MAG does not Require NgR1, PirB or Sialic Acid Binding to Inhibit Neurite Outgrowth

by
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Abstract

The Role of Gangliosides, NgR1, NgR2 and PirB Receptors in MAG Inhibition of Neurite Outgrowth

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Following injury, axons in the central nervous system (CNS) do not spontaneously regenerate, and this is due to several factors, one of which is the presence of myelin-associated inhibitors. There are three major myelin-associated inhibitors that have been identified, Nogo-66, myelin associated glycoprotein (MAG), and Oligodendrocyte myelin glycoprotein (OMgp). MAG is a member of immunoglobulin (IgG) super-family and contains 5 Ig-like domains in its extracellular domain. Like Nogo-66 and OMgp, MAG binds to a receptor complex consisting of NgR1- $p75^{NTR}$-Lingo-1 to inhibit neurite outgrowth. MAG is also a sialic acid binding protein and specifically binds to gangliosides GT1b and GD1a. Recently, NgR2 was also shown to be a sialic acid–dependent binding receptor for MAG. Recently, paired immunoglobulin B (PirB) was also identified as a novel receptor for MAG, Nogo-66 and OMgp.

Previously, we showed that the sialic acid binding activity of MAG is not necessary for its inhibitory effects. We mapped the sialic acid binding site on MAG to Arg 118 in the first Ig-domain. When this site is mutated, sialic acid binding is lost but MAG, when expressed by CHO cells, still retains its ability to inhibit neurite outgrowth. Also, we showed that a soluble form of MAG consisting of the MAG extracellular domain fused to the Fc portion of human IgG (MAG-Fc), and a truncated soluble form of MAG consisting

only of the first three Ig-like domains (MAG (d1-3)-Fc), both bind to neurons in a sialic acid–dependent manner; however, only MAG-Fc inhibits neurite outgrowth. In addition, MAG mutated at Arg118 (MAG (R118A)-Fc), does not bind to neurons and could not inhibit neurite outgrowth. Recently, we mapped the inhibition site on MAG to Ig-domain 5, which is distinct from the sialic acid binding site.

Others have reported that gangliosides are functional binding partners for MAG and are necessary for inhibition by MAG when expressed in immobilized membranes. They reported that neurons from mice deficient in the B1, 4-N-actylgalactosaminyltransferase (GalNAcT) gene, which lack all complex gangliosides including GT1b and GD1a, are not inhibited by MAG in immobilized membrane. Others have also shown that clustering gangliosides with antibodies in the absence of MAG is sufficient to inhibit neurite outgrowth via a mechanism engaging p75 ^{NTR} receptor.

Here we show that clustering MAG (d1-3)-Fc can inhibit neurite outgrowth in neurons from wild type mice but not from GalNAcT deficient mice. We also show that MAG can inhibit neurite outgrowth independent of NgR1, PirB, and sialic acid binding. We show that neurons from GalNAcT deficient mice are inhibited by MAG as effectively as neurons from wild type mice. Also, we show that neurons from NgR1 deficient mice are inhibited by full length MAG and mutated MAG (MAG R118A) that cannot bind sialic acid residues. In addition, in the presence of PirB antibodies, both MAG- and mutated MAG (R118A)-expressing CHO were able to inhibit neurite outgrowth of neurons from NgR1 deficient mice and wild type mice. Taking all these results together, MAG interacts with another as yet unknown receptor(s), in addition to NgR's, PirB and sialic acid to inhibit neurite outgrowth.

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Table of Contents

List of Figures

Abbreviations

AC adenylate cyclase

BDNF brain derived neurotrophic factor

C3 c3 transferase

cAMP cyclic adenosine monophosphate

CGN cerebellar granule neurons

CHO Chinese hamster ovary

CNS central nervous system

CSPGs chondroitin sulfate proteoglycans

CST corticospinal tract

dbcAMP dibutyryl cAMP

DRG dorsal root ganglia

ECL enhanced chemiluminescence

ECM extracellular matrix

ELISA enzyme-linked immunosorbent assay

ER endoplasmic reticulum

FBS fetal bovine serum

GAG glycosaminoglycan

GFAP glial fibrillary acidic protein

GPI glycosyl phospatidyl inositol

HN hippocampal neurons

IgG immunoglobulin

ITIM immunoreceptor tyrosine-based inhibitory motifs

KDa Kilo Dalton

LINGO-1 LRR and Ig domain-containing Nogo Receptor-interating protein

LRR leucine rich repeat

MAG Myelin-associated glycoprotein

mTOR mammalian target of rapamycin

NEP1-40 Nogo extracellular peptide, residues1-40

NGF Nerve Growth Factor

NgR1 Nogo receptor-1

NgR2 Nogo receptor-2

NgR3 Nogo receptor-3

NOG neurite outgrowth assay

NTs neurotrophins

OMgp oligodendrocyte myelin glycoprotein

$p75^{NTR}$ p75 neurotrophin receptor

P post-natal day

P4 ganglioside biosynthesis inhibitor

PAGE poly-acryamide gel electrophoresis

PBS phosphate buffered saline

PCR polymerase chain reaction

PDE4 phosphodiesterase 4

PirB paired immunoglobulin-like receptor B

PI-PLC phosphatidylinositol-specific phospholipase C

PLL Poly L-lysine

PNS peripheral nervous

SCs Schwann cells

SCI spinal cord injury

SDS sodium dodecyl sulfate

Siglec sialic acid binding Ig-like lectin

siRNA small interfering RNA

TNFR tumor necrosis factor receptor

Trk tyrosine kinase receptor

VCN *vibrio cholerae neuraminidase*

WD Wallerian degeneration

Y27632 Rho kinase inhibitor

Chapter I: Introduction

1.1 The Failure of CNS Axonal Regeneration Following Injury

It is well established that severed axons from the central nervous system (CNS) do not regenerate after injury to the spinal cord. This failure to regenerate is known to be caused by two major factors: the inhibitory environment of the CNS compared to a permissive peripheral nervous system (PNS) environment, and to a decreased or lost capacity of the neurons to grow past the injury site.

1.2 The Inhibitory Environment in The Adult Mammalian CNS

During development, neurons extend axons tipped by growth cones to reach their synaptic targets. In the adult nervous system, traumatic injury can cause severing of axons and thus the loss of functional connections. The distal portion of the axons, disconnected from the neuronal cell bodies, undergoes “Wallerian degeneration “ and degenerates over several weeks or months in the CNS. The proximal portion of the axon reforms a growth cone and attempts to regenerate (Ramon y Cajal, 1928).

In the PNS, macrophages are recruited to the injury site and myelin debris is rapidly cleared (Brown et al., 1991). In addition, Schwann cells contribute to regeneration by de-differentiating, down regulating their myelin proteins and starting to proliferate, therefore the attempt of the growth cone to regenerate is often successful. This permissive environment in the PNS does not exist in the CNS. The spontaneous attempt of axons to regenerate fails and the cut axon tips assume “end bulb” morphology, which reflect a failed attempt at elongation (Ramon y Cajal, 1928, Schwab and Bartholdi, 1996).

Based on intensive studies over the past two decades, it is now clear that there are two obstacles for axonal re-growth in the CNS: myelin-associated inhibitors and the glial scar. The glial scar takes several weeks to form, mature, and secretes other inhibitory molecules. Therefore it could be more beneficial to identify myelin-associated inhibitors and their receptors and elucidate their mechanism in order to design peptides or antibodies to neutralize and block their inhibitory signals.

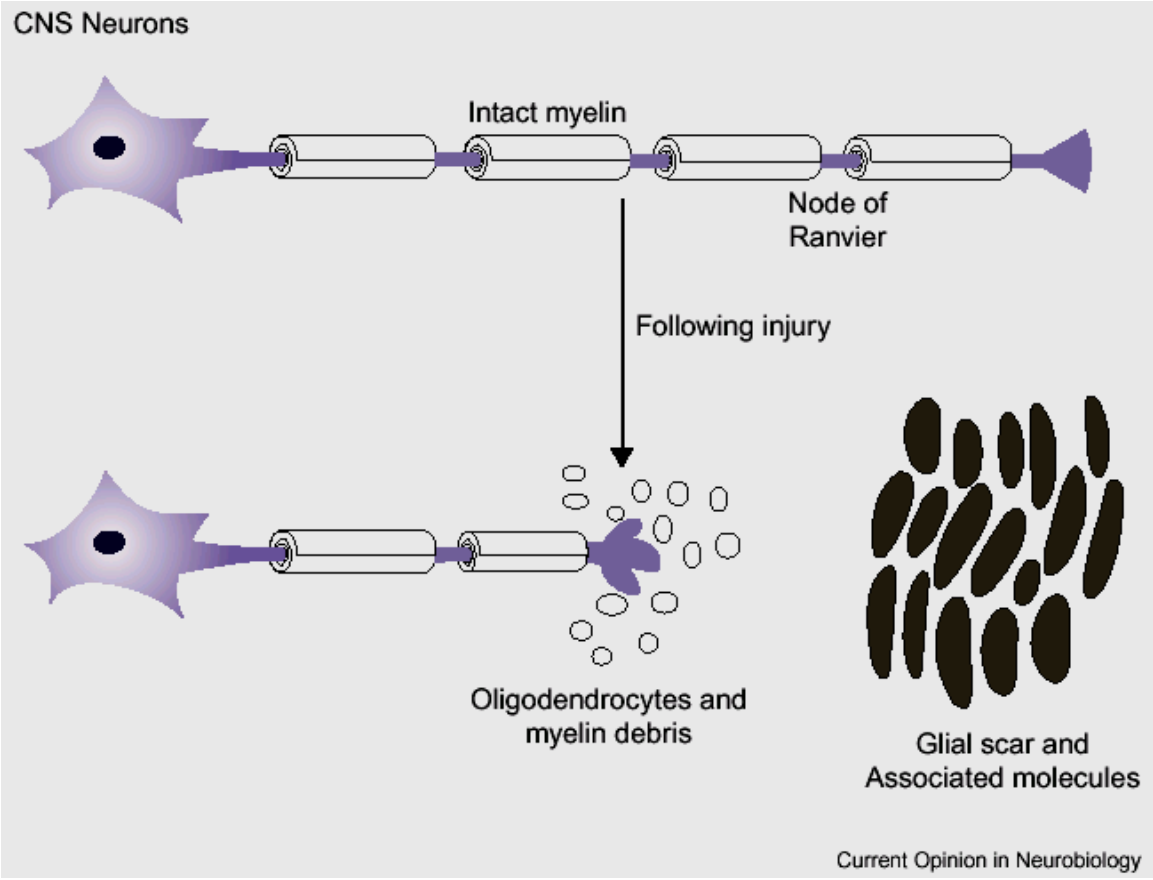


Fig (1.1): Physical and Molecular Barriers to Axonal Regeneration In The CNS. Following injury, the CNS has to overcome two main obstacles: inhibitors in myelin and the glial scar.

1.2.1 Inhibitors In Myelin:

1.2.1.1 Nogo

Martin Schwab and his colleagues first identified two protein enriched fractions from myelin extraction that had inhibitory activity. The two fractions molecular weights were 35kDa termed (NI-35) and 250kDa termed (NI-250) (Caroni and Schwab, 1988b). Next, they raised a monoclonal antibody termed IN-1 antibody, against the purified NI-250 fraction. The IN-1 antibody recognizes both NI-35 and NI-250 (Caroni and Schwab, 1988b). This IN-1 antibody was effective in neutralizing the inhibitory activity of CNS myelin *in vitro*, and also enhanced the regeneration of the corticospinal tract (CST) fibers *in vivo* with partial functional recovery (Caroni and Schwab, 1988a). It was not until 2000 that Nogo, the antigen for IN-1 antibody, was cloned from a partial sequence of IN-250 (Chen et al., 2000, GrandPre et al., 2000, Prinjha et al., 2000). There are three different isoforms of Nogo (-A, -B and -C), created by alternative splicing and different usage of the promoter. All three isoforms have a common 188-amino acid residue, a C-terminus homologous to reticulon protein; therefore Nogo belongs to the reticulon family of molecules, which are associated with the endoplasmic reticulum (ER). All three isoforms contain a 66-amio acid extracellular loop called Nogo-66 (GrandPre et al., 2000). Nogo-A, is expressed by both oligodendrocytes and neurons, and is localized to the inner and outer loop of myelin and on the surface of oligodendrocytes (Chen et al., 2000, GrandPre et al., 2000, Huber et al., 2002). Nogo-B is expressed in many tissues and cell types including neurons. Nogo-C is expressed outside the nervous system. The Nogo-66 domain of Nogo-A inhibit neurite outgrowth and cause growth cone collapse from

DRG neurons (Chen et al., 2000, GrandPre et al., 2000, Prinjha et al., 2000). In addition to Nogo-66, Nogo-A has an amino terminal domain, which is also inhibitory to neuronal re-growth. However, little is known about the receptor or the mechanism by which Amino-Nogo mediates it's inhibitory effect.

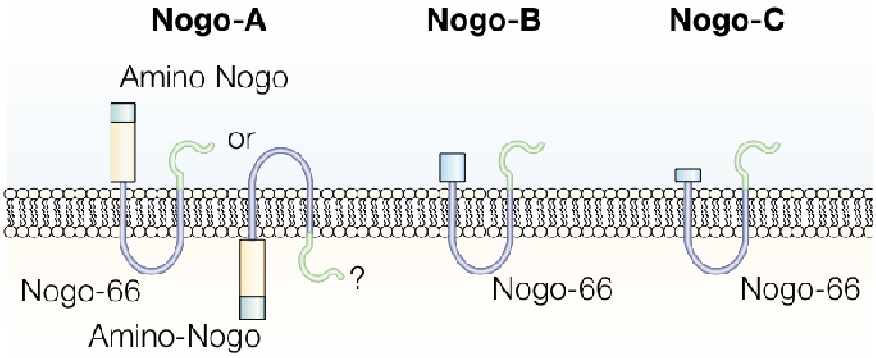


Fig (1.2): Structures of Nogo Three Isoforms-A, -B and –C. Only Nogo-A is enriched in oligodendrocytes and it carries two inhibitory domains, Nogo-66 and Amino-Nogo.

1.2.1.2 Myelin Associated Glycoprotein (MAG):

MAG is one of the first proteins expressed when myelination commences. It is a minor component of myelin, comprising only 1% of total protein in the adult CNS, and 0.1 % in the PNS (Trapp, 1990). MAG is expressed in the interface between myelinated axons and the periaxonal myelin membrane (Trapp et al., 1989). It is located in the first myelin wrap around the axon, as well as in areas of uncompacted myelin such as the paranodal loops (Trapp, 1988). Because MAG is expressed early in development, it was suggested that MAG plays a role in the initiation of myelination, however studies from MAG-deficient mice, showed that normal compact myelin is formed (Li et al., 1994, Montag et al., 1994). However, with time, MAG-deficient mice display both myelin degeneration and axonal degeneration (Fruttiger et al., 1995). This suggests that MAG interacts with the axon and is necessary for long-term maintenance.

During development, and in the intact adult CNS, growth cones are not normally exposed to MAG, because MAG is located at the paranodes or periaxonal leaflet of the membrane. However, when myelin is damaged, growth cones come into contact not only with MAG but also with other inhibitors in myelin. Our lab (Mukhopadhyay et al., 1994), and others (McKerracher et al., 1994) identified MAG as a potent inhibitor of axonal regeneration. When postnatal cerebellar (CGN) or adult DRG neurons are grown on a monolayer of MAG- expressing cells, axonal outgrowth was greatly reduced compared to neuronal outgrowth on control cells not expressing MAG. We showed that MAG inhibits neurite outgrowth from a variety of neurons: spinal, retinal, superior cervical ganglion (SCG), and hippocampal neurons (HN). Furthermore, inhibition on MAG-expressing cells was reversed by the addition of MAG antibodies to the co-cultures (Mukhopadhyay et al.,

1994). In addition, it was demonstrated that immunodepletion of MAG from CNS myelin resulted in a 60% recovery of neurite outgrowth (McKerracher et al., 1994).

In contrast to its potent inhibitory properties, MAG can also promote neurite extension, depending on the type and age of the neuron. We (Mukhopadhyay et al., 1994, DeBellard et al., 1996) and others (Johnson et al., 1989) have shown that young DRG neurons (newborn of postnatal day 1) grown on MAG-expressing CHO cells have neurite extensions as long as neurons grown on control CHO cells. This switch occurs by postnatal day (4-5); where MAG-expressing CHO cells inhibit the DRG neurons.

MAG is a member of the immunoglobulin (Ig) super-family and contains five Ig-like domains in its extracellular sequences, a single transmembrane domain, and a short cytoplasmic domain (Lai et al., 1987b, Salzer et al., 1987, Salzer et al., 1990). Importantly, MAG is a member of the sialic acid binding Ig-like lectin (Siglec) family of proteins. MAG (Siglec-4) is a sialic acid binding protein and shares 45-50% amino acid sequence similarity in its first four N-terminal domains with the domains of both CD22 and Sialoadhesin (Kelm et al., 1994). We mapped the sialic acid site in MAG to the first Ig –like domain Arg118 (a conserved sequence in all Siglecs) and we demonstrated that the sialic acid binding site is distinct from the inhibition site (Tang et al., 1997). In addition, 10 years later, our lab demonstrated that the inhibitory site on MAG resides at Ig-domain-5 (Cao et al., 2007). Also, we showed that the extracellular domain of MAG is secreted from damaged white matter and inhibits axonal regeneration (Tang et al., 2001). I will discuss MAG in more detail later in this chapter.

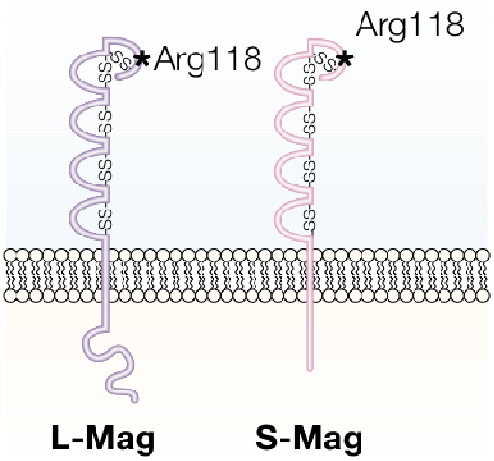


Fig (1.3): Structures of MAG. MAG exists in two isoforms that differ only in their cytoplasmic domain, Long MAG (L-MAG) and Short MAG (S-MAG). MAG has five immunoglobulin (Ig)-like domains in its extracellular segment. The sialic acid binding site on MAG is mapped to Arg118 in the first Ig-like domain, and the inhibitory site on MAG is mapped to Ig-like domain 5.

1.2.1.3 Oligodendrocyte Myelin Glycoprotein (OMgp)

OMgp was originally identified in 1988 (Mikol and Stefansson, 1988). Although its role during development is unknown, it was suggested that OMgp could be involved in the regulation of oligodendrocytes growth, the arrest of myelination or compaction of myelin. OMgp is expressed by oligodendrocytes and is localized to paranodal loops, adjacent to the node of Ranvier. In the CNS, OMgp is expressed by both

oligodendrocytes and neurons such as the pyramidal cells of the hippocampus, the Purkinje cells of the cerebellum, and the motor neurons in the brainstem (Habib et al., 1998). In 2002, OMgp was identified as a potent inhibitor of neurite outgrowth in myelin (Kottis et al., 2002, Wang et al., 2002b). OMgp is a glycosylphosphatidyinositol (GPI)-linked protein. It consists of a leucine-rich repeat (LRR) domain and a serine/threonine region. OMgp is the third major myelin- inhibitor to be discovered and together with Nogo-66 and MAG it was shown to bind to the NgR1-p75^{NTR}/Troy-Lingo-1 receptor complex. Very recently, the three myelin-associated inhibitors were shown to mediate their inhibitory activity of neurite outgrowth by binding to the Paired Immunoglobulin like receptor B (PirB) (Atwal et al., 2008).

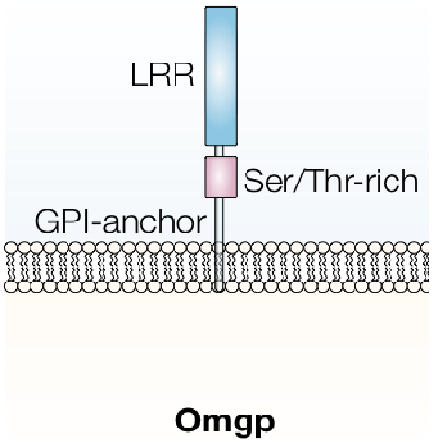


Fig (1.4): OMgp Structure. OMgp is a glycosyl phosphatidylinositol (GPI)-linked protein that carries a leucine- rich repeat (LRR) region and a serine/threonine(Ser/ Thr) rich region

1.2.2 Receptors for The Myelin Inhibitors:

1.2.2.1 Nogo-66 Receptor1 (NgR1)

In 2001, using a soluble form of Nogo-66 to screen an expression library, Strittmatter and colleagues cloned a binding partner for Nogo-66, which they called Nogo receptor (NgR) (Liu et al., 2002). NgR1, mediated inhibition of axonal regeneration in the injured CNS, while playing a physiological role in restricting plasticity in the intact CNS (Lee et al., 2008).

NgR1 is a GPI-linked protein that lacks a transmembrane domain and therefore requires co-receptor(s) to transduces the intracellular growth inhibitory signal. The co-receptors, $p75^{NTR}$ or Troy and Lingo-1 were identified as components of the NgR1 receptor complex.

The NgR1 receptor consists of 473 amino acids. Mutation analysis and the resolution of NgR1 crystal structure (Barton et al., 2003, He et al., 2003) demonstrated that the receptor ligand interaction localized to the LRR motifs. The multiple LRR motifs result in a concave groove, which contains a putative degenerate binding site, therefore it accounts for the similar interaction with different ligands. The crystal structure and the deletion studies also suggested a binding site in the unique C-terminal to a co-receptor.

NgR1 function as a common receptor complex that mediates the inhibition of axonal growth by the three myelin based inhibitors Nogo-66, MAG and OMgp (Fournier et al., 2001, Domeniconi et al., 2002, Liu et al., 2002, Wang et al., 2002b). All three unrelated ligands bind to NgR1 with high affinities. MAG binding to NgR1 is sialic acid – independent (Domeniconi et al., 2002, Liu et al., 2002). Cleaving GPI anchored NgR1

abolished inhibition *in vitro*. Genetic removal of NgR1 blocks acute growth cone collapse but does not relive chronic inhibition of neurite outgrowth by myelin inhibitors (Chivatakarn et al., 2007). Also, genetic deletion of NgR1 does not enhance regeneration of corticospinal tract axons (CST) (Zheng et al., 2005).

1.2.2.2 The p75 Neurotrophin Receptor ($p75^{NTR}$)

As mentioned earlier, NgR1 is a GPI linked receptor and therefore it requires the presence of co-receptors to transduce the signal initiated. Early during development $p75^{NTR}$ is expressed in a wide variety of cells within the CNS and PNS, as well as many non-neuronal tissues such as kidney, testis, lung and muscle (Ryffel and Mihatsch, 1993). In postnatal animals, $p75^{NTR}$ levels are reduced in most tissue and are restricted to a narrow range of cells (Ryffel and Mihatsch, 1993) however, after trauma in the CNS $p75^{NTR}$ expression is induced in many cells (Ebadi et al., 1997). Increased levels in mRNA and protein of $p75^{NTR}$ are demonstrated in motor cortex, corticospinal tract and hippocampus, including neurons, oligodendrocytes, Schwann cells, purkinje cells, microglia and macrophages (Ebadi et al., 1997). The $p75^{NTR}$ is a member of the tumor necrosis factor receptor (TNFR) super-family, class type I transmembrane protein with significant homology in their extracellular domains, which contains one or more cysteine-rich domains (CRDs) (Locksley et al., 2001). The $p75^{NTR}$ was first identified as a low affinity neurotrophin receptor and it has been suggested to play a role in cell death signaling (Rabizadeh and Bredesen, 2003).

