective effects in axonal regeneration in a redundant manner. Activation of either pathway alone is sufficient to overcome MAG and myelin inhibition and encourage axonal regeneration.

Finally, these results not only reveal a novel mechanism for overcoming neurite outgrowth inhibition by MAG and myelin, but they also point out new and specific therapeutic strategies for altering the intrinsic ability of neurons to grow through an inhibitory CNS environment and consequently encourage regeneration *in vivo*.

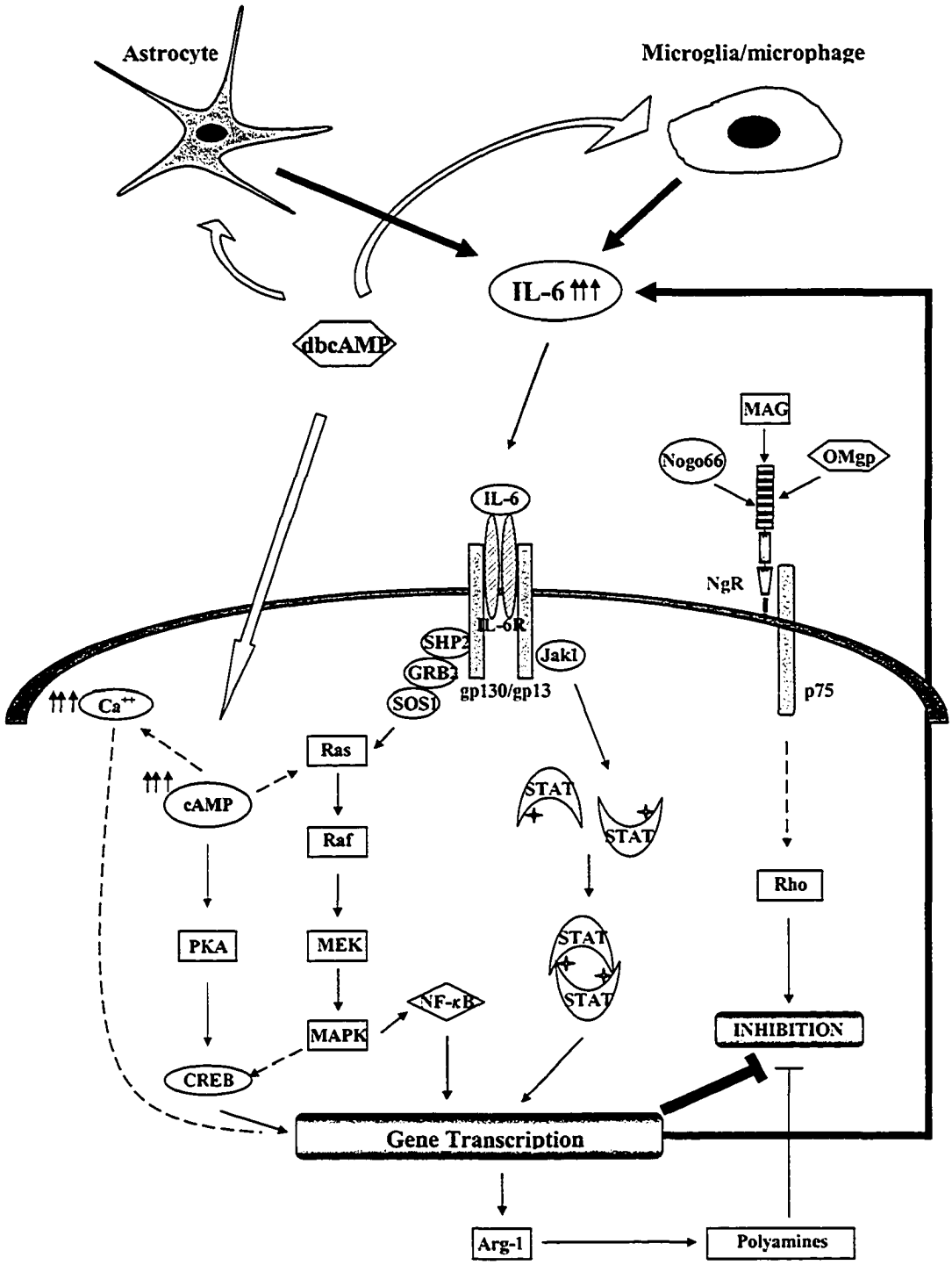


Figure 6.6: Schematic representation of the molecular mechanisms involved in the cAMP-induced elevation of IL-6 expression and the IL-6-mediated block of MAG/myelin inhibition of axonal regeneration.

CHAPTER VII

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