In 2002, The Yamashita group was first to report that $p75^{NTR}$ is a transducer for MAG. They showed that DRG and CGN neurons form $p75^{NTR}$ deficient mice are not inhibited

by MAG. Also, they showed that $p75^{NTR}$ co-precipitated with MAG from neurons, but they were unable to show a direct interaction (Yamashita et al., 2002). Later in 2002, Wang et al demonstrated that the inhibition of axonal regeneration induced by all three myelin-associated inhibitors Nogo-66, MAG and OMgp are dependent on the association of NgR1 with the $p75^{NTR}$ receptor. This was evident by using neurons from $p75^{NTR}$ null mice which were insensitive to myelin-associated inhibitors (Wang et al., 2002a). Wang et al further elucidated that NgR1 is present when $p75^{NTR}$ was precipitated with MAG, Nogo-66 and OMgp. Also, they demonstrated that the inhibitory effect of myelin–associated inhibitors or myelin is abolished if the NgR1-$p75^{NTR}$ interaction is disrupted by soluble $p75^{NTR}$-Fc, or by dominant negative NgR1 which lacks the binding site for $p75^{NTR}$. In addition, over-expression of a truncated form of $p75^{NTR}$ that lacks the intracellular domain attenuates the inhibitory activity and suggests a role for intracellular $p75^{NTR}$ in NgR1 mediated inhibition of neurite outgrowth.

1.2.2.3 Troy

Similar to $p75^{NTR}$, Troy is a member of the TNFR family. Since $p75^{NTR}$ is not expressed in all neurons the presence for other co-receptor for NgR1 or a $p75^{NTR}$ homolog was predicted. In 2005, two different groups independently demonstrated Troy to interact with NgR1 and to substitutes for $p75^{NTR}$ to activate RhoA (Park et al., 2005, Shao et al., 2005). Genetic deletion, over-expression of dominant negative, or soluble form of Troy can also promote neurite outgrowth on myelin-associated inhibitors. Moreover, neurons isolated from Troy deficient mice are resistant to the inhibitory effects of OMgp and Nogo-66.

1.2.2.4 Lingo-1

In 2004, another component for the NgR1 receptor complex was identified, Lingo-1 (Mi et al., 2004). Lingo-1 is a transmembrane protein consisting of 12 LRR motifs, one Ig-like domain, a transmembrane domain, and a short cytoplasm tail. Lingo -1 expression is specific to the CNS and it binds to both NgR1 and $p75^{NTR}$ to activate RhoA. Deletion of Lingo-1 reduces the inhibitory effect of myelin-associated inhibitors. Expression of dominant negative Lingo-1 or addition of soluble Lingo-1 attenuates myelin inhibition in CGN neurons. In addition, transfecting CGN neurons with full length Lingo-1 render them more sensitive to myelin (Mi et al., 2004).

1.2.2.5 NgR2 and NgR3

Beside NgR1, two other isoforms homologous to NgR1 were discovered, NgR2 and NgR3. In 2005, The Giger's group reported that NgR2 receptor is specific only for MAG. The mechanism by which NgR2 mediates MAG inhibitory activity of neurite outgrowth is still unknown. However, MAG binds to NgR2 in a sialic acid-dependent manner (Venkatesh et al., 2005), unlike NgR1, which binds Nogo-66, MAG and OMgp in a sialic acid-independent manner (Domeniconi et al., 2002). Also, they showed that NgR3 does not bind any of the three known myelin-associated inhibitors.

1.1.2.6 Paired Immunoglobulin Like Receptor B (PirB)

Very recently, a new receptor for myelin inhibitors was identified as a functional receptor for myelin-associated inhibitors of axonal regeneration (Atwal et al., 2008). Using expression cloning to screen an array library of cDNA pool, they identified 2 receptors for Nogo-66: the NgR1 receptor and human leukocyte immunoglobulin (Ig)-like receptor

B2 (LILRB2). LILRB2 consists of 5 homologues members in human, but in mice there is only a single ortholog, Paired Immunoglobulin like receptor B (PirB). PirB shares about 50% amino acid homology with LILRB2 and contains six Ig-like repeats instead of four Ig-Like repeats in LILRB proteins. They demonstrated that Ap-Nogo binds PirB receptor similar to NgR1. In addition, they also demonstrated that MAG-Fc and Ap-OMgp binds PirB receptor with high affinity (Atwal et al., 2008). Functionality of PirB receptor was shown in neurite outgrowth inhibition by using PirB antibody and PirB mutant mouse (PirBTM). PirB antibody partially blocks Nogo-66, MAG and total myelin inhibition of neurite outgrowth in CGN and DRG neurons. Neurons isolated from PirBTM mutant mice are less inhibited by Nogo-66, MAG, OMgp and myelin. They further investigated if PirB and NgR1 function together to mediate inhibition of neurite outgrowth. They reported that CGN neurons from NgR1 deficient mice are not inhibited by myelin in the presence of PirB antibody. However, when Nogo-66 was used, inhibition was only partially reversed by PirB antibody in CGN neurons from NgR1 deficient mice. To date, the mechanism by which PirB inhibits neurite outgrowth has not been elucidated. It will be interesting to investigate axonal regeneration in NgR1-PirB double knockout mice.

In the CNS, PirB is expressed throughout the brain and has been reported to restrict plasticity in the visual cortex (McGee et al., 2005, Syken et al., 2006). In mice deficient for PirB, cortical-occular dominance plasticity persisted through adulthood (110 days) instead of being terminated by postnatal day 32 (Syken et al., 2006). Suggesting that like NgR1 it has a physiological role in restricting plasticity in the intact CNS.

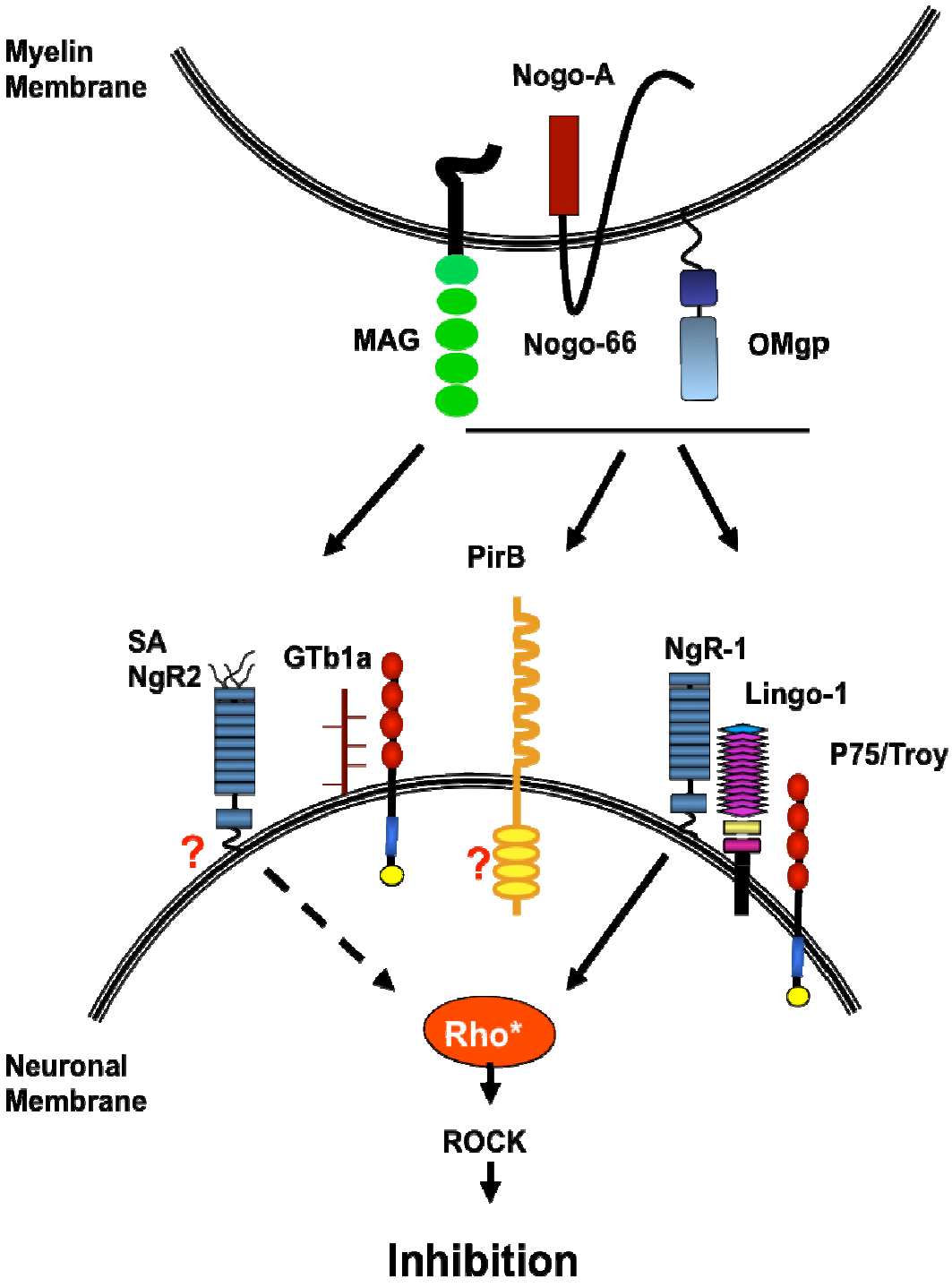


Fig (1.5): Myelin Inhibitors and their receptors. Nogo-66, MAG and OMgp interact with both NgR1-p75/Troy-Lingo1 receptor complex and PirB receptor. In addition, MAG can specifically bind to NgR2 in sialic acid-dependent manner and to gangliosides GT1b and GD1.

1.2.3 Other Inhibitors In Myelin

Ephrin-B3 is a known vertebrate axon guidance molecule that functions during development as a midline repellant for axons of the CST (Kullander et al., 2001). In 2005, Ephrin-B3 was identified as a myelin-based inhibitor of neurite outgrowth (Benson et al., 2005). They showed that Ephrin-B3 was expressed in the adult spinal cord in postnatal myelinating oligodendrocytes, and it is inhibitory to axonal growth equivalent to the other three myelin-based inhibitors, MAG, Nogo-66 and OMgp.

Sema4D is a member of a protein family called Semaphorins, which are expressed in the CNS. They exist as membrane-bound or secreted molecules. Sema4D is selectively expressed in oligodendrocytes and myelin and its expression is up-regulated following injury (Moreau-Fauvarque et al., 2003). It was shown that Sema4D inhibits neurite outgrowth of CGN and DRG neurons (Moreau-Fauvarque et al., 2003). Also, Sema4D induces growth cone collapse in CNS neurons (Swiercz et al., 2002).

1.2.4 Glial Scar

1.2.4.1 The Formation of The Glial Scar

Following injury, severed neurons from the CNS are exposed to both myelin associated inhibitors, and the glial scar. The glial scar forms within weeks of injury primarily by reactive astrocytes (Reier et al., 1983, Reier and Houle, 1988, Jakeman and Reier, 1991). After CNS injury, the processes of reactive astrocytes becomes tightly interlinked and form a mechanical barrier, which prevents regenerating neurons from crossing the injury site.

1.2.4.2 Inhibitors in the Glial Scar

Besides forming a mechanical barrier, the condensed reactive astrocytes also forms a chemical barrier by expressing and secreting a number of inhibitory molecules including tenascin, keratin, semaphorin III and chondrotin sulfate proteoglycans (CSPG). Recently PTPσ, a transmembrane protein tyrosine phosphatase, was identified as a receptor that binds with high affinity to neuronal CSPGs (Shen et al., 2009). All of these molecules inhibit axonal regeneration *in vitro* (McKeon et al., 1991, Smith-Thomas et al., 1994, Niederost et al., 1999).

1.2.5 Signaling By Myelin Inhibitors

Following injury in the CNS, myelin-associated inhibitors present in the myelin (Nogo-66, MAG and OMgp) bind to NgR1-$p75^{NTR}$/Troy-Lingo receptor complex. Also it could bind to the PirB receptor. In addition, the presence of other inhibitors in myelin such as EphB3 and Sema4D or glial scar associated inhibitors, initiates an inhibitory signaling cascade that elevates Ca^{2+} and activates the small GTPase RhoA (Schwab et al., 1993, Lehmann et al., 1999, Winton et al., 2002, Yamashita et al., 2002). RhoA activation mediates inhibitory signaling by regulating cytoskeleton rearrangements. RhoA activation results in growth cone collapse and inhibition of axonal growth (Dergham et al., 2002, Winton et al., 2002). Previously, we reported that PKC regulates $p75^{NTR}$ cleavage. Upon MAG binding to NgR1 receptor complex, intramembrane proteolysis of $P75^{NTR}$ occurs, and subsequently, RhoA is activated in a PKC-dependent manner (Domeniconi et al., 2005). The mechanism by which PirB inhibits axonal growth is still not understood.

1. 3 The Intrinsic Neuronal State

It is well established that the environments of the CNS prevents axonal growth after injury, due to the obstacles that we discussed earlier (the presence of myelin-associated inhibitors, and the glial scar). However, embryonic neurons transplanted into the injured spinal cord are able to regenerate over long distance (Bates and Stelzner, 1993, Hasan et al., 1993). When neurons from embryonic tissues were planted on the CNS injury site, these neurons were able to regenerate and cross the injury site, which indicates that the intrinsic state of this young neurons are different from the mature CNS neurons and have a higher growth capacity and do not recognize inhibitors.

1.4 Overcoming Myelin Inhibition

1.4.1 Blocking Inhibitors with Antibodies and Peptides

IN-1 antibody was raised against NI-35 and NI-250 inhibitory fractions (Caroni and Schwab, 1988a). Application of IN-1 antibody in vivo, improved regeneration of injured fibers and functional recovery (Schnell and Schwab, 1990, Bregman et al., 1995). Another interesting study reported improved regeneration following dorsal column lesion in adult mice that were vaccinated with myelin-associated proteins (Huang et al., 1999).

Since all three myelin-associated inhibitors (MAG, Nogo-66 and OMgp) binds to the same receptors, the NgR1-p75^{NTR}/Troy-Lingo receptor complex or PirB, this redundancy mechanism could provide potential targets for therapeutic intervention to encourage regeneration before the formation of the glial scar. Using anti-NgR antibody in vitro

improved neurite outgrowth growing on purified myelin (Domeniconi et al., 2002). Other studies used small antagonistic peptides such as (NEP1- 40), which consist of the first 40 amino acids of Nogo-66 domain, which will bind NgR1 receptor and block the inhibitory signaling of the endogenous Nogo-66 (GrandPre et al., 2002). Also other studies used non-signaling peptides from the receptor complex to compete out and compromise the ligand-receptor binding such as $p75^{NTR}$-Fc (Wang et al., 2002a), Lingo-Fc (Mi et al., 2004) and PirB (Atwal et al., 2008), all these peptides promotes neurite outgrowth *in vitro*.

1.4.2 Myelin-Associated Inhibitors and Receptors - Knockout Mice

Genetically modified mice were used to further elucidate the role of myelin-associated inhibitors and their receptors. Two different groups made MAG deficient mice. One group reported little or no regeneration of optic nerve and CST tract (Bartsch et al., 1995), and another group reported a small but significant amount of regeneration in the CST fibers compared to wild type mice (Li et al., 1996). The difference in this finding could be explained by the different background strains between these two mice lines. In addition, the presence of the glial scar and other inhibitors in myelin could explain the modest regeneration results seen with these mice.

Three different groups independently generated Nogo knockout mice, and again there was a lot of discrepancy (Kim et al., 2003, Simonen et al., 2003, Zheng et al., 2003). The Strittmatter's group generated mice that lack Nogo-A and Nogo-B but not Nogo-C by inserting a retroviral gene trap insertion to disrupt exon Nogo-A. After spinal cord injury,

this group reported robust axon sprouting and regeneration in addition to improved functional recovery (Kim et al., 2003). Schwab's group also disrupted Nogo-A with a conventional knockout approach, and they reported an up-regulation of Nogo-B in the CNS of these mice. Nevertheless, they reported enhanced regeneration after dorsal hemisection in these Nogo-A knockout mice. The third group, Tessier-Lavigne's group generated two mice lines, one lacking Nogo-A and -B and another lacking all three isoforms of Nogo A-, B-, and C-. Their results contradict Strittmatter's group, because they observed no regeneration after CST injury. All this variability in results could be explained by the different backgrounds and experimental techniques used between the groups (Woolf, 2003).

The Strittmatter and Tessier-Lavigne's groups also individually generated NgR1 deficient mice. Although, there was some regeneration in raphespinal and rubrospinal tract with some recovery of motor function after dorsal hemisection in Strittmatter's NgR1 knockout mice (Kim et al., 2004), both studies observed no regeneration in the CST tract after injury (Zheng et al., 2003, Kim et al., 2004). This suggests that indeed other receptors for Nogo-66, MAG and OMgp could be present, such as PirB. PirB is a novel receptor that needs to be further investigated *in vivo* to elucidate its role in inhibition of axonal regeneration.

1.4.3 Changing The Intrinsic State of Neurons

1.4.3.1 Cyclic AMP, Neurotrophins and Conditioning Lesion

Cyclic AMP (cAMP) is a second messenger and has many roles in a variety of signaling pathways. cAMP levels in the neurons could be elevated by, non-hydrolysable cAMP

analogs such as dibutyryl cAMP (dbcAMP) or prior exposure of neurons to neurotrophins, "priming", such as NGF, BDNF, and GDNF, and by inhibiting phosphodiesterases (PDEs) such as rolipram (inhibits PDE4) improve regeneration and functional recovery (Nikulina et al., 2004)

Previously, our lab demonstrated that cAMP levels of embryonic neurons are significantly higher than that of adult neurons, and this elevation could account for their ability to extend axons in the inhibitory CNS environment. In addition, we demonstrated that cAMP levels in young neurons remains high until the developmental switch occurs which also results in spontaneous regenerative loss in the CNS inhibitory environment (Cai et al., 2001). Elevating intracellular cAMP levels, by using the chemical analog dbcAMP or priming with neurotrophins such as brain derived neurotrophic factor (BDNF), in the postnatal neurons results in overcoming inhibition by MAG and Myelin (Cai et al., 1999). Our lab showed that this priming effect is PKA- and transcription-dependent through CREB. In addition, cAMP up regulates several genes that are associated with axonal regeneration, such as Anginas 1 (Arg 1) (Cai et al., 2002, Deng et al., 2009), and interleukin-6 (IL-6) (Cao et al., 2006).

DRG neuron extends two branches, the central branch extends into the spinal cord, and the peripheral branch extends to the periphery. Spontaneous regeneration in the CNS is observed by first lesioning the peripheral branch and 1 day or 7 days subsequent to that a dorsal column lesion is performed (Neumann and Woolf, 1999). This conditioning lesion effect is cAMP-dependent and a single injection of dbcAMP into the DRG prior to dorsal column lesioning could mimic this conditioning effect (Neumann et al., 2002, Qiu et al., 2002).

1.4.3.2 Blockage of RhoA Small GTPase

It is well established that Myelin-associated inhibitors, Nogo-66, MAG and OMgp, in addition to CSPGs activates RhoA (Lehmann et al., 1999, Kottis et al., 2002, Winton et al., 2002, Borisoff et al., 2003, Monnier et al., 2003). It was shown that inactivation of RhoA with C3 transferase promotes axonal regeneration after optic nerve crush in adult rats (Lehmann et al., 1999). Blocking RhoA with C3 transferase also promotes regeneration of CST axons and improved functional recovery in adult rats after spinal cord hemisection injury (Dergham et al., 2002). In addition to blocking RhoA, blocking ROCK a down stream effecter with Y27632 promotes regeneration of CNS CST axons (Dergham et al., 2002, Fournier et al., 2003).

1.5 Structural and Physiological Roles of MAG

MAG is a 100-KDa glycoprotein that belongs to the immunoglobulin super-family (Williams and Barclay, 1988), located in the periaxonal membranes of myelin-forming oligodendrocytes and Schwann cells. MAG is one of the first proteins expressed when myelination commence. It is a minor component of myelin. It compromises about 1 % of total myelin proteins in the CNS and 0.1 % of total myelin proteins in PNS myelin (Trapp, 1990). In this section, we will take a more in depth look into MAG's structural and physiological roles.

1.5.1 Structural characteristics of MAG

Three different groups independently cloned MAG (Arquint et al., 1987, Lai et al., 1987a, Lai et al., 1987b, Salzer et al., 1987). They found that MAG is an integral membrane protein with five Ig-like domains in its extracellular N-terminal segment, a single transmembrane domain and a C-terminal cytoplasmic tail.

Two different isoforms exists for MAG generated by alternative splicing from a single gene that consists of 13 exons: the large form of MAG (L-MAG) contains 607 amino-acids with a mass of 72 KDa, and the small form of MAG (S-MAG) contains 563 amino acids with a mass of 67 KDa. L-MAG and S-MAG are identical in their N-terminal extracellular segment and transmembrane domains, but differ in the C-terminal cytoplasmic domains. In the rodent CNS, most of the MAG synthesized during myelination is L-MAG.

The first Ig domain contains an Arg-Gly-Asp (RGD) tri-peptide that is known to be a binding site to integrin type receptors on cell surfaces (Ruoslahti and Pierschbacher, 1986), suggesting that MAG binding to cells could involve integrin receptors. However, this tri-peptide is inaccessible and therefore MAG binding to integrin via this RGD sequence is unlikely (Pedraza et al., 1990, Sadoul et al., 1990, Yim et al., 1995).

1.5.2 MAG as a member of Siglec Family

The Siglec family of proteins (sialic acid-binding, immunoglobulin-like lectins) are subgroup of the Immunoglobulin super-family, that are known by their shared amino acid sequence similarity among the first four-Ig-like domains and their sialic acid–dependent binding to cells (Kelm et al., 1998). Sialoadhesin are the first member of Siglecs family

that was identified (also named Siglec-1, Marine macrophage restarted cell surface molecule). Other members of the Siglec family include, CD22 (Siglec-2, B cell adhesion molecule), CD33 (Siglec 3, lymphoid differentiation antigen) and MAG (Siglec-4) (DeBellard et al., 1996, Tang et al., 1997).

All members of the Siglec family bind sialic acid, and the specificity of binding defer between each other. MAG prefers to bind α 2,3-linked sialic acid residues attached to O-linked glycoconjugates (Kelm et al., 1994). 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 et al., 1994, Cornish et al., 1998).

MAG has a low-binding affinity to sialic acid, and needs to aggregate in order for binding to be measured. This implies that MAG clusters in the periaxonal myelin membranes and interacts with multiple sialic acid residues on the opposing axonal membrane to exert stable binding in order to initiate a functional response *in vivo*.

1.5.3 Roles of MAG in Formation and Maintenance of Myelin

Since MAG is expressed early in development, and it is exclusively and preferentially localized to the interface between myelinated axons and the periaxonal myelin membrane (Trapp et al., 1989), it has been hypothesized that MAG plays a role in axon-glial interactions (Schachner and Bartsch, 2000). Also, it could influence myelin formation. Genetic depletion of MAG showed a modest alternation in myelination, reduced axon caliber, and reduced neurofilament spacing and phosphorylation, and progressive axonal degeneration (Li et al., 1994, Montag et al., 1994, Fruttiger et al., 1995, Yin et al., 1998).

Therefore it was concluded that MAG plays an important role in axon-myelin interaction and is required for long-term myelin maintenance and stability.

This was confirmed in the MAG-deficient mice, which have normal myelination, but with age, they develop both demyelination and axonal degeneration phenotypes.

Using genetically modified approaches, MAG-deficient mice were used to further clarify the differential roles MAG plays in the CNS and PNS. During development, MAG-deficient mice exhibit delays in the formation of compact myelin in the optic nerves of young postnatal animals. The density of retinal ganglion cell axons surrounded by compact myelin was also significantly reduced compared to control animals from the same age (Montag et al., 1994). The ultra-structure of compact myelin was not affected in MAG-deficient mice, but they exhibit a dilated periaxonal space and abnormal formation of periaxonal cytoplasmic collar (Li et al., 1994, Montag et al., 1994). In addition, some axons in MAG-deficient mice are surrounded by more then one myelin sheath (Bartsch, 1996). Moreover, in the adult MAG-deficient mice, substantial portions of oligodendrocytes undergo degenerative alternations in the distal processes either in the periaxonal space or within compact myelin. This feature is common to immune-mediated demyelinating diseases, including multiple sclerosis (Rodriguez-Pena et al., 1993, Lassmann 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-term maintenance of oligodendrocyte structure and myelin integrity. However, in the PNS, MAG only seems to be involved in the formation of intact myelin, and long-term maintenance of myelin structure, but not in the initiation of myelination.

In the PNS, MAG-deficient mice exhibit deletion of the periaxonal space and loss of the periaxonal cytoplasmic collar in myelinating axons (Trapp and Quarles, 1984, Li et al., 1994, Montag et al., 1994). In addition, degeneration of both Schwann cells and axons occurs more frequently, similar to the CNS (Fruttiger et al., 1995). However the speed of myelination in the PNS was no different between MAG-deficient mice and wild type control mice (Montag et al., 1994).

Collectively, it is clear from the cell culture experiments and from the MAG-deficient mice that MAG is 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 the axons.

1.6 Gangliosides

1.6.1 Characteristic and Physiological Role of Gangliosides

In 1935, Klenk extracted from the brain of a Niemann-Pick disease patient (a group of inherited diseases in which lipids collect in the cells of spleen, liver and brain) something new that he called substance X (Klenk 1935). In 1939 he understood that substance X was a mixture of compounds and he named them gangliosides (Klenk 1939). In 1947 the structure of sphingosine was elucidated for the first time (Carter et al., 1947), and in 1955 the structure of silaic acid was also elucidated (Gottschalk 1955). Finally, in 1963 the first ganglioside structure was described (Kuhn and Wiegandt, 1963). Today, gangliosides are considered to be functional molecules involved in the modulation of

enzyme properties and of cell signaling, cell adhesion, and protein sorting. Also, gangliosides are the binding sites for viruses, bacteria, and toxins (Karlsson, 1989).

In vertebrates, glycolipids are predominantly glycosphingo lipids, which are glycan on a ceramide lipid core. Gangliosides are glycosphingolipids that contain one or more sialic acid residues in their oligosaccharide structure (Sonnino et al., 2007). They are components of all animal cell membranes and are particularly abundant in the plasma membranes of neurons. Gangliosides are complex lipids with a strong amphiphilic big saccharide head-group and a double tailed hydrophobic moiety. The lipid moiety of gangliosides, shared with all sphingolipids, is called ceramide (Fig1.6) and is constituted by a long-chain amino alcohol termed sphingosine (Karlsson, 1970) connected to fatty acids by an amide linkage. The ceramide lipid moieties of gangliosides are embedded in the outer leaflet of the plasma membrane and their complex glycans extend into the extracellular space (Sonnino et al., 2007). Sialic acid is the sugar that differentiates gangliosides from neutral glycosphingolipids and sulfatides. It defines all derivatives of 5- amino-3, 5-dideoxy-d-glycero-d-galacto-non-2-ulopyranosonic acid or neuraminic acid (Schauer, 1982) (Fig 1.7). Gangliosides are positioned to interact laterally (*cis*) with other molecules in their own membrane and with molecules on opposing cell membranes (*trans*) (Lopez and Schnaar, 2009). Gangliosides are typically anchored in the outer leaflet of the plasma membrane where the long saturated hydrocarbon chains or ceramide drive gangliosides to partition laterally into lipid rafts, which are membrane micro-domains that contain other sphingolipids, cholesterol, and selected signaling molecules. This lateral *cis*- interaction in the membrane normally results in gangliosides-mediated regulation of membrane proteins such as receptor kinases. Ganglioside glycans extend

outward from the cell surface, where their sialoglycans participate in intermolecular interactions. This *trans*- interaction with proteins on opposing membranes results in gangliosides–mediated cell-cell- recognition such as myelin-axon interaction.

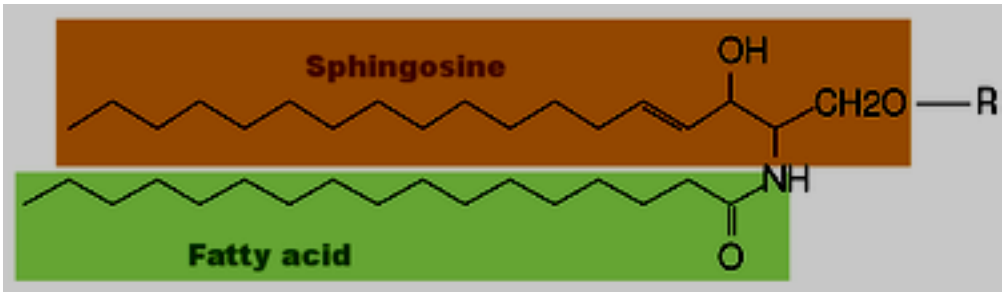


Fig (1.6): The Structure of Ceramide. Ceramide is a component of glycosphingolipids, composed of a long-chain amino alcohol (sphingosine) and an amide-linked fatty acid (modified from AOCS lipid library).

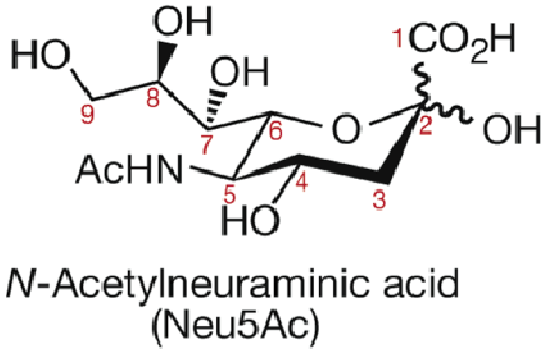


Fig (1.7): The Structure of Sialic Acid. Sialic acids are family of acidic sugars with nine-carbon backbone, of which the most common is N-acetylneuraminic acid, in vertebrates (modified from Varki A., in essential of glycobiology, second edition, chapter 14, Fig 1).

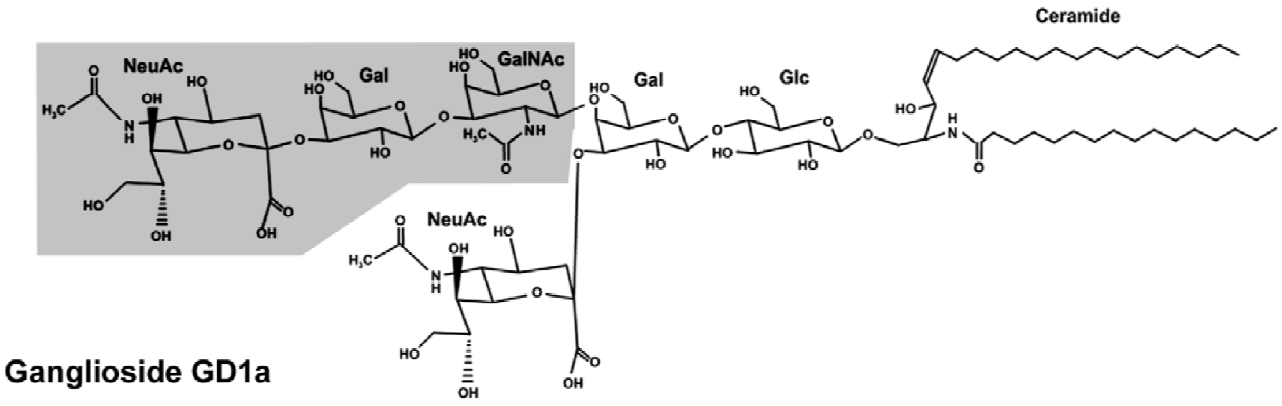


Fig (1.8): Schematic structure of the ganglioside GD1a. GD1a structure with MAG-binding determinant (NeuAc αn2-3 Gal β 1-3 GalNAcT) shaded. (Modified from Schnaar 2010).

1.5.2 Biosynthetic Pathway:

As mentioned earlier, Gangliosides are glycosphingolipids that have one or more sialic acid residue(s) in their glycan structure. Gangliosides biosynthesis starts with the formation of ceramide in the membrane of the ER. Ceramide is the common precursor of glycosphingolipids and sphingomyelin and is transported to the Golgi apparatus by unknown mechanisms.

Glycosphingolipids are synthesized by the stepwise addition of monosaccharide sugars first to ceramide, then to the growing sugar. Ganglioside biosynthesis is stepwise, starting with transfer of glucose to ceramide then, subsequent addition of galactose, sialic acid, and N-acetylgalactosamine from their nucleotide sugar donors to the growing saccharide chain generate penta, hexa and hepta saccharide glycans (Kolter et al., 2002)

As shown in Fig (1.9), gangliosides biosynthetic pathway, in the sequential order of glycosylation, occurs by two main pathways, designated "a" series (GM2, GM1a, GD1a) and "b" series (GD2, GD1b, GT1b) (Fig 1.9) (van Echten and Sandhoff, 1993). Each ganglioside is structurally more complex than its precursor molecule, and the stepwise addition of monosaccharide or sialic acid residues by specific membrane bound glycosyltransferases in the Golgi apparatus is catalyzed by the same glycosyltransferases in both pathways.

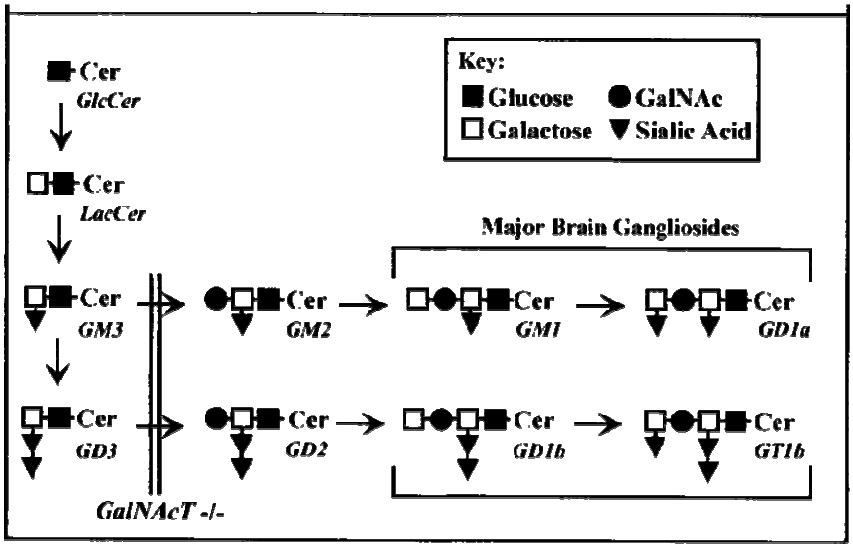


Fig (1.9): Biosynthetic pathway for major brain gangliosides:
The schematic biosynthetic relationship between major brain gangliosides and their precursors is shown. Disruption of the GalNAcT locus blocks ganglioside biosynthesis including GT1b and GD1a that MAG binds to. The GalNAcT -/- mice lack all complex gangliosides; instead it expresses an increase in simpler gangliosides GM3 and GD3. Modified from (Sheikh et al 1999).

1.6.3 GalNAcT Deficient Mice

Gangliosides are considered to play important roles in the development and differentiation of the nervous system. They are abundantly expressed in the nervous system of many vertebrates. Gangliosides are synthesized by consecutive addition of monosaccharide to ceramide by multiple glycosyltransferases in the Golgi apparatus and are then transferred to the outer leaflet of the plasma membrane. Among the many glycosyltransferases β1,4 GalNAc-transferase (β1,4 GalNAc-T; GM2/GD2 synthase; EC2.4.1.92) plays an important role in biosynthesis of almost all complex gangliosides.

Two different groups independently targeted disruptions in the β1,4 GalNAcT gene, which consists of 10 exons, to generate the GalNAcT deficient mice. The Aizawa's group wanted to address the role of gangliosides in neuronal development and physiological events in the neural tissue. They disrupted exon 4 of the β1,4 GalNAcT gene by inserting a neomycin-resistant plasmid. They reported that GalNAcT deficient mice express no complex gangliosides but higher concentration of the simpler gangliosides GD3 and GM3. These knockout mice develop only subtle neurological defects, which are restricted to decreased central conduction velocity (Takamiya et al., 1996). They suggested that complex gangliosides are required in neuronal function but not in the morphogenesis and organogenesis of the brain. And the higher levels of GM3 and GD3 expressed in the brains of these mutant mice may be able to compensate for the lack of complex gangliosides.

The Proia's group generated an independent strain of GalNAcT deficient mice (GM2/GD2 synthase). A replacement vector in which exon 6, 7, and part of exon 8 were deleted, and replaced with the MC1NeoPolyA selection cassette disrupted the GalNAcT

gene. They reported that these mice display decreased central myelination and axonal degeneration in both central and peripheral nervous system (Sheikh et al., 1999). These GalNAcT deficient mice lack the NeuAcα3Galβ3GalNAc terminus, and instead express only the relatively simple gangliosides GD3 and GM3. These knockout mice are viable and have a normal life span (although the knockout males are sterile). Proia's group wanted to determine if these mice lacking gangliosides have morphologic changes similar to MAG-deficient mice. Indeed, they reported that similar to MAG-deficient mice, the GalNAcT deficient mice have unusual myelin abnormalities, such as doubly myelinated axons with cytoplasm between the two compact myelin sheaths. GalNAcT deficient mice express normal levels of MAG in younger animals, but MAG levels decline in both the CNS and PNS to about 30% of normal levels. In addition these mice develop Wallerian degeneration (Sheikh et al., 1999).

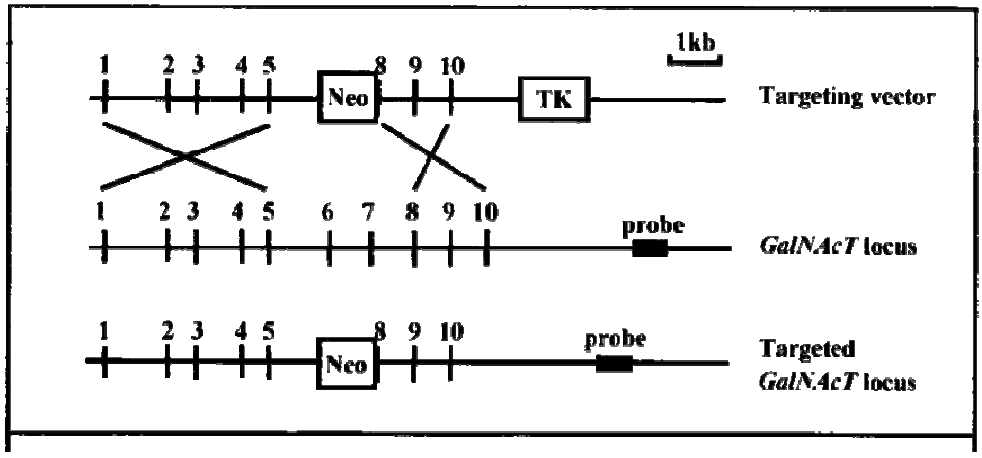


Fig (1.10): Targeted disruption of the GalNAcT locus generated by the Proia group. Targeting vector (top), GalNAcT locus (middle), and predicted homologous recombined locus (bottom). Exons 6, 7 and part of exons 8 of the GalNAcT gene was deleted and replaced with the targeting vector. Modified from (Sheikh et al., 1999).

Proia's group attributed this controversy to the fact that the Aizawa's group animals were examined at a young age (10 weeks), however, is the morphological abnormal changes in the MAG-deficient mice became more prominent with increasing age. They argued that since conduction velocity depends on both myelination and axonal diameter, the decreased central conduction velocity seen by the Aizawa's group could be attributed to myelination defects, and axonal atrophy.

1.6.4 Implication of Gangliosides in MAG-Mediated Neurite Outgrowth Inhibition

MAG binds preferentially to the sequence NeuAcα2-3Galβ1-3GalNAc (Kelm et al., 1994, Crocker et al., 1998) (Crocker and Feizi, 1996, Crocker et al., 1996), which is the terminus of major brain gangliosides GT1b and GD1a (Fig 1.8) These gangliosides are abundant on the neuronal cell surface and all along the axons. Mice lacking GalNAcT have grossly impaired myelination and progressive neurodegeneration.

Previously, we have shown that removal of sialic acid residues by sialidase treatment does not affect MAG's inhibitory activity (Tang et al., 1997). Also, the addition of IgM Fab fragments generated from anti-GT1b or anti-GD1a antibodies mimics MAG inhibitory effect in the outgrowth assay, which suggest that this interaction is not specific to MAG pathway. Our model Fig (2.1) suggested that the recognition of sialylated glycans are not essential for inhibition of neurite outgrowth by MAG, although it may result in a weak transient activation of the inhibitory pathway independent of the NgR1 – dependent inhibition effects. Our lab showed that the sialic acid binding property of

MAG is not sufficient to inhibit neurite outgrowth, and gangliosides become necessary only for the soluble form of MAG (Tang et al., 1997). However, sialic acid binding alone is not sufficient to induce inhibition of neurite outgrowth. Collectively, MAG my have two discrete functions: first, to hinder aberrant sprouting and generate structural axon-glia stability via interaction with gangliosides, and second, to strongly inhibit neurite outgrowth in interaction with a functional high-affinity receptor such as NgR1, NgR2 or PirB.

In contradiction to us, others have reported that sialylated glycans (gangliosides) are mediators of MAG inhibition of neurite outgrowth, and that MAG inhibition is a result of carbohydrate recognition (Vinson et al., 2001, Vyas et al., 2002). In 1999, Collins and Schnaar reported that treatment of nerve cell surface with neuraminidase, which converts MAG-binding gangliosides GD1a and GT1b to the non-binding gangliosides GM1, reversed MAG-mediated inhibition of neurite outgrowth (Collins et al., 1999). In 2001, Mary Vinson and Frank S. Walsh reported that recognition of sialylated glycans is essential for the inhibition of neurite outgrowth by MAG, and that MAG interacts with gangliosides GT1b to inhibit neurite outgrowth (Vinson et al., 2001). Similar to our findings they reported that a mutation of R118 residue in the first Ig-like domain reduces the potency of MAG inhibition, and this residue activity is a result of the carbohydrate recognition. They showed that MAG specifically binds both gangliosides GT1b and GD1a, expressed at the surface of MAG responsive neurons. In addition, they reported that antibody cross-linking of GT1b but not GD1a mimics the effect of MAG and activates RhoA. Multivalent IgM antibody cross-linking of cell surface GT1b mimics the effect of MAG. Pre-incubation with increasing concentration of alpha methyl sialic acid 3

sialyllactose to HN neurons before addition of MAG-Fc resulted in blocking of MAG-Fc inhibition by small sugars. Also, pre-incubation of HN neurons with purified GT1b and GD1a gangliosides blocks MAG-and MAG (R118A)-Fc inhibition of neurite outgrowth. Anti-GT1b antibodies inhibit neurite outgrowth from HN neurons, and this inhibition is dose-dependent. Also, pre-incubation of GT1b antibody with purified GT1b but not other purified gangliosides blocks inhibition of neurite outgrowth by anti-GT1b antibodies in the absence of MAG. They stained gangliosides on the surface of primary neurons anti-GT1b and -GD1a antibodies. Finally, a Rho kinase inhibitor (Y27632) blocks inhibition of neurite outgrowth by MAG or anti-GT1b antibody in both HN and CGN neurons. They concluded that MAG interaction with GT1b on the neuronal cell surface is a potential mechanism for inhibition of neurite outgrowth by MAG.

In apparent controversy to our previous result, they reported that MAG-R118A-Fc inhibits neurite outgrowth at higher concentration (Vinson et al., 2001). In 2001, Vayas and Schnaar claimed that brain gangliosides are functional ligands for myelin stability and the control of nerve regeneration. They reported that CGN neurons from rat are attenuated by neuraminidase treatment of the neurons. In addition, neurons from the GalNAcT deficient mice were not inhibited by MAG. Furthermore, highly multivalent clustering of GD1a or GT1b by using pre-complex anti-ganglioside antibodies mimics neurite outgrowth inhibition.

1.7 The Goal of My Work:

It is now well established that the presence of myelin-associated inhibitors in myelin are the major obstacle for regeneration in the CNS, and there is a window of opportunity to

interfere and neutralize this inhibitory signal before the formation and maturation of the glial scar. In order to understand growth inhibition, first we need to identify and characterize the neuronal receptors to growth inhibitory molecules present in myelin. The goal of this work is to further characterize the known receptors specific for MAG and to assess if there is still an unknown receptor for MAG.

MAG is a potent inhibitor of neurite outgrowth after injury, and like Nogo-66 and OMgp, it mediates its inhibitory effect by binding to NgR1-p75^{NTR}/Troy-Lingo-1 receptor complex. However, there is evidence that MAG can bind several other receptors to mediate its inhibitory effect. In 2005, Giger's group reported that in some type of neurons, such as cortical neuron, retinal ganglion cells (RGC), and postnatal (PND14) DRG neurons, MAG can also binds to NgR2 to inhibit neurite outgrowth, yet the mechanism and the partners for NgR2 is unknown (Venkatesh et al., 2005). Recently, PirB, a new receptor for Nogo-66, MAG and OMgp, has been identified, yet the mechanism by which it exerts its inhibitory effect is unknown. It was reported before that complex gangliosides such as GT1b and GD1a are the functional receptors for MAG. By using neurons GalNAcT deficient mice and by utilizing immobilized membrane from MAG-expressing CHO cells or detergent extracted MAG from myelin, they claim that gangliosides are important for inhibition by MAG.

The goal of my work is to investigate the known receptors for MAG and to examine their role in neurite outgrowth utilizing wild type full length (MAG), truncated (MAG d1-3) or mutated MAG (R118A) proteins in their soluble forms or when expressed by CHO cells. Also, by using neurons from the NgR1 deficient mice or GalNAcT deficient mice, I will assess the role of those known receptors and search for the possibility of existence of

more unknown receptors for MAG. Another goal of my work is to elucidate the nature of MAG binding to the sialic acid residues, and to assess if sialic acid residues my act to assist MAG encounter neurons, in order to allow it to exert its inhibitory activity of neurite outgrowth.

Chapter II: Materials and Methods

2.1 Cell Culture

2.2 CHO Cells Maintenance

Stably transfect MAG- or MAG (R118A)-expressing CHO cells or control CHO cells (Mukhopadhyay 1994) were maintained in Dulbecco's modified eagle Medium (DMEM; Gibco) supplemented with 10% dialyzed fetal bovine serum (dFBS; Gibco), 40mg/L l-proline (Sigma), 7.5mg/L Glycine (Sigma), 0.73mg/L Thymidine (Sigma) and 0.29g/L L-Glutamine (Gibco). Cells were incubated at 37°C in 7.3% CO_2 and used as monolyers in the membrane bound neurite outgrowth assay (NOG).

2.3 Monolayer Preparation

Permanox 8 well chamber glass slides (Lab-Tek) was coated with 20ug/ml poly-L-lysine (>300KD; Sigma) for 1 hr at room temperature, then Poly-L-lysine was removed and slides were coated with 20ug/ml fibronectin (Sigma) for 2hours at 37°C. Control CHO, MAG- or MAG (R118A)-expressing CHO were plated at 200,000 cells/ml. Slides were then incubated overnight at 37°C.

Permanox 8 well chamber slides (Lab-Tek) were coated with 20μg/ml poly-L-lysine (Sigma) for 30 minutes at room temperature. After incubation, poly-L-lysine was removed and the slides were treated with 20μg/ml fibronectin (Sigma) for at least 2 hours at 37ºC. Monolayers of control and MAG-expressing CHO cells (passage number 5-20) were plated in the individual chambers of an 8-well tissue culture slide (Lab-Tek) at the

following concentrations: control-CHO: 6 x 10^4 cells/well; MAG-CHO: 5.5 x 10^4 cells/well. The slides were then incubated overnight at 37º C and grown to confluence, prior to the plating of primary neurons on top of the CHO monolayers.

2.4 Isolation of Primary Neurons

Cerebellar Neurons (CGN):

Cerebellum were taken from each animal at the age from postnatal day 5 (P5) to day 7 and dissociated in 6ml of 0.025% trypsin (Life Technology) by pipetting. 10ml DMEM containing 10% FBS was then added to terminate trypsinization. Cells were centrifuged at 1000 rpm for 5 minutes at 4°C and re-suspended to a single-cell suspension in SATO (progesterone, 200nM; selenium, 224nM; insulin, 4µg/ml; BSA, 0.35mg/ml; L-thyroxin, 0.4µg/ml; tri-iodo-thyronine, 0.34µg/ml).

Dorsal Root Ganglion (DRG)

For DRG neurons, ganglia were removed from mice aging from P5 to P14 and incubated in 6ml of L15 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 trypsinization was stopped by adding 10ml of DMEM containing 10% FBS, centrifuged at 1000 rpm for 5 minutes and resuspended in SATO (DeBellard et al., 1996).

2.5 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 et al., 1997a). An 8-chamber slide (Lab-Tek) was first coated with 20μg/ml poly-L-lysine (Sigma) in sterile water for 1 hour at room temperature, and then washed twice with freshly made 0.1M bicarbonate buffer. Wells were then incubated with anti-human Fc antibody (Sigma) 15 μg/ml in 0.1M bicarbonate buffer for 2 hours at 37°C. After washing three times with DMEM, L1-Fc was immobilized onto anti-Fc by incubating 30 μg/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 25 μg/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

2.6 Soluble MAG-Fc Assay

An 8-chamber slide (Lab-Tek) was first coated with 100μg/ml poly-L-lysine (Sigma) in sterile water for 1 hour at room temperature. Cerebellar granular neurons were suspended in SATO medium with 2% FBS into a concentration of $5x10^4$ cells/well, and incubated with 20 μg/ml of MAG-Fc protein at 37°C for 18 hours. The fixation and Staining procedures were the same as neurite outgrowth on CHO cell monolayers.

2.7 MAG (d1-3)-Fc Clustering Assay

An 8-chamber slide (Lab-Tek) was first coated with 100μg/ml poly-L-lysine (Sigma) in sterile water for 1 hour at room temperature. Cerebellar granular neurons were suspended in SATO medium with 2% FBS into a concentration of $5x10^4$ cells/well, and incubated with 20 μg/ml of truncated MAG (d1-3)-Fc protein at room temperature for 1 hour. 5 μg/ml of anti human-Fc IgG antibody was add to cluster truncated MAG (d1-3)-Fc. Wells were incubated at 37°C for 18 hours. The fixation and Staining procedures were the same as neurite outgrowth on CHO cell monolayer

2.8 Solid-Phase Binding Assay With Immobilized Fc-Chimeras

An Immulon-3 96 well ELISA plate (Dynatech) was first coated with 15 ug/ml anti-Fc for 2 hr at 37^o C in 0.1M Sodium bicarbonate buffer. The plate was then coated with 20 ug of MAG-Fc, MAG (R118A)-Fc and MAG (d1-3)-Fc. Prior to the binding assay, Cerebellar neurons from Wild Type mice, NgR-1 KO mice or GalNAcT KO mice were isolated and labeled with fluorescent dye, 10 uM calcein AM (molecular probe) for 15 min at 37^oC, then washed and re-suspended in PBA buffer. The fluorescently labeled cerebellar cells ($2X10^5$) were added to each well and incubated at room temperature for 1 hour. The plate was washed 4 times under gravity with cold PBS contaning 0.25% BSA. Bound cells were measured by Fluor-Imager (Molecular Dynamics).

2.9 Genotyping

Tail Tipping and DNA Isolation:

Cut 0.4-0.6 cm length of tail. Isolate DNA from tails using Dneasy^{R} Blood and Tissue from Qiagen.

2.10 GalNAcT (GM2/GD2) Deficient Mice

The GalNAcT KO mice were generated by the Proia's group and are a gift from Dr. Walkley at Einstein University.

Primers

Forward (GM2KO-F): TACCAGGCCAACACAGCA

Reversed (GM2KO-R): CAGGTCCAGGGGCGTCTT

PCR reagents from Roche Extend Long Template PCR, System Cat # 1 681 842 are used to make 2 different master mixes reactions:

Mix 1

H_2O	16.9µl
3 Primer	0.3µl
5 Primer	0.3µl
DNTPs (2.5 mM)	7.0µl
Total	24.5µl per tube

Mix 1

H_2O	19.25µl
10X PCR Buffer	5.0µl

5 Primer 0.3µl

Taq 0.75µl

Total 25µl per tube

On ice, add 0.5µl from Easy DNA extraction in tubes to 24.5µl Mix#1. Add 25 µl Mix#2 and run PCR reaction ass follows:

1. 4^{O}C x 10 Sec
2. 94^{O}C x 2 Min
3. 94^{O}C x 20 Sec
4. 61^{O}C x 30 Sec
5. 68^{O}C x 2 Min
6. Repeat step 2-4 9 times
7. 94^{O}C x 20 Sec
8. 61^{O}C x 30 Sec
9. 68^{O}C x 2.20 Min
10. Repeat step 7-9 19 times
11. 4^{O}C infinity

Mix 18µl PCR product with 2 µl loading buffer

Run on a 1% agarose gel at 100 V for one hour. The wild type band is the upper band and the lower is the mutant band.

2.11 NgR1 Deficient Mice

Nogo Receptor PCR and Primers:

WT size	Mutant size
325	210

Primer1	NR3F1	tcggcacatcaatgactctcc
Primer2	NR3R3	tatgtacacacacctggtggc
Primer3	bpA2	TGGGCTCTATGGCTTCTGAG

PCR GENOTYPING PROTOCOL

1) Make sure tail is fully dissolved in TE (pH8.0).

2) Make ready the PCR machine. Run program as follows:

   1) 94^{O}C for 5 minutes

   2) 94^{O}C for 30 seconds

   3) 62^{O}C for 45 seconds

   4) 72^{O}C for 1 minute

   5) Repeat 2-4 for 39 times

   6) 72^{O}C for 5 minutes

   7) 4^{O}C infinity

3) Set up 15µl PCR reaction in PCR tubes

   -Add 1 µl of DNA sample to each tube

   -Make cocktails according to the ff. Recipe.

   -For each DNA samples, you need:

   1.5µl 10X green PCR buffer (containing MgC12)

0.3µl 10M primer 1

0.3ul 10µM primer 2

0.3ul 10µM primer 3 (if required)

0.3ul 10mM dNTP

11.15 µl autoclaved Q-H20

0.15µl (5 U/ µl) Taq polymerase

15µl TOTAL

4) Aliquot 14µl of cocktail to each tube

5) Close the lid and put the tubes in PCR machine. It takes about 2-2.5 hours.

2.12 Statistical Analysis

After fixation, neurons were stained for β-III-tubulin. The length of the longest neurite from 500-800 neurons was quantified using Metamorph imaging analysis software. Prism GraphPad software was used to perform one-way ANOVA, followed by multiple comparisons using the Turkey's test.

1.13 RT-PCR:

Cortical neurons (P1) neurons isolated from NgR1 deficient mice were plated in 6 well tissue culture plates coated with 100 µg/ml of PLL at a density of 500,000 cells/ml and incubated at 37^{O}C. Media was removed from plate and cells were lysed in lysis buffer from RNeasy RNA isolation kit (QIAGN). Total RNA was quantified and 2 mg of total RNA was used to generated poly A RNA using Micro Poly (A) Purist Kit according to

the manufacturer's protocol (Ambion). Purified poly (A) RNA was re-suspended in RNase/DNase-free water and used to synthesize cDNA with oligo dt and reverse transcriptional enzyme (Stratagene) at 37^{O}C for 1 hour. NgR2 CDNA was amplified using primers forward (F) 5$^{/}$ CTC GGT GAC AAC CGG CAC CT and reverse (R) 5$^{/}$. GCG CGC TGG AAC CAA GC Using the polymerase chain reaction (PCR) from (Stratagene). Amplification of GAPDH from the same sample was used as control and primers used were F: 5$^{/}$ ATG GTG AAG GTC GGT GTG AAC G 3$^{/}$, and R: 5$^{/}$ TGG TGA AGA CGC CAG TAG ACT C 3$^{/}$. PCR was preformed according to manufacture's protocol. DNA was analyzed in 1.5 % agarose gel and stained with ethidium bromide.

2.14 siRNA

NgR2 siRNA sequences for the sense strand:

Sense 5′(5′ Th) AAAUGGGCCCCGCAAAACdTdT3.

Antisense 3′ dtdtUUUACCCGGGGCGUUUUG 5′

The siRNA duplexes were generated with a thiol on the sense strand and HPLC purified (Dharmacon). Annealed siRNA duplexes were re-suspended in the RNAase-free water. An equimolar ratio of Penetratin-1 (q-Biogene) was added and the mixture was heated at 65^{O}C for 15 min and then at 37^{O}C for 1 hour. The coupled 300 ηM Penetratin- siRNA were then added to cultured cortical (P1) neurons and incubated for 48 hours at 37^{O}C. Neurons were then transferred onto monolayers of control-, MAG- and MAG (R118A)-expressing CHO cells for neurite outgrowth assay or analyzed for NgR2 expression by RT-PCR.

Chapter III: The Sialic Acid-Binding Property of MAG is not Required for MAG-Mediated Inhibition of Neurite Outgrowth

Introduction

Since identification of MAG as a potent inhibitor for CNS axonal regeneration (McKerracher et al., 1994, Mukhopadhyay et al., 1994), enormous efforts were directed at finding receptors for MAG. It is well established that NgR1 and recently PirB are functional receptors for MAG (Domeniconi et al., 2002, Liu et al., 2002, Atwal et al., 2008). In addition, MAG as a sialic acid binding protein interacts with complex gangliosides GT1b and GD1a (Vinson et al., 2001). However, the role of this interaction in mediating neurite inhibition remains controversial. Since, identification of MAG receptors is critical to develop therapeutic targets to promote regeneration, it is most important to clarify the role of gangliosides in MAG-induced inhibition of neurite outgrowth. Our data suggest that the sialic acid binding property of MAG is not sufficient to inhibit neurite outgrowth when MAG is expressed by CHO cells (Tang et al., 1997), and gangliosides became necessary only for the soluble form of MAG. However, sialic acid binding alone is not sufficient to induce inhibition of neurite outgrowth (Tang et al., 1997).

MAG is a member of the Siglec (sialic acid –binding immunoglobulin-like lectins) family of molecules. Siglecs are a family of animal lectins that bind to sialic acid containing glycans (Crocker et al., 2007). Each Siglec has multiple Ig-like polypeptide domains, including an N-terminal V-set Ig-like domain with sialic acid-binding site.

MAG (Siglec-4) prefers to bind the "NeuAcα 3 Gal β3 Gal-NAc " glycan structure. Because of these characteristics, MAG can only bind to gangliosides GD1a and GT1b (Kelm et al., 1994, Vinson et al., 1996, Collins et al., 1997, Crocker et al., 1998, Vinson et al., 2001).

MAG's extracellular domain consists of 5 Ig-like domains. We mapped the sialic acid binding site to Arg118 on the first Ig-like domain (Tang et al., 1997), and we mapped the inhibitory site to Ig-like domain-5 distinct from the sialic acid binding site (Cao et al., 2007). We generated CHO-expressing cells that are stably transfected to express either full length MAG, MAG mutated at Arg 118 (MAG R118A), truncated forms of MAG that contains the sialic acid binding site (MAG d1-3) or contains the inhibitory site (MAG d3-5). Also, we used soluble forms of MAG consisting of either full length MAG fused to Fc (MAG-Fc), or full length mutated MAG (MAGR118A)-Fc, or truncated form of MAG that contains the silaic acid binding site, but not the inhibitory site (MAG d1-3)-Fc (Fig 3.1).

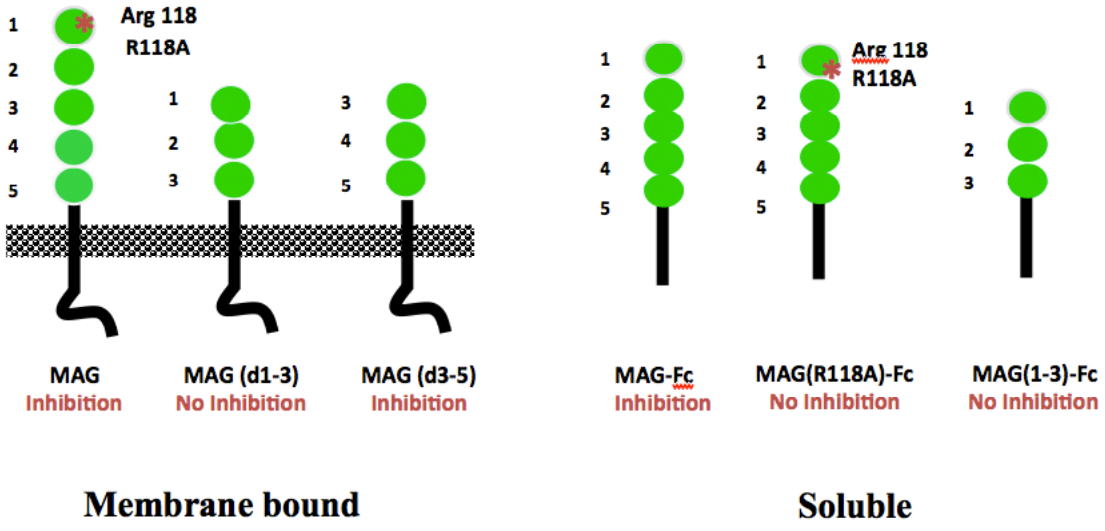


Fig (3.1) Membrane bound and soluble schematic forms of MAG.
Membrane Bound MAG: Full-length wild type MAG, and mutant MAG at the sialic acid site in the first Ig-like domain- expressing CHO Cells.
Soluble MAG: Full -length wild type MAG consists of the 5- extracellular domains of MAG, mutant MAG (R118A) which can no longer bind sialic acid, and (MAG d1-3) a truncated form of MAG consisting of the first 3 domains of MAG which can bind to sialic acid but does not have the inhibitory Ig-domain 5 required for neurite outgrowth inhibition.

Previously, we showed that MAG is a potent inhibitor of axonal regeneration (McKerracher et al., 1994, Mukhopadhyay et al., 1994). In addition, we mapped the sialic acid binding site Arg 118 on MAG to the first Ig-like domain (Tang et al., 1997). This amino acid is conserved in a number of Siglecs and is essential for sialic acid binding. Surprisingly, mutations of this amino acid to either Alanine (Ala) or Aspartate (Asp) did not affect MAG's ability to inhibit neurite outgrowth when stably transfected on CHO-cells. Therefore, it was concluded that the MAG sialic acid binding property is not required for MAG inhibition by MAG-expressing CHO-cells. However, when these mutants were expressed in a soluble form of MAG (R118A)-Fc or MAG (R118D)-Fc, inhibition of neurite outgrowth was abolished compared to wild type MAG-Fc. Therefore, the sialic acid binding property of MAG becomes necessary only when MAG is in its soluble form. Consistent with this finding, we also showed that a truncated form of MAG that contains the first three Ig-like domain of MAG called (MAG d1-3-Fc) which binds neurons in a sialic acid –dependent manner, did not inhibit neurite outgrowth. We concluded that there are two sites on MAG, the first site is the sialic acid binding site in Arg 118, which is distinct from the inhibitory site. In 2007, our lab showed that the MAG inhibition site is within Ig- domain 5 and is distinct from the sialic acid binding site on Ig-domain-1 (Cao et al., 2007). Based on those findings, we propose a two-site model for MAG inhibition (Fig 3.2).

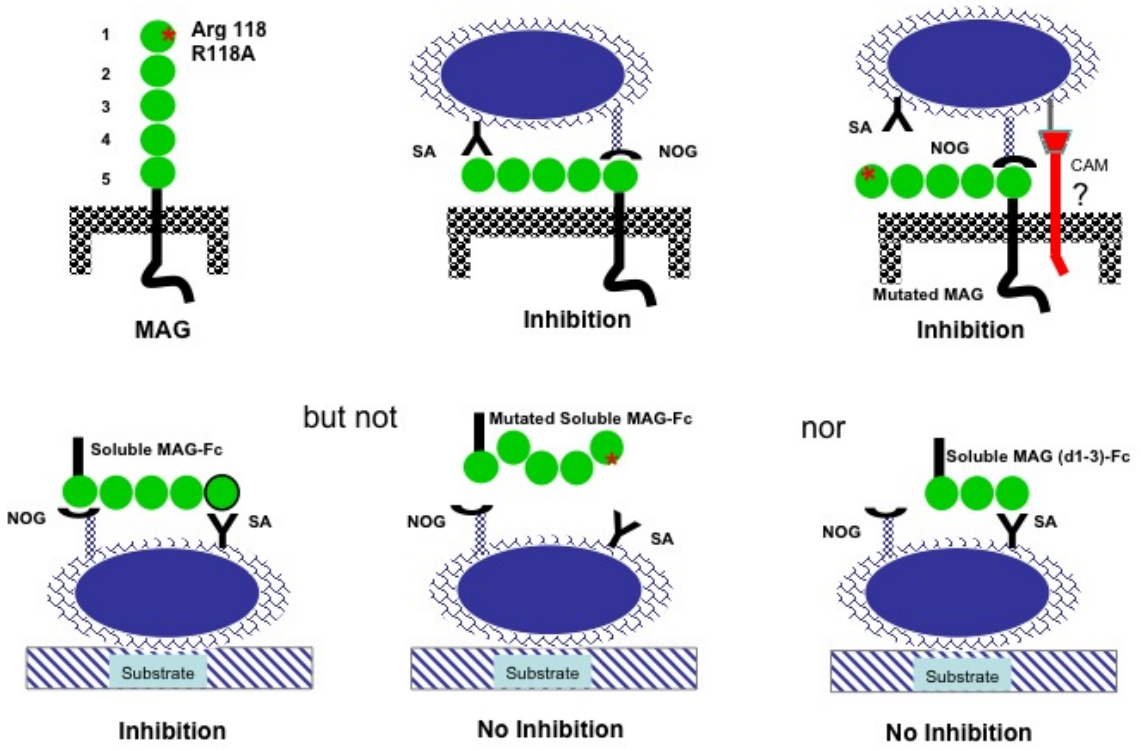


Fig (3.2): The Sialic Acid binding on MAG R118 is critical for inhibition of axonal regeneration by soluble MAG-Fc but not MAG expressed by CHO cells. When CHO cells express MAG, both sites engage the neuron and the neurite outgrowth are inhibited. When MAG is mutated at its sialic acid binding site and expressed in CHO cells, another cell adhesion molecule such as (N-CAM) present on the cell surface could engages the neuron along with the inhibitory site and neurite outgrowth is still inhibited. When soluble MAG-Fc is added to neurons, both the sialic acid and the inhibition sites engage the neurons and neurite outgrowth is inhibited. However, when MAG-Fc is mutated at the sialic acid binding site (R118A) and added to neurons, it cannot bind to neurons and consequently the inhibition site cannot engage and there is no inhibition of neurite outgrowth. In contrast, when MAG (d1-3)-Fc is added to neurons it binds through its sialic acid binding site but does not inhibit axonal growth because the inhibition site is absent (Tang et al., 1997).

In apparent contradiction to us, others have reported that gangliosides are functional receptors for MAG-mediated inhibition of neurite outgrowth. They reported that neurons from GalNAcT deficient mice are not inhibited in their immobilized-membrane extracted neurite outgrowth assay (Vyas et al., 2002). In addition, another group showed that clustering GT1b but not GD1a mimics MAG-mediated inhibition of neurite outgrowth in the absence of MAG (Vinson et al., 2001).

Taking advantage of genetic approaches and MAG chimerics proteins we wanted to determine the role of gangliosides in MAG-mediated inhibition of neurite outgrowth.

MAG binds to complex gangliosides GT1b and GD1a. Complex gangliosides synthesis requires the enzyme GalNAc Transferase. The GalNAcT deficient mice lack all complex gangliosides including GT1b and GD1a (Fig 1.10). We wanted to assess if neurons isolated from GalNAcT deficient mice are inhibited by MAG-or MAG (R118A)-expressing CHO cells in our neurite outgrowth assay. If gangliosides mediate MAG inhibition, MAG- or MAG (R118A) -expressing CHO cells should not inhibit neurons from GalNAcT deficient mice. However, based on our model (Fig 3.2) we predict that MAG- and MAG (R118A)-expressing CHO cells inhibit neurons from GalNAcT deficient mice similar to neurons from wild type. In addition, we established before that soluble MAG-Fc inhibits neurite outgrowth in a sialic acid -dependent manner; therefore we predict that neurons from GalNAcT deficient mice are not inhibited by soluble MAG-Fc or by soluble MAG (R118A)-Fc.

In addition, it was reported that clustering gangliosides GT1b but not GD1a mimics MAG inhibition of neurite outgrowth (Vinson et al., 2001) and it was also reported that MAG-Fc binding to gangliosides induces the association of the p75 ^{NTR} to lipids rafts and

signal transduction. Therefore, we predict that clustering truncated MAG (d1-3)-Fc will inhibit neurite outgrowth of neurons from wild type but not from GalNAcT mice.

Results:

3.1 Gangliosides are not necessary for neurite outgrowth inhibition by MAG-expressing CHO cells

To assess the role of gangliosides in inhibition of membrane MAG-expressing CHO cells and sialic acid mutated MAG (R118A)-expressing CHO cells, we isolated CGN and DRG neurons from GalNAcT deficient and wild type mice and preformed the neurite out growth assay on MAG-expressing CHO cells, MAG (R118A)-expressing CHO or control CHO cells. Fig (3.3) and (3.4) shows that CGN and DRG neurons from GalNAcT deficient mice are inhibited by MAG-expressing CHO cells similar to wild type neurons. In addition, mutated MAG (R118A) expressing CHO cells can also inhibit CGN neurons isolated from GalNAcT deficient mice Fig (3.5).

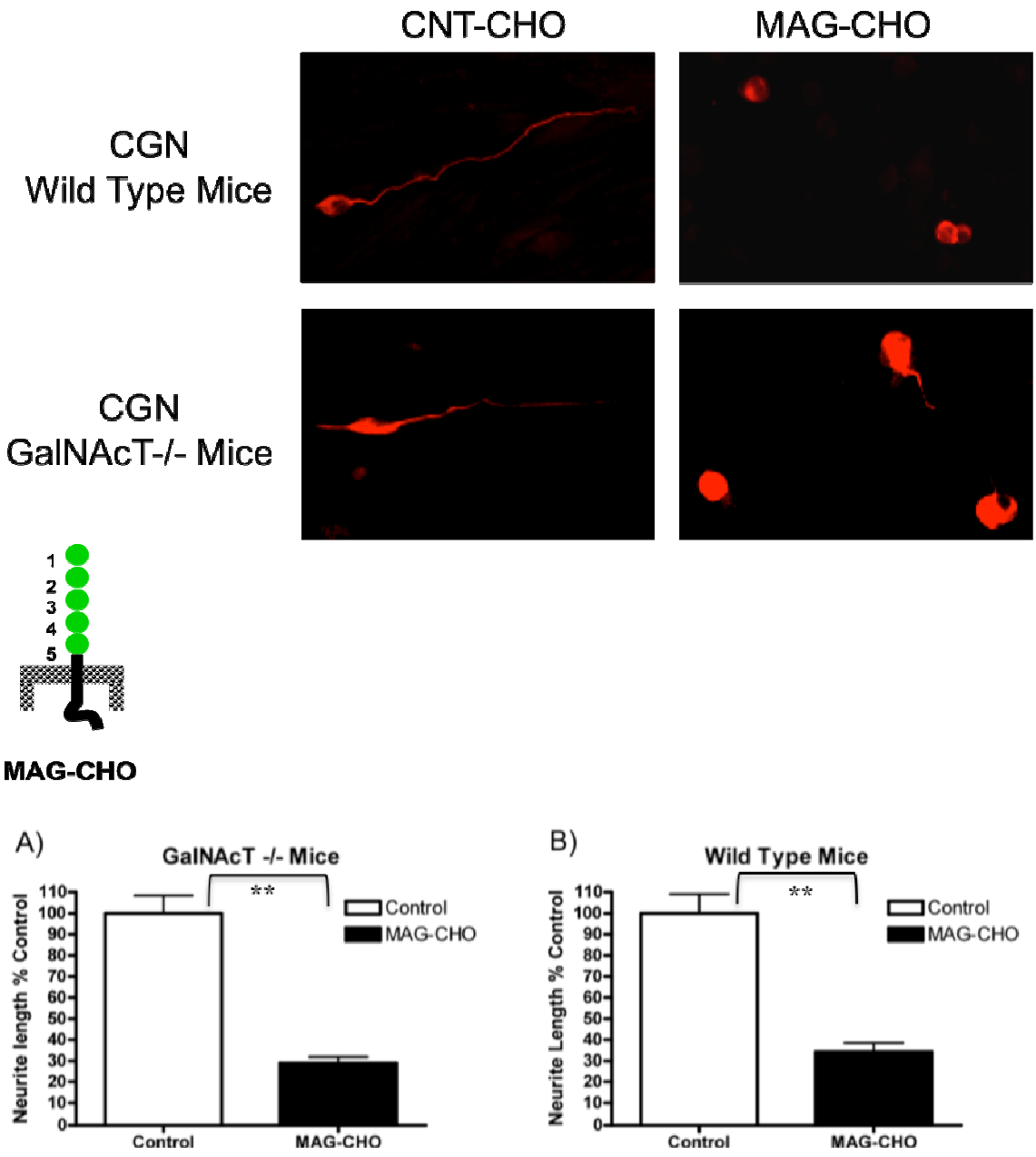
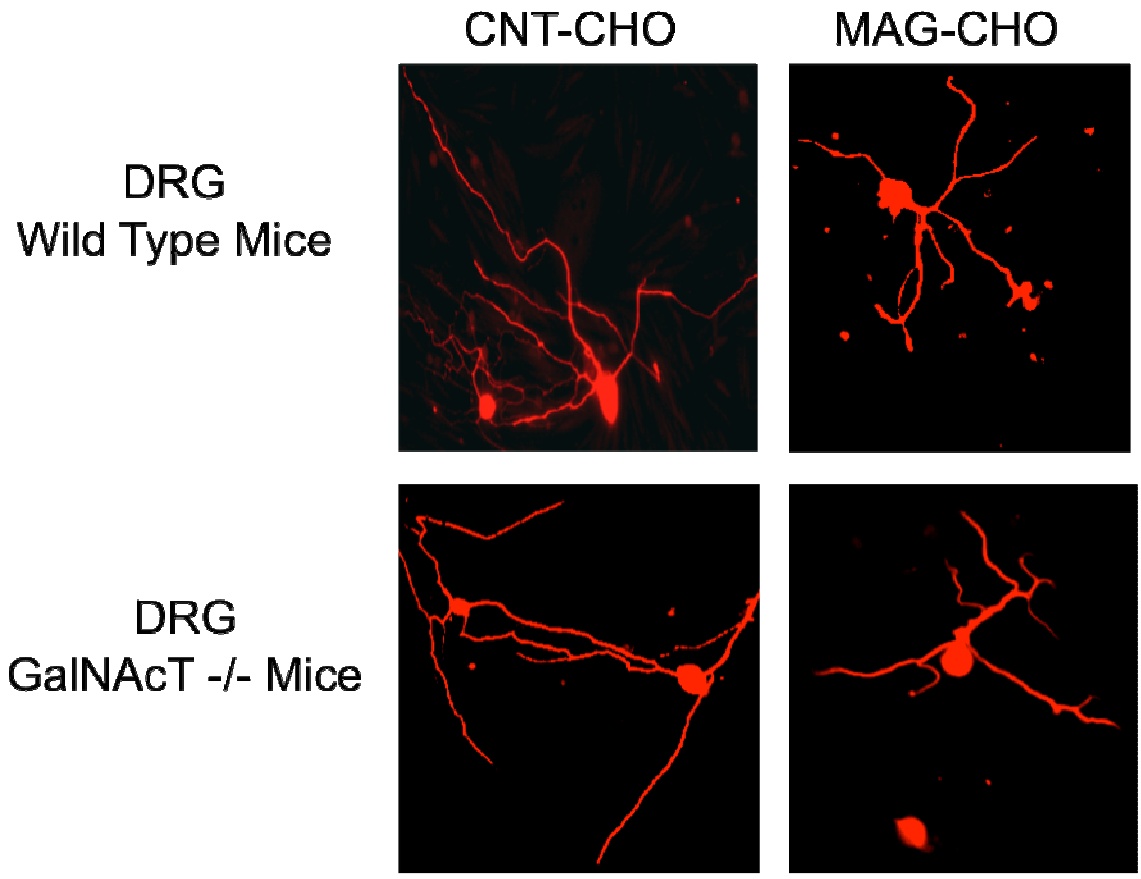
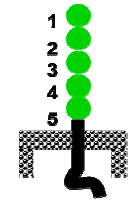


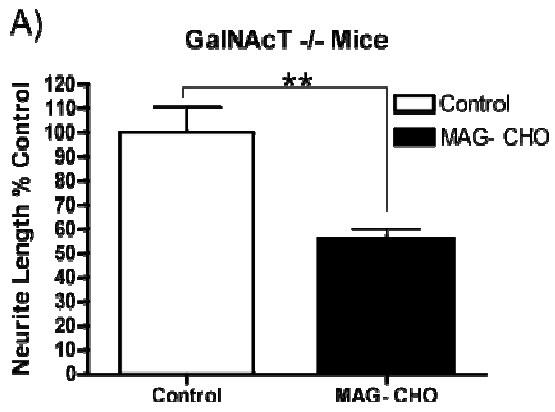
Fig (3.3): MAG-expressing CHO cells inhibit CGN neurons isolated from GalNAcT deficient and wild type mice: neurite outgrowth assay of CGN neurons from A) GalNAcT deficient or B) wild type mice are isolated and re-suspended to single neuronal culture before plating onto monolayers of MAG-expressing or control-CHO cells. Cells were incubated overnight, fixed, and stained for βIII tubulin. Results represents the percentage of the average length of the longest neurite over the control ±SEM. **P<0.01.

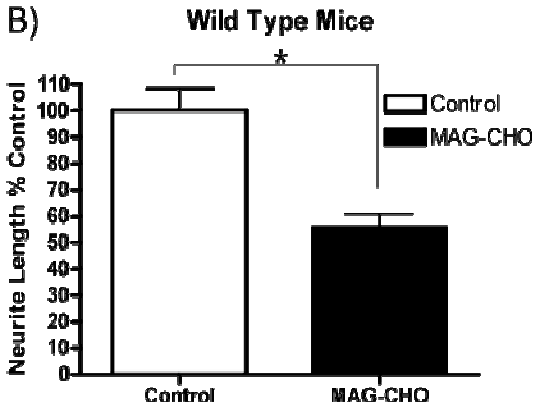
Fig (3.4): MAG-expressing CHO cells inhibit DRG neurons isolated from GalNAcT deficient or wild type mice: neurite outgrowth assay of DRG neurons from A) GalNAcT deficient or B) wild type mice are isolated and re-suspended to single neuronal culture before plating onto monolayers of MAG-expressing or control-CHO cells. Cells were incubated overnight, fixed, and stained for βIII tubulin. Results represents the percentage of the average length of the longest neurite over the control ±SEM. *P<0.05, **P<0.01.


CNT-CHO
MAG-CHO
DRG
Wild Type Mice
DRG
GalNAcT -/- Mice


1
2
3
4
5

MAG-CHO


A)
GalNAcT -/- Mice
**
Control
MAG- CHO
Neurite Length % Control
120
110
100
90
80
70
60
50
40
30
20
10
0
Control
MAG- CHO


B)
Wild Type Mice
*
Control
MAG-CHO
Neurite Length % Control
110
100
90
80
70
60
50
40
30
20
10
0
Control
MAG-CHO

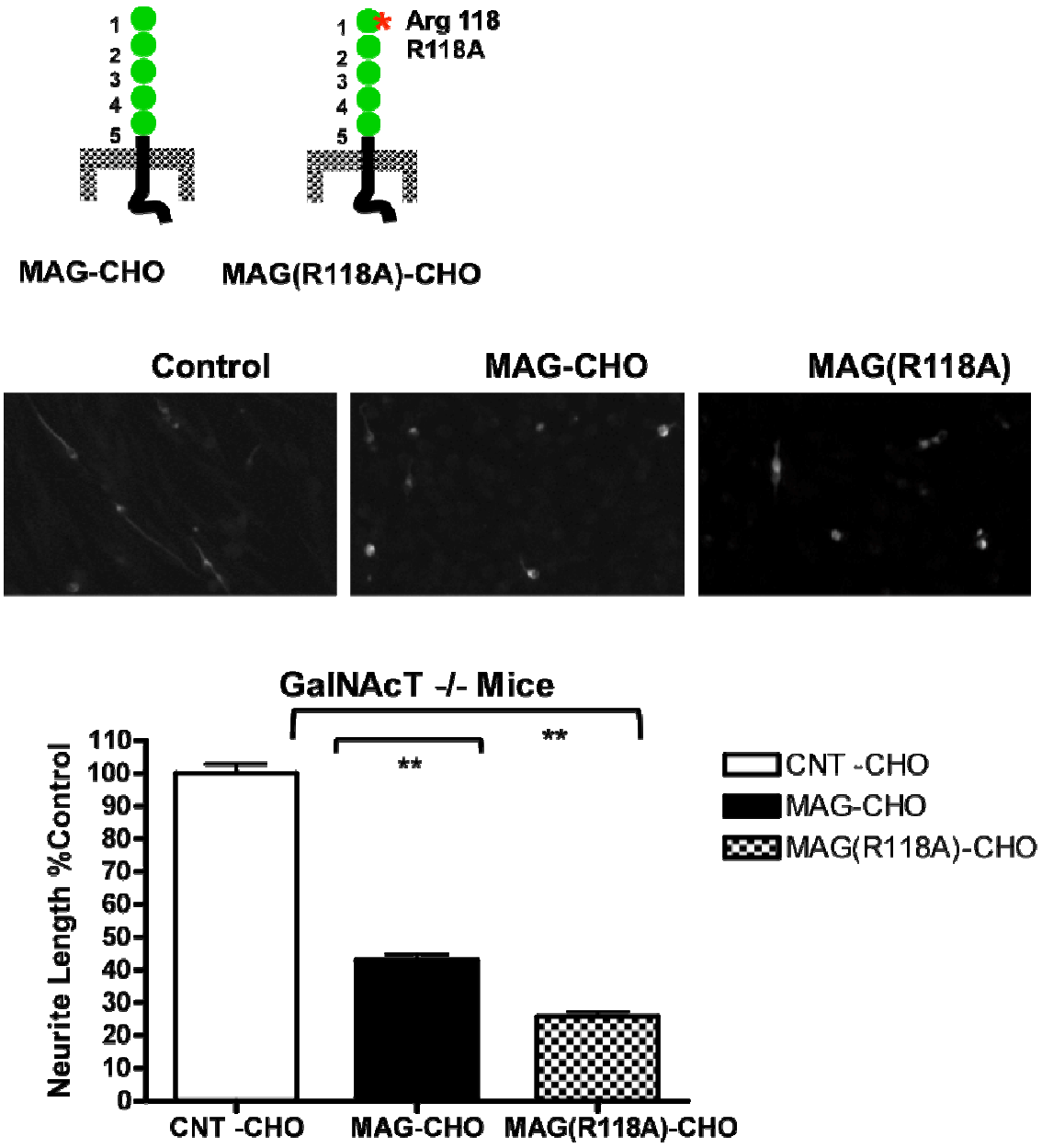


Fig (3.5): MAG (R118A)-expressing-CHO cells inhibits neurite outgrowth of CGN neurons isolated from GalNAcT deficient mice. Neurite outgrowth assay of CGN neurons from GalNAcT deficient or are isolated and re-suspended to single neuronal culture before plating onto monolayers of wild type MAG-, mutated MAG (R118A)- or control-expressing CHO cells. Cells were incubated overnight, fixed, and stained for βIII tubulin. Results represents the percentage of the average length of the longest neurite over the control ±SEM. **P<0.01.

3.2 Gangliosides are necessary for inhibition of neurite outgrowth by soluble MAG (MAG-Fc).

Previously, we reported that the extracellular domain of MAG is released from white matter, and it inhibits neurite outgrowth. Here, we wanted to investigate the role of gangliosides in inhibition of soluble MAG.

Cerebellar neurons from GalNAcT deficient and wild type mice were isolated and utilized in a soluble neurite outgrowth assay. Only neurons from wild type mice were inhibited (Fig 3.6). This result confirms our previous reports that soluble MAG-Fc requires sialic acid binding in order for the inhibitory site to engage. Moreover, it suggests that ganglioside potentiate inhibition by MAG-Fc.

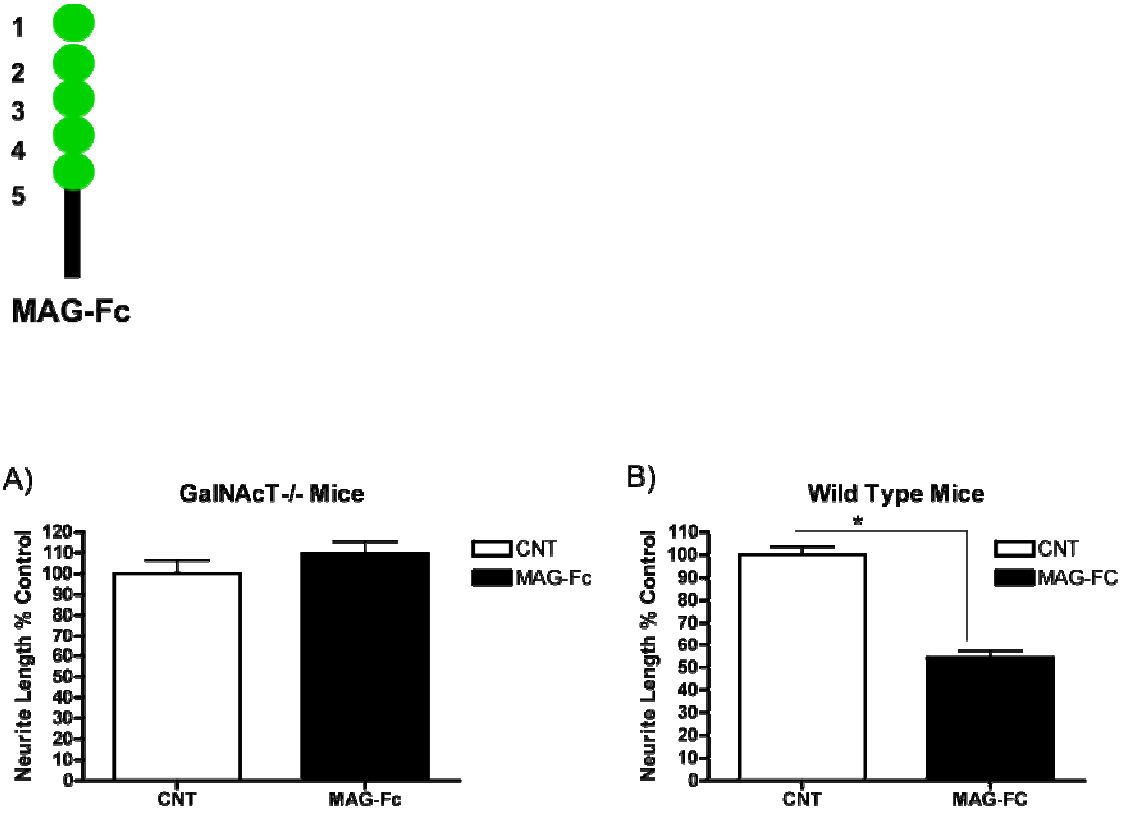


Fig (3.6): Cerebellar neurons from GalNAcT deficient mice are not inhibited by MAG-Fc. CGN neurons from GalNAcT deficient or wild type mice are isolated and plated on 8- well slides that were pre coated with 100μg/ml poly-lysine for 1 hour. 20 μg/ml MAG-Fc was added and incubated with the neurons. Slides were incubated for 18 hours and fixed and stained for βIII tubulin. Inhibition was observed only with wild type neurons, GalNAcT deficient neurons were not affected. Results represents the percentage of the average length of the longest neurite over the control ±SEM. *P<0.05.

3.3 Clustering Gangliosides by MAG (d1-3)-Fc Inhibits Neurite Outgrowth in Neurons from Wild Type, but not from GalNAcT deficient mice

Previously, the Yamashita group reported that binding of soluble MAG-Fc to gangliosides induces the association of p75NTR to lipid rafts. They showed that inhibition is $p75^{NTR}$-dependent and activates RhoA. They suggested that lipids rafts are sufficient to inhibit neurite outgrowth through RhoA. In addition Vinson et al reported that antibodies cross linking to GT1b but not GD1a mimic the effect of MAG and activate RhoA kinase. They concluded that MAG interaction with GT1b on the neuronal cell surface is a potential mechanism for inhibition of neurite outgrowth by MAG. On the other hand we reported that MAG (d1-3)-Fc binds neurons in a sialic acid-dependent manner, but does not inhibit neurite outgrowth.

Based on these reports, we wanted to examine if clustering MAG (d1-3) utilizing anti-Fc IgG antibody will inhibit neurite outgrowth of neurons isolated from wild type but not from GalNAcT deficient mice. Fig (3.8) illustrates that clustering MAG (d1-3)-Fc only inhibits neurons from wild type but not from GalNAcT deficient mice.

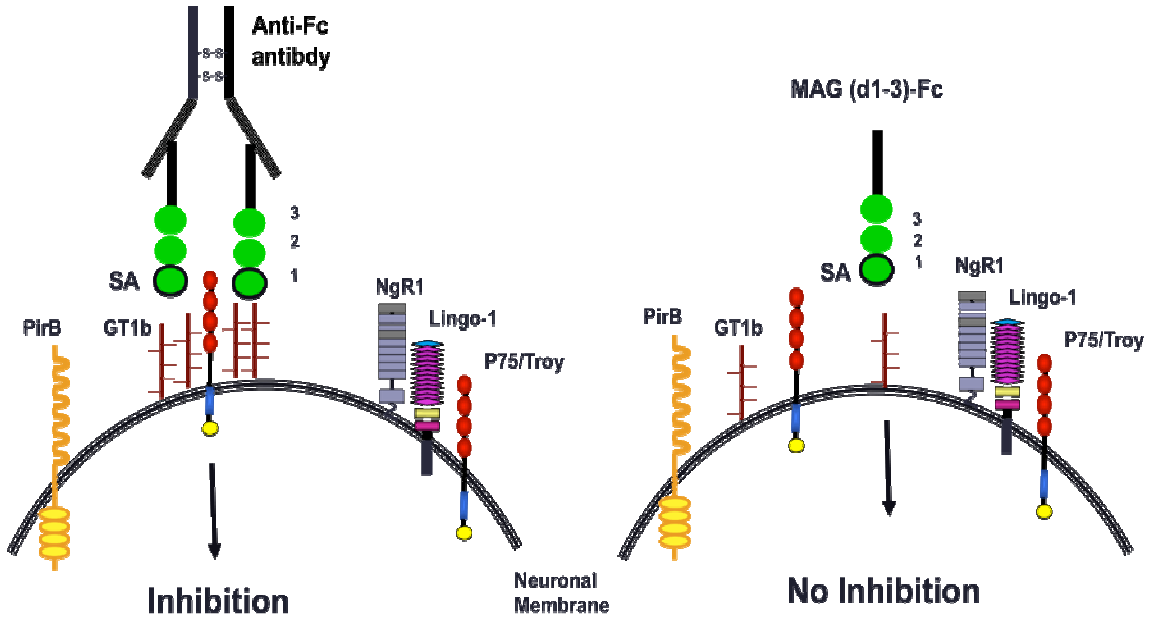


Fig (3.7): A Model of Neurite Inhibition by Clustering MAG (d1-3)-Fc. MAG binds GT1b and GD1a via its sialic acid binding site located in the first Ig-like domain. MAG (d1-3)-Fc binds neurons in sialic acid dependent manner but does not inhibit neurite outgrowth. Clustering MAG (d1-3)-Fc with anti-Fc antibody is sufficient to inhibit neurite outgrowth even in the absence of MAG's inhibitory site on Ig-like domain-5, by bringing sialic acid residues into close proximity of the p75 ^{NTR}.

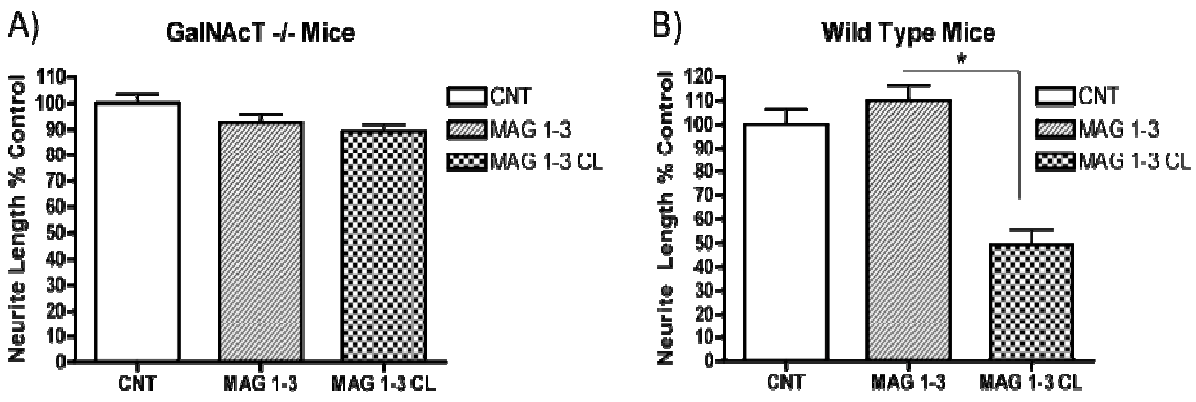


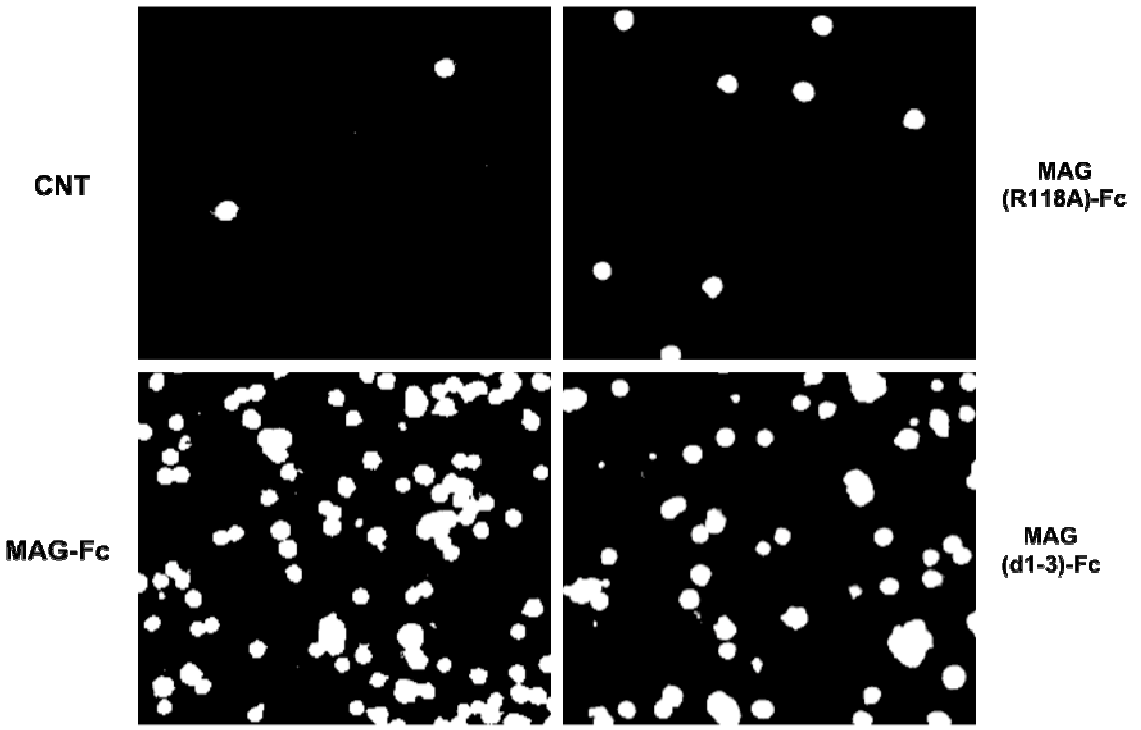
Fig (3.8): Clustering MAG (d1-3)-Fc inhibits neurite outgrowth of cerebellar neurons isolated from wild type but not from GalNAcT deficient mice. CGN neurons isolated from GalNAcT deficient or wild type mice are isolated, plated on 100μg/ml PLL, treated with 20μg MAG (d1-3)-Fc, and incubated at room temperature for 1 hour before adding anti-IgG antibody. Cells were incubated overnight, fixed, and stained for βIII tubulin. Results represents the percentage of the average length of the longest neurite over the control ±SEM. *P<0.05.

Next we wanted to assess if a truncated form of MAG consisting of the first 3 Ig-like domain (MAG d 1-3)-Fc, which binds to sialic acid only but does not inhibit neurite outgrowth, would cause neurite inhibition when clustered with anti-Fc IgG. As illustrated in the model Fig (3.7), neurons from GalNAcT deficient or from wild type mice were treated with MAG (d1-3)-Fc and then subsequently were clustered by anti human Ig- Fc. Neurons from wild type mice had shorter neurites when clustered with anti-human Ig –Fc compared to neurons from the GalNAcT deficient mice which lacks complex gangliosides (Fig 3.8). Therefore CGN neurons isolated from GalNAcT deficient mice does not express gangliosides GT1b or GD1a and the sialic acid binding site on MAG

(Arg 118) in the first Ig-domain is not engaged and therefore, MAG (d1-3)-Fc could not be clustered on CGN neurons isolated form GalNAcT deficient mice.

Next, we wanted to assess MAG-Fc binding to CGN neurons from GalNAcT deficient compared to wild type neurons. In our solid phase MAG-Fc binding assay, 20μg of MAG-Fc, MAG (R118A)-Fc and MAG (d1-3)-Fc were immobilized on an Immulon-3 96 well ELISA plate (Dynatech), which was first coated with 15 ug/ml anti-Fc for 2 hr at 37^{o} C in 0.1M Sodium bicarbonate buffer. Cerebellar neurons from wild type or GalNAcT deficient mice were isolated and labeled with fluorescent dye, 10uM calcein AM (molecular probe) for 15 min at 37^{o}C, then washed and re-suspended in PBA buffer. The fluorescently labeled cerebellar cells ($2X10^{5}$) were added to each well and incubated at room temperature for 1 hour. The plate was washed 4 times under gravity with cold PBS containing 0.25% BSA. Bound cells were measured by Fluor-Imager (Molecular Dynamics). CGN neurons from wild type mice binds MAG-Fc and MAG (d1-3)-Fc but not to MAG (R118A)-Fc. However, CGN neurons from GalNAcT deficient mice although it show similar binding to MAG-Fc, it results in week binding to MAG (d1-3)-Fc. This is excepted since GalNAcT deficient mice does not express gangliosides GT1b and GD1a, however, the full length MAG still have the inhibitory Ig-domain 5 which explains the binding shown with MAG-Fc but not MAG (d1-3)-Fc in CGN neurons from the GalNAcT deficient mice Fig (3.10).

A)



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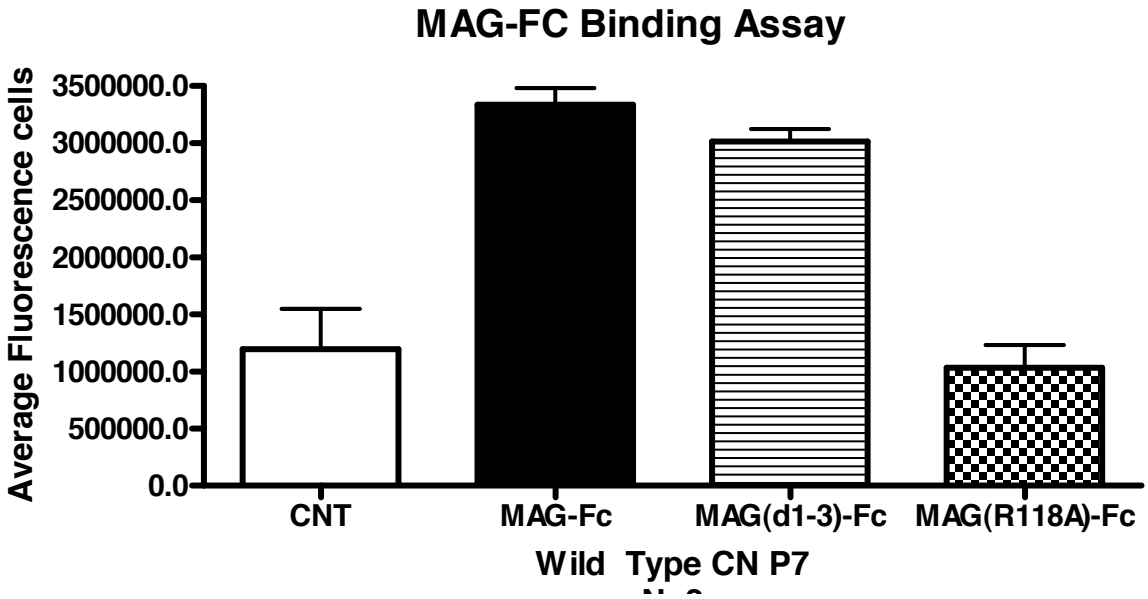
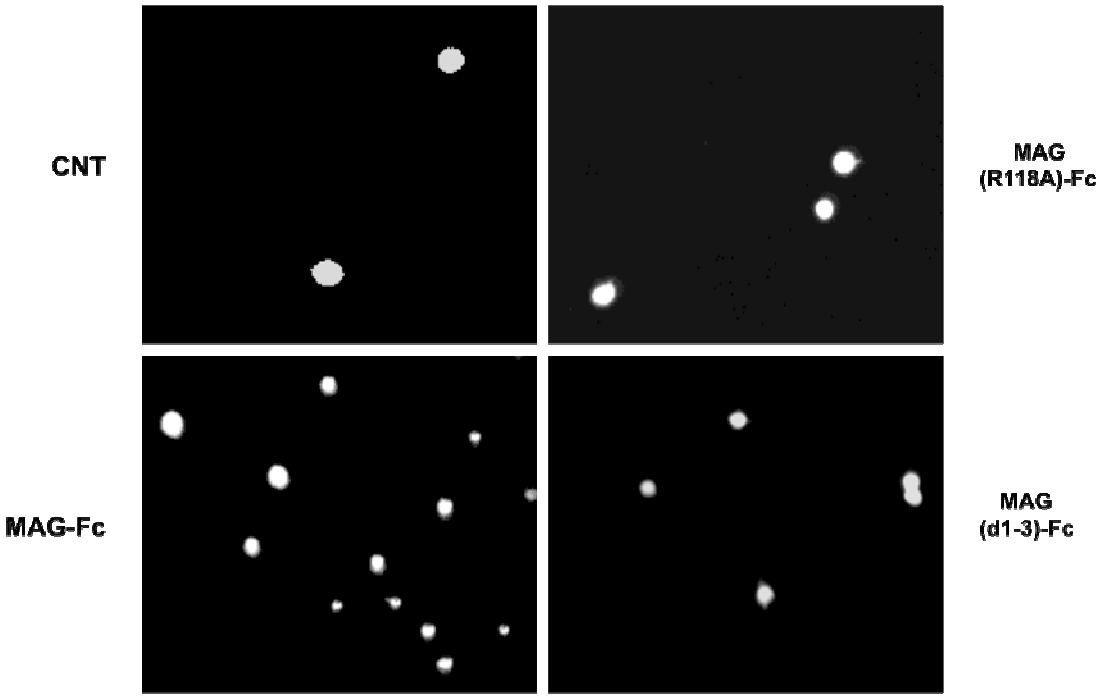


Fig (3.9): MAG-Fc Binding Assay. A) Pictures of fluorescently labeled wild type CGN neurons were plated on 96 well Elisa plate, which was pretreated with anti-Fc-IgG and then with MAG-Fc, MAG (d1-3)-Fc and MAG (R118A)-Fc. B) Quantification of average bound labeled cells per treatments.



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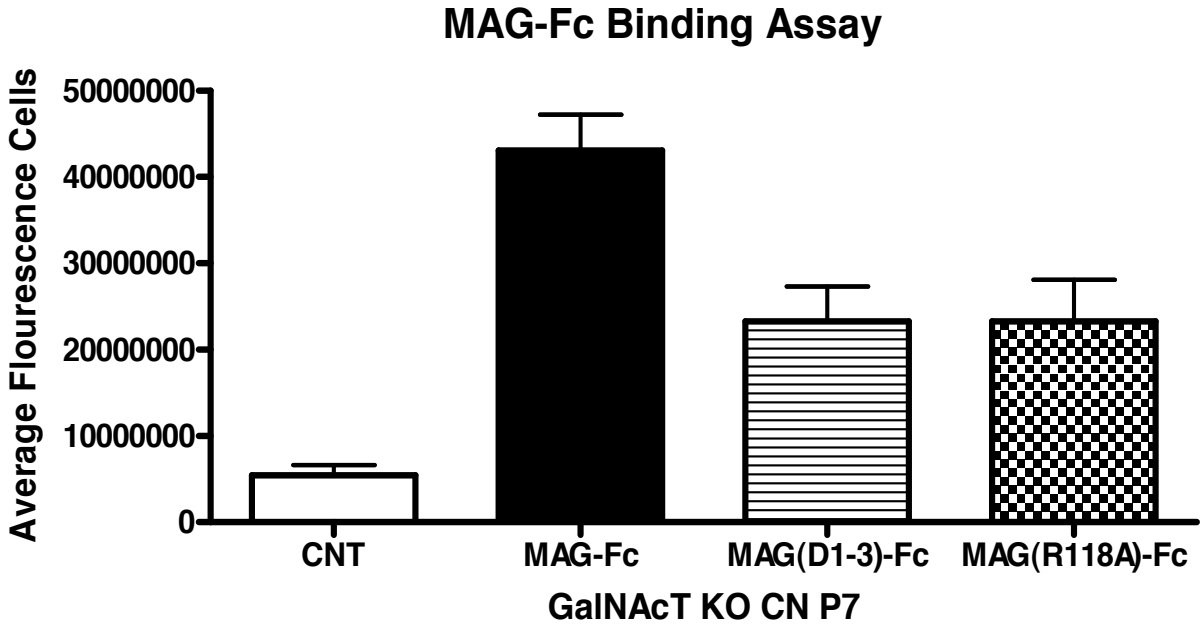


Fig (3.10) MAG-Fc binding assay CGN neurons isolated from GalNAcT deficient mice. Fluorescently labeled CGN neurons isolated from GalNAcT deficient mice were plated on 96 well Elisa plate, which was pretreated with anti-Fc-IgG first and then with MAG-Fc, MAG (d1-3)-Fc and MAG (R118A)-Fc. B) Quantification of average bound labeled cells per treatments.

Discussion:

Here, we report that gangliosides are not sufficient to inhibit neurite outgrowth (functional receptors for MAG). MAG-expressing CHO cells inhibited neurite outgrowth of cerebella granule, dorsal root ganglion, cortical and hippocampal neurons from GalNAcT mice similar to neurons from the wild type mice. Also, mutated MAG (R118A)-expressing CHO cells that can no longer bind to sialic acid still inhibit neurite outgrowth of neurons isolated from both GalNAcT deficient and wild type mice. Therefore, the sialic acid binding property of MAG is not sufficient to bring about inhibition of axonal growth. Also, it showed that gangliosides are not the functional receptor for MAG-mediated inhibition of neurite outgrowth.

MAG is a potent inhibitor of neurite outgrowth and a member of the Siglec family of sialic acid binding proteins. MAG preferentially binds to gangliosides GT1b and GD1a. Gangliosides are sialic acid containing glycosphingolpids and are abundant in the plasma membrane of neurons. Because of the sialic acid binding property of MAG and because of similarities between the MAG deficient and GalNAcT deficient mice, it was proposed that gangliosides are the functional receptors for MAG and mediators of its inhibitory effects. However, previous works from our lab showed that the sialic acid binding characteristic of MAG is not necessary for its inhibitory effect. We mapped the sialic acid-binding site to Arg 118 in the first Ig-domain of MAG (Tang et al., 1997), while the inhibitory site on MAG was mapped to Ig-domain-5 (Cao et al., 2007). Our hypothesis is that gangliosides are in the receptor complex of MAG, and that they potentiate MAG interaction with its receptors. The sialic acid binding of MAG in the first Ig-domain could bind first to GT1b or GD1a and bring the inhibitory site into close proximity to engage in

a proposed hairpin shape. We tested this hypothesis in the clustering studies we preformed. MAG (d1-3)-Fc binds to sialic acid but lacks the inhibitory domain and cannot inhibit neurite outgrowth. However, when we clustered MAG (d1-3)-Fc with antihuman IgG-Fc antibodies, neurons from the wild type, which have GT1b and GD1a gangliosides, were inhibited by MAG (d1-3)-Fc unlike neurons from GalNAcT deficient mice, which lack GT1b and GD1a. Moreover, neurite outgrowth is inhibited from GalNAcT deficient mice similar to wild type mice in MAG-expressing CHO cells, and in MAG (R118A)-expressing CHO cells. Collectively, these results show that gangliosides are not functional receptors for MAG, which contradicts a report that claims gangliosides are functional receptors for MAG.

This controversy could be explained by the different way that experiments were conducted. In our case, we use live CHO cells that are stably transfected with MAG. We plate those live cells as a monolayer overnight before plating neurons on top. The Schnaar group utilized our CHO cells to extract membranes and this changes the properties of MAG to a rigid form similar to soluble MAG-Fc. Also, other adhesion molecules such as N-CAM, which are normally present in live cells, are no longer present in their assay. Moreover, they use detergent extracted myelin, which also changes its properties to a more rigid form (Vyas et al., 2002), while we use non-detergent purified myelin, which is more similar to the physiological state of myelin debris after injury.

In addition, results from MAG-Fc soluble assay, confirms that sialic acid is needed for MAG-Fc inhibition of neurite outgrowth, since CGN neurons from GalNAcT mice were not inhibited by MAG-Fc unlike wild type CGN neurons.

In the soluble form of MAG, the sialic acid binding site in the first Ig-domain is necessary for MAG's inhibitory effect. When we mutated this site from Arg to Ala or Asp, inhibition of neurite outgrowth was affected. However, sialic acid alone is not sufficient to inhibit neurite outgrowth, because a truncated soluble form of MAG consisting of the first 3 Ig-like domains MAG (d1-3)-Fc does not inhibit neurite outgrowth. Moreover, a mutated soluble form of MAG that consists of the full length MAG that is mutated at Arg118 to Ala118 can no longer bind to sialic acid, but retains its inhibitory domain. This form of MAG (R118A)-Fc did not inhibit neurons from GalNAcT nor from wild type mice. This result could be explained as follows; when MAG is expressed at the surface of CHO cells other adhesion molecules such as N-CAM are present in the plasma membrane of those cells, and therefore gangliosides are not needed to engage the inhibitory site. However, when MAG is in its soluble form or absorbed in an immobilized membrane, interaction with sialic acid bearing gangliosides is needed in order for the inhibitory site to engage.

The previous results confirmed the existence of two functional sites on MAG, the sialic acid recognition site, and a neurite outgrowth inhibition site. The sialic acid recognition site are common to Siglecs family, each family member have a distinct binding preference for specific sialic acid and also for specific glycoconjugates linkages. This sialic acid binding specificity may be important for Siglecs proteins in recognition and communication with other target cells. For example 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 neurons in sialic acid dependent manner similar to

MAG, but does not inhibit neurite outgrowth (Tang et al., 1997, Shen et al., 1998). This suggest that sialic acid recognition site on Siglecs proteins may only function in a general way for binding of proteins to their target cells, whereas the biological effects of such interactions are carried out by other parts of the protein.

MAG, together with Nogo-A and OMgp, interact with the same receptor complex NgR1-p75/Troy-Lingo-1 (Domeniconi et al., 2002, Liu et al., 2002, Wang et al., 2002a, Wang et al., 2002b). Recently, NgR2, another member of the NgR family, has been shown to bind MAG, but not Nogo-A or OMgp (Venkatesh et al., 2005). This high affinity binding of MAG to NgR2 was sialic acid -dependent.

Others have also reported that sialylated glycans are mediators of MAG inhibition of neurite outgrowth and that MAG inhibition is a result of carbohydrate recognition (Vinson et al., 2001, Vyas et al., 2002). MAG specifically binds gangliosides GT1b and GD1a, which are both expressed on the surface of MAG-responsive neurons. It is also known that multivalent IgM antibody cross-linking of cell surface GT1b mimics the effect of MAG (Vinson et al., 2001). However, other studies indicated that removal of sialic acid residues by sialidase treatment does not affect MAG's inhibitory activity since the addition to outgrowth assays of IgM Fab fragments generated from either anti-GT1b or anti GD1a antibodies mimics MAG inhibitory activity, it is likely that this interaction is not specific for the MAG pathway. Current data supports a model in which the recognition of sialylated glycans, while not essential for inhibition of neurite outgrowth by MAG, may result in a weak, transient activation of the inhibitory pathway, independent of the NgR1 –dependent effects (or PirB). Furthermore, *in vivo* the effects of gangliosides–mediated binding of MAG may serve to potentiate the inhibitory effects of

the NgR1/PirB-mediated pathway. One possible interpretation of these events is that MAG has two discrete functions: first, to hinder aberrant sprouting and generate structural axon-glial stability through interaction with gangliosides, and second, to strongly inhibit outgrowth through interaction with functional high affinity receptors such as NgR1 or PirB.

Chapter V: The Existence of Other Unknown Receptors for MAG Beside NgR1, NgR2 and PirB

Introduction

Immediately after CNS injury and before the formation of glial scar, myelin-associated inhibitors are the major obstacles for axonal regeneration. Therefore, it is important to identify and characterize myelin-associated inhibitors and their receptors in order to successfully develop therapeutic strategy to block the inhibitory signaling initiated by these inhibitors. MAG, together with Nogo-66 and OMgp are the major myelin associated inhibitors and despite the difference in homology, they bind the same receptors NgR1-p75^{NTR}/Troy-Lingo1 and PirB. However, MAG is a sialic acid binding protein, and because of this property, MAG can also bind to NgR2 and gangliosides GT1b and GD1a. In the previous chapter, we examined MAG's interaction with gangliosides. Here, we will further characterize MAG-mediated inhibition though its interaction with NgR1, NgR2, PirB receptors, and to investigate if blocking all these receptors will transmit MAG- induced inhibition of neurite outgrowth. The persistence of the inhibition effect of MAG would strongly suggest the existence of yet another unidentified receptor(s).

In 2001, the Strittmatter group used a soluble form of Nogo-66 to screen an expression library for proteins that bind Nogo-66 with high affinity. They cloned a GPI-linked binding partner for Nogo-66, which they called Nogo receptor (NgR1) (Fournier et al., 2001). They demonstrated that direct interaction between Nogo-66 and NgR1 is required to induce growth cone collapse, and neurons that are normally unresponsive to Nogo-66 became responsive when they were transfected with an NgR1-expressing vector (Fournier et al., 2001). When a reverse strategy was applied and a soluble form of NgR1 consisting of the entire protein was used to screen an expression library for binding

proteins, MAG was cloned as a ligand for NgR1 receptor (Liu et al., 2002). At the same time, we also identified NgR1 as the receptor for MAG (Domeniconi et al., 2002). Both studies demonstrated that NgR1 is required for MAG-mediated inhibition of neurite outgrowth. A soluble form of MAG co-precipitates NgR1 from primary neurons, and both soluble NgR1 and NgR1 antibody block MAG's inhibition of neurite outgrowth. In addition, a dominant negative form of NgR1 blocks MAG's inhibitory effects. Importantly, both studies showed that the interaction of MAG with NgR1 is sialic acid-independent (Domeniconi et al., 2002, Liu et al., 2002). Around the same time, He's group reported that NgR1 is the receptor for OMgp; the third identified myelin-associated inhibitor of axonal regeneration. They reported that OMgp and NgR1 co-precipitated, and that a soluble form of NgR1 blocks growth cone collapse by OMgp. Also, a dominant-negative mutant NgR1 blocks the inhibition of neurite outgrowth by OMgp (Wang et al., 2002b).

NgR1 is expressed in a variety of brain regions, including the hippocampus, dorsal thalamus, cerebellum granule cell layer and the olfactory bulb. NgR1 is also expressed by pyramidal neurons that project axons into the CST.

Two different groups, the Strittmatter and Tessier-Lavigne's group, independently generated NgR1 deficient mice (Kim et al., 2004, Zheng et al., 2005). The NgR1 gene has two exons, with the second exon being the primary coding exon that is separated from the first exon by 23 KB. In the current study, we used NgR1 deficient mice from the Tessier-Lavigne group at GenenTech. Figure (4.1) shows the targeting strategy of generating NgR1 deficient mice. A targeting vector was utilized to replace most of the coding sequence in exon 2 with an *IRES-Tau-LacZ* reporter gene. Any mutant transcript,

if translated would encode only the first 19 amino acid residues of the NgR1 protein. The targeted allele was obtained in AB2.2 ES cells derived from 129S7 mouse strain. This mutation in 129S7 mouse was bred to C57BL/6 mice. Homozygous mutants were obtained and were viable, fertile, and morphologically indistinguishable from their wild type littermates.

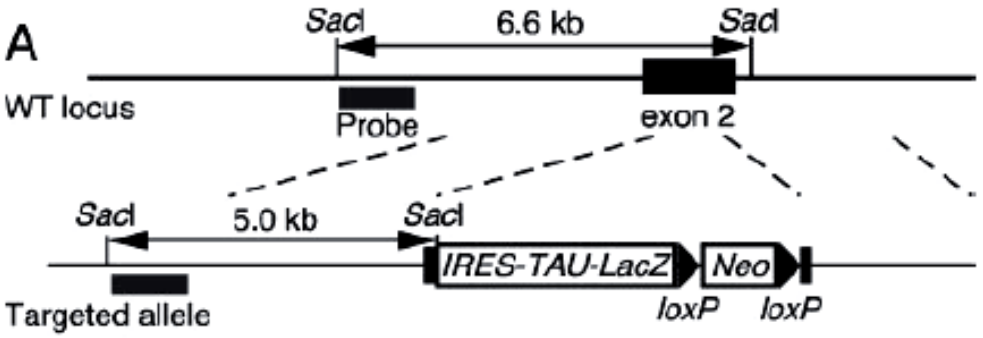


Fig (4.1): Generation of NgR1 Deficient Mice -Targeting Strategy. The targeting vector; *IRES* (internal ribosomal entry site); *LacZ* (β-gal gene); *Neo* (neomycin resistance gene). The black bars indicate exons (modified from Zheng, B et al; 2005).

It was reported that genetic deletion of NgR1 did not relieve the chronic inhibition of neurite outgrowth by myelin inhibitors *in vitro* (Chivatakarn et al., 2007), and did not enhance regeneration of CST axons after dorsal hemisection *in vivo* (Zheng et al., 2005), although some regeneration of raphespinal and rubrospinal tracts after spinal cord injury were reported (Kim et al., 2004). These data suggest that NgR1 is important for mediating some of the inhibitory activity of myelin inhibitors, but also other binding receptor(s) for these inhibitors remains to be identified.

In 2005, the Giger's group identified NgR2 as a specific functional receptor for MAG and it was shown that MAG's binding to NgR2 is sialic acid-dependent. However, the mechanism by which NgR2 mediates MAG inhibitory effect is still unknown, although it appears that NgR2 function is independent of $p75^{NTR}$, Troy or Lingo-1.

Recently, PirB was identified as a functional receptor for myelin inhibitors of axonal regeneration (Atwal et al., 2008). The PirB receptor was identified by the expression-cloning screen of a library of human cDNA. Nogo-66 binds both NgR1 and the human leukocyte immunoglobulin (Ig)-like receptor B2 (LILRB2). LILRB2 is part of B type subfamily of LILR receptors, which in humans consist of five highly homologous family members (Martin et al., 2002). In mice, there is only one single ortholog called paired immunoglobulin-like receptor B (PirB) (Kubagawa et al., 1997). PirB contains six (Ig)-like repeats apposed to four (Ig)-like repeats in LILRB2, and shares with it a 50% homology. Blocking PirB with a soluble ectodomain of PirB (PirB-His) or with an antibody specific to PirB (anti-PirB-1 antibody) reverses inhibition of P7 CGN on both Ap-Nogo-66 and myelin. Anti PirB-1 antibody was directed against the extracellular domain of PirB and specifically interferes with Ap-Nogo-66, MAG and myelin binding to PirB. In addition, the PirBTM mouse was generated. This mice line carries a loss of function allele in which four exons encoding the transmembrane domain and part of the PirB intracellular domain has been removed (Syken et al., 2006). Both CGN and DRG neurons isolated from the PirBTM mice were utilized to further confirm that PirB is a functional receptor mediating inhibition of neurite outgrowth. However, loss of PirB activity does not fully rescue growth in the neurite outgrowth assay, which suggests that another unknown receptor(s) may exist to mediate neurite inhibition of myelin inhibitors.

In addition, it is possible that PirB and NgR1 function together to mediate inhibition of neurite outgrowth, which necessitates the need for PirB/NgR1 double knockout mice.

First, to assess the real contribution of NgR1 in MAG-mediated inhibition of neurite outgrowth, we utilized NgR1 deficient mice on our neurite outgrowth assay. We wanted to determine if soluble MAG-Fc inhibits neurite outgrowth in CGN neurons isolated from NgR1 deficient mice. Also, we want to determine if MAG- or mutated MAG (R118A)-expressing CHO cells inhibits neurite outgrowth of neurons isolated from NgR1 deficient mice.

Secondly, to assess if NgR2 plays a role in MAG-mediated inhibition of neurite outgrowth in the absence of NgR1, we preformed neurite outgrowth assay from NgR1 deficient mice on mutated MAG (R118A)-expressing CHO, which cannot bind to sialic acid and hence cannot bind to NgR2 receptor (Venkatesh et al., 2005). In addition, to assess if blocking both receptors will abolish MAG inhibition we knocked-down NgR2 receptors in neurons isolated from NgR1 deficient mice.

Finally, to assess the role of PirB receptor and to investigate if blocking all three receptors NgR1, NgR2 and PirB is sufficient to overcome MAG-mediated inhibition. We utilized neurons the NgR1 deficient mice on MAG-and mutated MAG (R118A)-expressing CHO cells to exclude effects of NgR1 and NgR2-sialic acid-dependent signaling. We performed neurite outgrowth assay in the presence and absence of anti-PirB-1 antibody to address the above questions, and to determine the existence of other receptors beside NgR1, NgR2 and PirB that mediates MAG Inhibitory activity.

Results

4.1 Soluble MAG-Fc inhibits neurons from NgR1 Deficient Mice:

First we assessed if CGN neurons from NgR1 deficient mice were inhibited by MAG-Fc in our soluble neurite outgrowth assay. Our result demonstrates that neurite length of postnatal day 7 (P7) CGN neurons from NgR1 deficient mice growing in the presence of MAG-Fc is 50% shorter compared to poly L-lysine control (Fig 4.2).

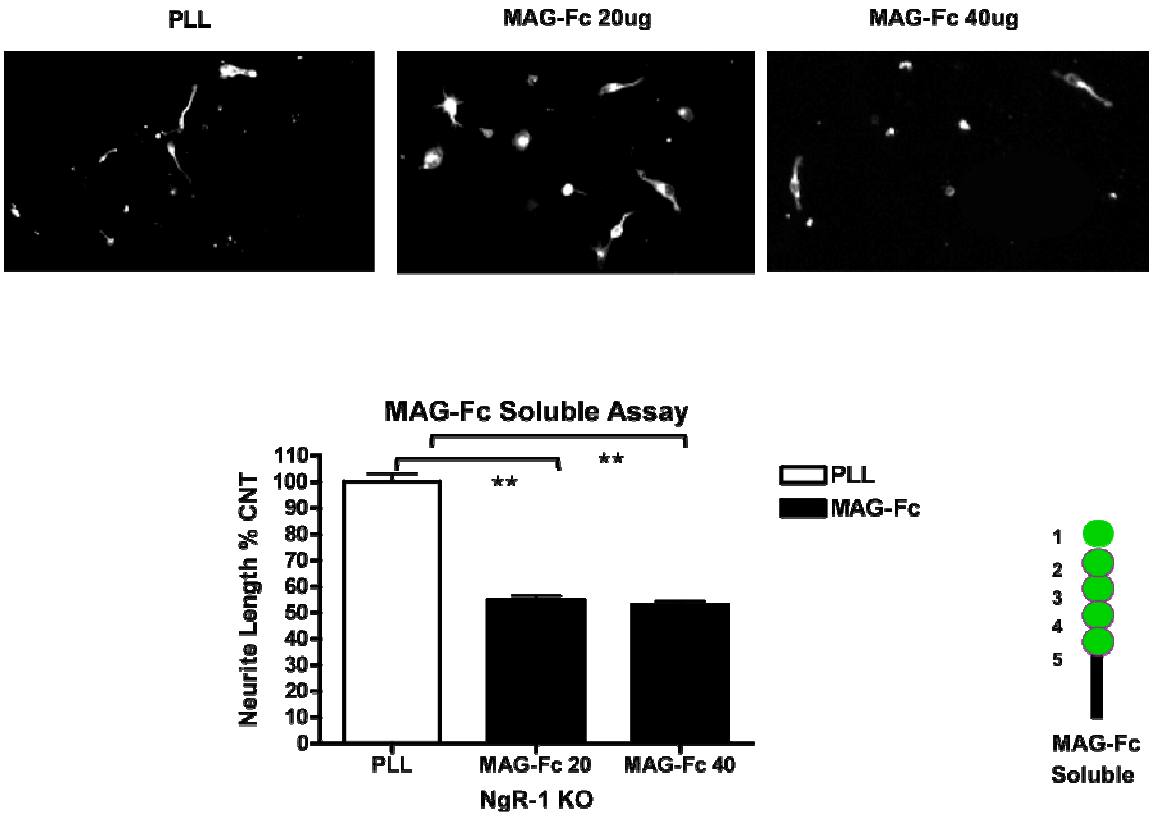


Fig (4.2): The effect of Soluble MAG-Fc on neurite outgrowth from NgR deficient CGN neurons. P7 CGN neurons from NgR1 deficient mice were dissociated and cultured for 18 hours on poly L-lysine (PLL) in the presence of 20 μg/ml or 40μg/ml MAG-Fc. Cells were fixed, and stained for βIII tubulin. Results represents the percentage of the average length of the longest neurite over the control ±SEM. **P<0.001. Neurons are inhibited 50% by MAG-Fc compared to PLL control.

4.2 Blocking NgR1 and PirB receptors is not sufficient to reverse inhibition of CGN neuron from wild type and NgR1 deficient mice growing on MAG- and MAG (R118A)-expressing CHO cells.

Next, we asked if MAG- and MAG (R118A) -expressing CHO cells inhibit CGN neurons from NgR1 deficient mice and if this inhibition is reversed by anti-PirB-1 antibody. CGN neurons from wild type or NgR1 deficient mice were isolated, dissociated and plated on top of control-, MAG- or MAG (R118A)-expressing CHO cells monolayers. Co-culture slides were incubated overnight at $37^{O}C$, fixed and stained for neuronal marker βIII tubulin. Both MAG- and mutated MAG (R118A)-expressing cells inhibits neurons from NgR1 deficient mice (Fig4.3), as well as CGN from wild type mice (Fig 4.4) even in the presences of anti-PirB-1 (PB) antibody which was shown before to effectively block PirB receptor (Atwal et al., 2008). However, treatment of CGN neurons from NgR1 deficient or wild type mice with anti-PirB-1 antibody induces moderate reversal of MAG mediated inhibition than the absence of NgR1 alone. However, this reversal is not obvious with DRG neurons from NgR1 deficient mice.

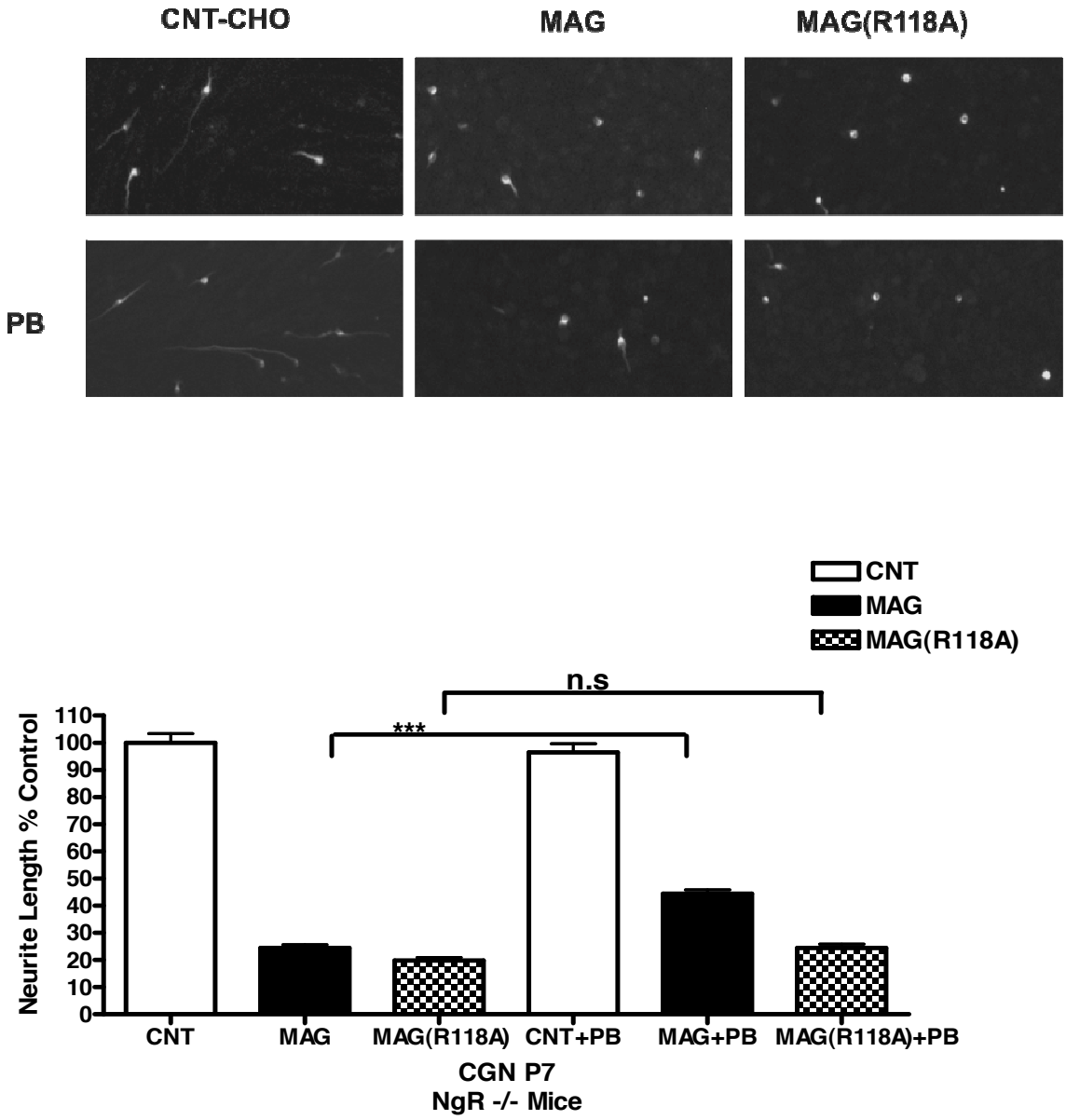


Fig (4.3): MAG- AND MAG-R118A-EXPRESSING CHO cells inhibit CGN neurons from NgR1 deficient mice in the presence and absence of anti-PirB-1 antibody (PB). Results represents the percentage of the average length of the longest neurite over the control ±SEM. ***P<0.0001.

Fig (4.4): Blocking PirB in wild type CGN neurons is not sufficient to reverse MAG and MAGR118A inhibition of neurite outgrowth. MAG- MAG (R18A)- expressing CHO cell in the presence of anti-PirB-1 antibody (PB), inhibits CGN neurons from wild type mice. Results represents the percentage of the average length of the longest neurite over the control ±SEM. ***P<0.0001.

Next, we asked if blocking PirB receptors with PirB antibodies in DRG neurons from NgR1 deficient mice will abrogate MAG-mediated inhibition in both MAG- and mutated MAG (R118A)-expressing CHO cells. Our results shows that DRG neurons isolated from NgR1 deficient mice are inhibited by both MAG- and MAG (R118A)-expressing CHO cells even in the presence of anti-PirB-1 antibody (Fig 4.5).

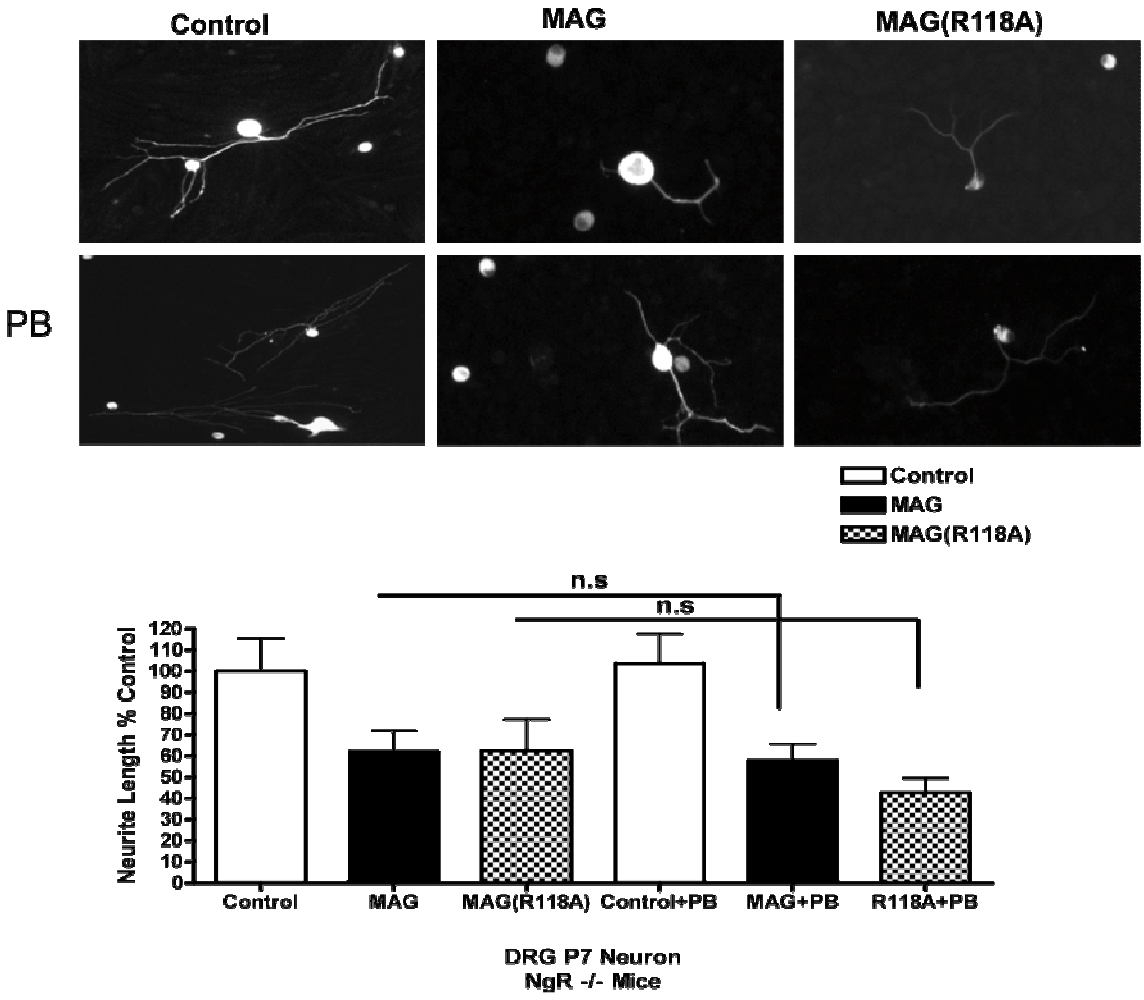


Fig (4.5): Both MAG- and MAG (R118A)-expressing CHO cells inhibit DRG neurons from NgR1 deficient mice in the presence and absence of anti-PirB-1 antibody (PB). Results represent the percentage of the average length of the longest neurite over the control ±SEM. *P<0.05, n.s: not significant.

4.3. CGN neurons from NgR1 deficient mice bind to MAG-Fc, MAG (d1-3)-Fc but not to MAG (R118A)-Fc

Next, we want to determine the extend of MAG-Fc binding to each of our chimaric soluble wild type MAG-Fc, truncated MAG (d1-3)-Fc, and mutated MAG (R118A)-Fc. We preformed our MAG-Fc binding assay to compare binding of wild type and NgR1 deficient neurons MAG-Fc. Our result demonstrates that like wild type CGN neurons from NgR1 deficient mice bind to MAG-Fc and MAG (d1-3) Fc but not to mutated MAG (R118A)-Fc. This result suggests that like wild type, NgR1 deficient mice requires sialic acid binding in order for MAG-Fc- to binds CGN neurons.

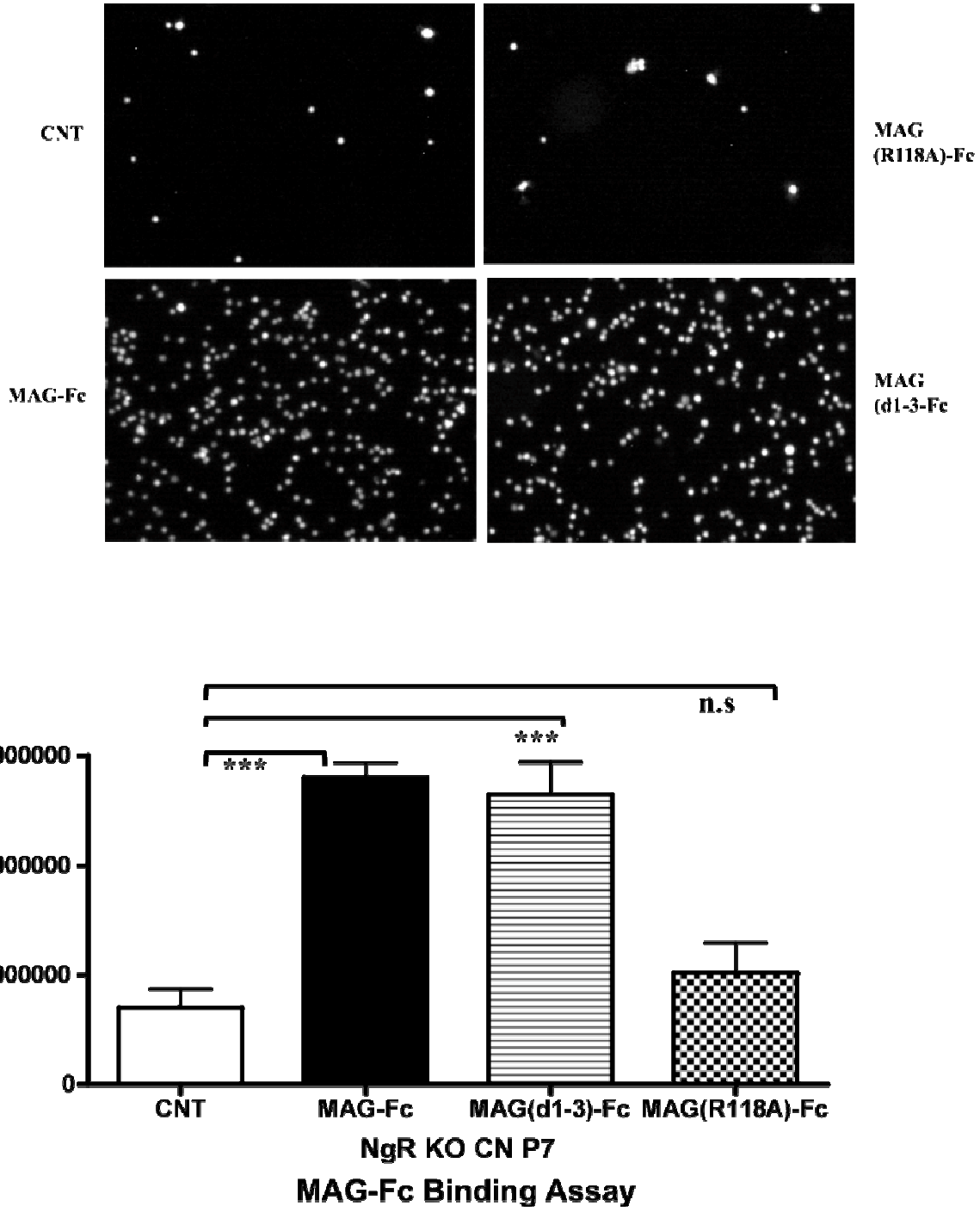


Fig (4.6): CGN neurons from NgR1 deficient mice bind to MAG-Fc and MAG (d1-3)-Fc but not to MAG (R118A)-Fc. Dissociated labeled CGN neurons were plated on immobilized MAG-Fc, truncated MAG (d1-3)-Fc and mutated MAG (R118A)-Fc. A) 4x pictures of CGN neuron (4x). B) Quantification of bound cells per treatment. ±SEM ***P<0.001, n.s: not significant.

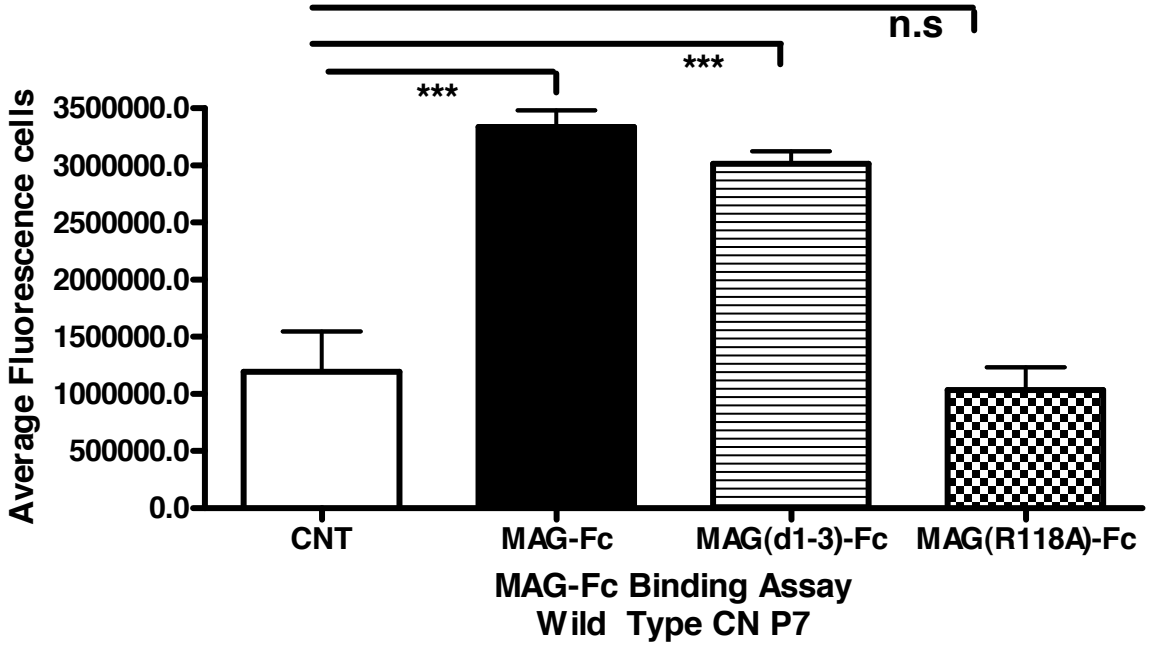


Fig (4.7): CGN neurons isolated from wild type mice bind to MAG-Fc and MAG (d1-3)-Fc but not to MAG (R118A)-Fc. Dissociated labeled CGN neurons were plated on immobilized MAG-Fc, truncated MAG (d1-3)-Fc and mutated MAG (R118A)-Fc. Quantification of bound cells per treatment. ±SEM ***P<0.0001, n.s: not significant.

4.4. Knocking down NgR2 in neurons from NgR1 deficient mice is not sufficient to overcome MAG-mediated inhibition:

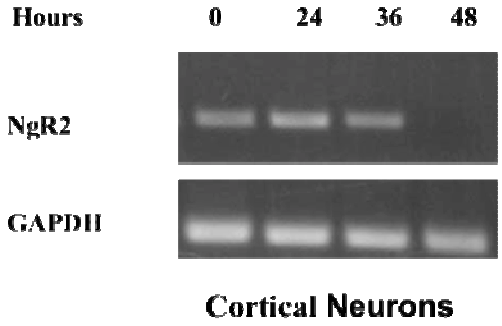
In 2005, NgR2 was identified as a sialic acid-dependent specific receptor for MAG (Venkatesh et al., 2005). In the CNS, NgR2 is expressed in cortical neurons while CGN neurons lack NgR2 expression. Unlike cortical neurons NgR2 is not expressed in DRGs until postnatal day 14 (P14). This limitation of NgR2 expression and specification of binding only to MAG, unlike its homologue NgR1 which was shown to be a functional receptor not only for MAG but Nogo-66 and OMgp, lead us to take a more detailed investigation to further assess the role of NgR2 in MAG-mediated inhibition of neurite outgrowth. We utilized P2 cortical neurons that lack NgR1 from NgR1 deficient or wild type mice, and knocked down NgR2 by siRNA coupled to Penetratin-1 (PN1).

NgR2 was successfully knocked down by 48 hours of treatment with 300 ηM siRNA-PN-1. In Figure (4.8A) RT-PCR result shows that endogenous NgR2 mRNA levels went down to 0.32 compared to control. Our neurite outgrowth assay results show that knocking-down NgR2 in addition to knocking-out NgR1 expression even in the presence of mutated MAG (R118A)-expressing CHO cells did not reverse MAG-mediated inhibition of neurite outgrowth (Fig 4.8 B, C and D). This result clearly shows a limitation of NgR2 role in MAG mediated inhibition of neurite outgrowth; also it suggests the existence of other receptors beside NgR1, and NgR2 that mediate MAG inhibition.

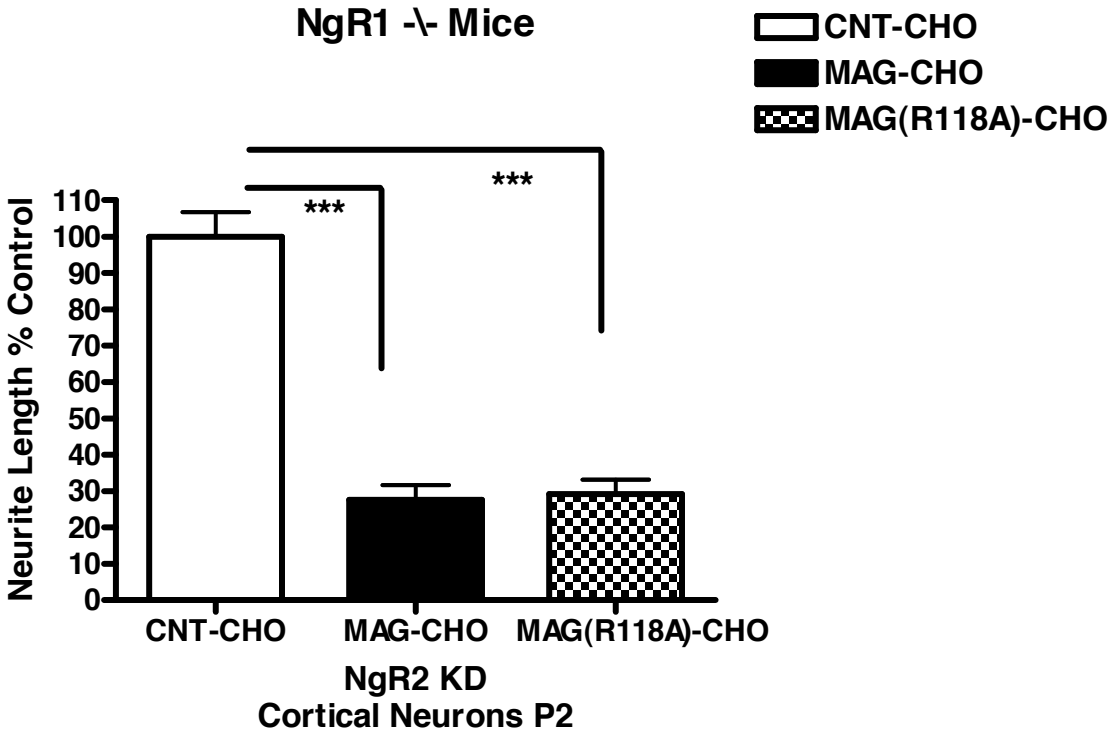
Fig (4.8): NgR2 knockdown in P2 cortical neurons.

A) NgR2 knockdown in NgR1 deficient cortical neurons: PN1-NgR2-siRNA time course treatment: RT-PCR indicates successful knockdown of NgR2 endogenous levels within 48 hours of treatment of PN1-NgR2-siRNA compared to control. B) Neurite outgrowth assay of P2 cortical neurons from NgR1 deficient mice treated with PN1-NgR2 siRNA. NgR2-KD did not reverse inhibition of MAG- or MAG (R118A)-expressing CHO cells. C) Neurite outgrowth assay of P2 cortical neurons from wild type mice treated with PN1-NgR2-siRNA shows no effect on MAG-mediated inhibitory activity. Results represents the percentage of the average length of the longest neurite over control ±SEM. ***P<0.001.

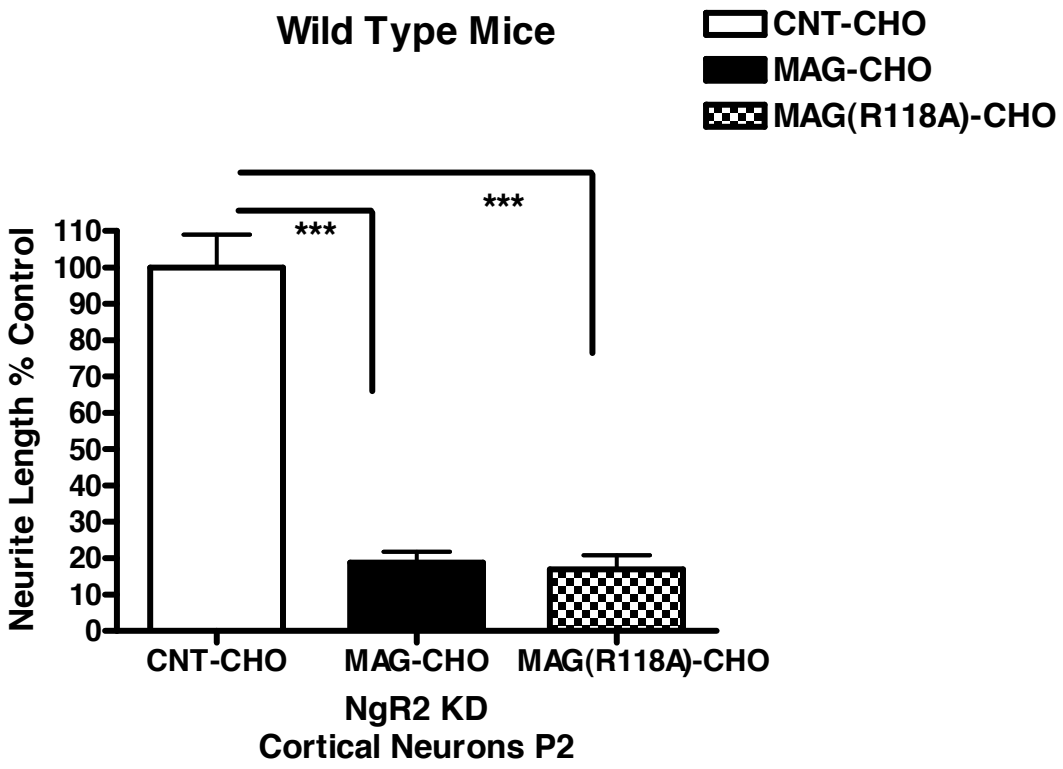
A)



B)



C)


Wild Type Mice
CNT-CHO
MAG-CHO
MAG(R118A)-CHO
***
***
Neurite Length % Control
110
100
90
80
70
60
50
40
30
20
10
0
CNT-CHO
MAG-CHO
MAG(R118A)-CHO
NgR2 KD
Cortical Neurons P2

Discussion

Here we show that blocking all known receptors for MAG does not relieve MAG-mediated inhibition of neurite outgrowth. These results strongly suggest that MAG exerts its inhibitory activity through another unidentified receptor(s) beside NgR1, NgR2 and PirB.

In our neurite outgrowth, MAG-expressing CHO cells inhibit neurons from wild type mice. This inhibition is mediated by NgR1-$p75^{NTR}$/Troy-Lingo-1 receptor complex, NgR2 or PirB. In this study, we utilized CGN, DRG and cortical neurons from wild type and NgR1-deficient mice. CGN neurons are a population of largely unmyelinated neurons that lack NgR2 protein expression (Venkatesh et al., 2005, Zheng et al., 2005), while cortical neurons and P14 DRG neurons express NgR2. However, CGN neurons from wild type mice are still inhibited by mutated MAG (R118A)-expressing CHO (Fig 4.4). This inhibition could be due to the presence of NgR1 and PirB but not NgR2. Also, NgR2 binding to MAG was shown to be sialic acid–dependent (Venkatesh et al., 2005), therefore by utilizing mutated MAG (R118A)-expressing CHO, we can exclude MAG-mediated inhibition though NgR2 receptor. To further investigate the role of NgR2 in MAG-mediated inhibition, we used P2 cortical neurons from NgR1 deficient mice, and knocked-down NgR2 by PN1-NgR2 siRNA. Our results demonstrate that MAG-expressing CHO cells inhibit PN1-NgR2siRNA similar to control scramble siRNA (Fig 4.8). Moreover, since binding of MAG to NgR2 is sialic acid-dependent (Venkatesh et al., 2005), and MAG (R118A)-expressing CHO cells inhibits P2 cortical neurons from both wild type and NgR1 deficient mice, it suggests a limited role for NgR2 in MAG-mediated inhibition.

In addition, CGN and DRG neurons from NgR1 deficient mice are still inhibited by MAG (R118A)-expressing CHO cells even in the presence of anti-PirB-1 antibody (Fig 4.3 and 4.5), which was shown to interfere with PirB receptor and to improve neurite outgrowth in the presence of myelin inhibitors (Atwal et al., 2008). Treatment of CGN neurons from NgR1 deficient and wild type mice with anti-PirB-1 antibody induced limited reversal of inhibition of neurite outgrowth on MAG-expressing CHO cells. This result suggests that in CGN PirB mediated inhibition of neurite outgrowth plays more prominent role than NgR1-mediated inhibition.

In our current study, MAG-Fc inhibits neurons from NgR1 deficient mice (Fig 4.2). This result contradicts a previous study by Doherty's group, which reports that ganglioside inhibition of neurite outgrowth requires NgR1 function (Williams et al., 2008). They reported that MAG-Fc does not inhibit CGN neurons from NgR1 deficient mice. This contradiction in results could be explained by the two different mouse strains used in the studies.

Here, our MAG-Fc binding assay shows that CGN neurons from NgR1 bind to full length MAG-Fc, truncated MAG (d1-3)-Fc but not to mutated MAG (R118A)-Fc similar to CGN neurons from wild type mice (Fig 4.6 and 4.7).

Previously, Schnaar's group reported that gangliosides and Nogo receptors independently mediate MAG inhibition of neurite outgrowth in different neuronal cell types (Mehta et al., 2007). In an attempt to distinguish the role of gangliosides and NgR1 in MAG-mediated inhibition of neurite outgrowth from three nerve cell types, DRG, CGN and HN rat neurons. They preformed neurite outgrowth assay in which they used substrata adsorbed with immobilized full length MAG extracted from myelin. They reported that

in DRG neurons most of the MAG inhibition was through NgR1 while a small percentage was from gangliosides. Treatments of DRG neurons with phosphatidylinositol-specific phopholipase-C (PI-PLC), which cleaves GPI- anchors (including NgR1), reverse MAG-mediated inhibition. Moreover, they reported that CGN and HN neurons are inhibited exclusively through gangliosides. In HN neurons, inhibition was mostly reversed by sialidase to cleave GD1a and GT1b, or P4 an inhibitor of gangliosides biosynthesis (Mehta et al., 2007).

In addition, Giger's group also reported distinct and cell type-specific mechanisms for neurite outgrowth inhibition (Venkatesh et al., 2007). In their neurite outgrowth assay, they used *vibrio cholerae neuraminidase* (VCN), which removes sialic acid, and reported that it is not sufficient to release MAG inhibition of retinal ganglion cells (RGCs), however it attenuates MAG inhibition of CGN. Our results dispute these reports. Here we demonstrate that both MAG- and MAG (R118A)- expressing CHO cells inhibits more than one neuronal type. For example, CGN neurons from both wild type and NgR1 deficient mice (Fig 4.3) as well as DRG neurons from both wild type and NgR1 deficient mice were strongly inhibited by MAG-expressing and MAG (R118A)-expressing CHO cells (Fig 4.5). Both results show that MAG-and MAG (R118A) inhibits DRG and CGN neurons independently of NgR1, NgR2 or sialic acid. These different results between the groups could be explained by the different methodologies used to perform these experiments. In some of these studies, enzymes and pharmacological agents are used differently. Moreover, some of these studies were conducted before the identification of PirB as a functional receptor for all three major myelin inhibitors (Atwal et al., 2008).

Collectively, our data suggest the existence of other unknown receptors beside NgR1, NgR2 and PirB that mediates MAG's inhibitory effect

Chapter VI: Summary

First we showed that gangliosides are not functional receptors for MAG. Binding of MAG-expressing CHO cells to gangliosides is neither necessary nor sufficient to bring about inhibition of neurite outgrowth. However, the soluble form of MAG (MAG-Fc), sialic acid binding becomes necessary to bring about inhibition of neurite outgrowth. When MAG is in its soluble form interaction with sialic acid is needed in order to potentiate the inhibitory site on MAG to engage. This is not the case when MAG is expressed with at the surface of CHO cells, where other adhesion molecule such as N-CAM are present in the plasma membrane of CHO cells and therefore gangliosides are not needed.

Secondly, we show that excluding NgR2 receptor by utilizing mutated MAG (R118A)-expressing CHO that cannot bind to sialic acid and consequently cannot bind to NgR2 is not sufficient to overcome MAG inhibition even when NgR1 was ablated. Moreover, knocking-down NgR2 in neurons from NgR1 deficient mice was not sufficient to block MAG –mediated inhibition.

Finally, PirB was recently identified as a novel receptor for myelin-associated inhibitors including MAG. Anti-PirB-1 antibody raised against the extracellular portion of PirB was shown to block its inhibition effect. However, neurons from NgR1 deficient mice treated with anti-PirB-1 antibodies are still inhibited by MAG (R118A)-expressing CHO cells even when we used mutated MAG (R118A)-expressing CHO cells that can no longer bind to sialic acid.

Collectively, our results suggest the existence of other unknown receptor(s) for MAG beside NgR1, NgR2, PirB and sialic acid bearing glycosphingolipids and we propose the following new working model (Fig 5.1).

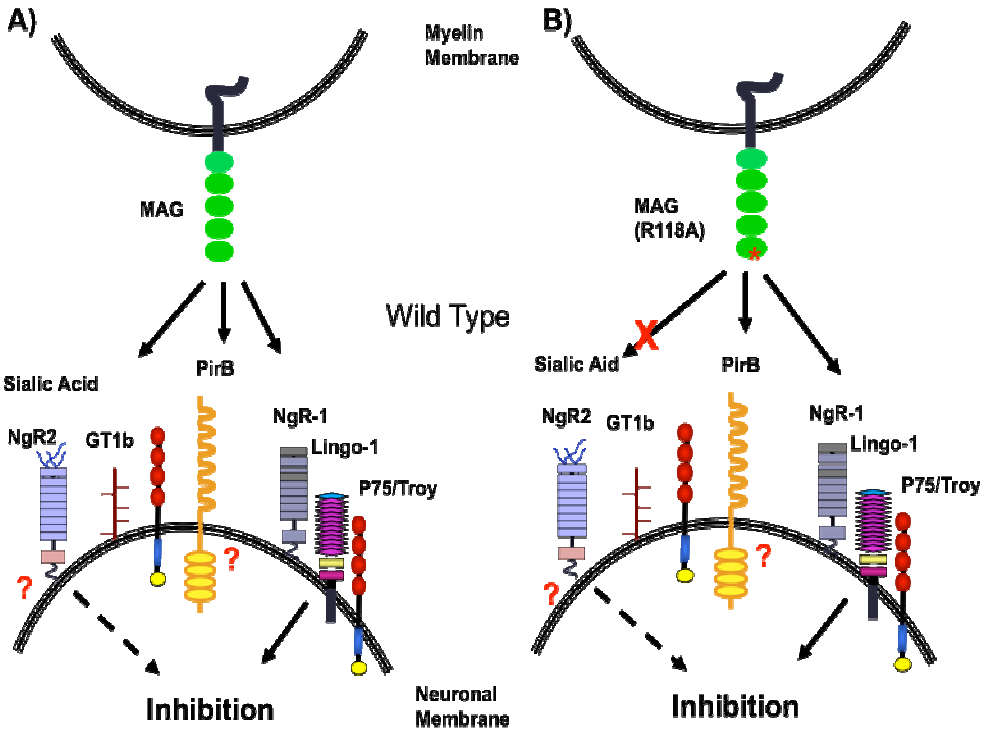
New Working Model:

Here, we evaluated the role of MAG's known receptors in MAG-mediated inhibition of neurite outgrowth.

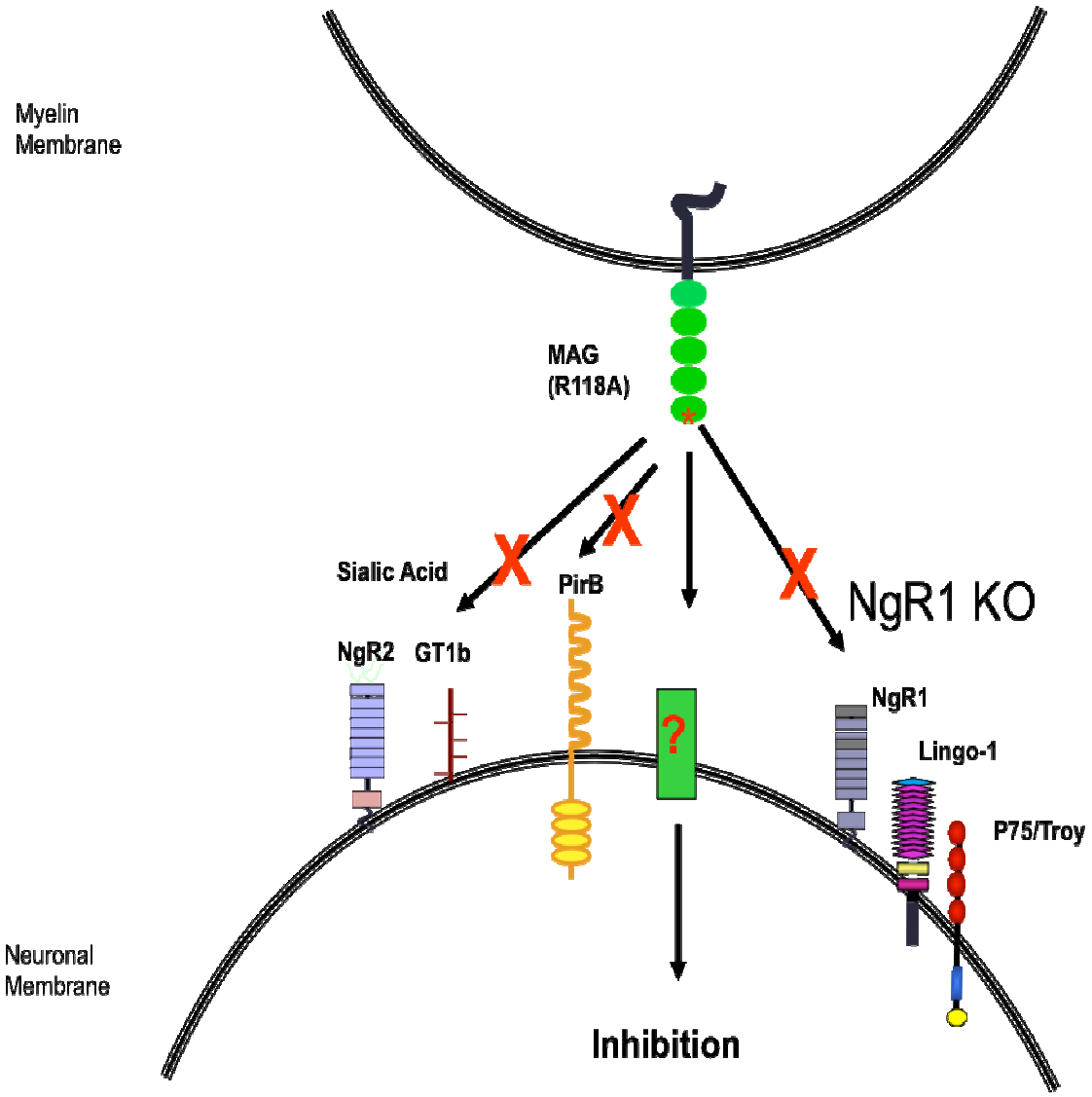
It was demonstrated before that MAG-expressing CHO cells inhibit neurite outgrowth of neurons isolated from wild type mice. This inhibition could be mediated by well-characterized receptors such as NgR1-p75NTR/TROY-Lingo-1 receptor complex, NgR2 and PirB.

In addition, MAG can also bind to sialic acid bearing gangliosides such as GT1b (Fig 5.1 A). However, when mutated MAG (R118A)-expressing CHO cells is used and consequently, the sialic acid binding property of MAG is excluded, MAG can no longer binds to NgR2 since it is sialic acid-dependent nor to gangliosides GT1b (Fig 5.1 B). Furthermore, mutated MAG (R118A)-expressing CHO when used with neurons from NgR1 deficient mice can no longer inhibit neurite outgrowth through NgR1 receptor complex. In addition, in the presence of anti-PirB-1 antibody PirB receptor is blocked and it can no longer mediate it is inhibitory effect. However, neurons from NgR1 deficient mice are still inhibited by MAG (R118A)-expressing CHO cells even in the presence of anti-PirB-1 antibody, which suggests the presence of other unknown receptor(s) (Fig 5.1 C).

Fig (5.1): New Model of neurite inhibition of axonal growth by MAG. A) In wild type neurons, MAG-expressing CHO mediates its neurite outgrowth inhibitory activity through NgR1-P75/Troy-Lingo receptor complex, PirB receptor or NgR2 receptor. B) Mutated MAG (R118A)-expressing CHO cells inhibits neurite outgrowth in wild type neurons through NgR1 and PirB but not NgR2 or gangliosides GT1b. C) NgR1 deficient neurons are inhibited by MAG (R118A)-CHO cells even in the presence of an anti-PirB-1 antibody, which suggest the presence of another unknown receptor(s) for MAG. (SA= sialic acid, PB= anti-PirB-1 antibody)



C)


Myelin
Membrane
MAG
(R118A)
Sialic Acid
PirB
NgR1 KO
NgR2
GT1b
?
NgR1
Lingo-1
P75/Troy
Neuronal
Membrane
Inhibition

Chapter VII: References

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