Myelin-Associated Glycoprotein (MAG) Protects Neurons from Acute Toxicity Using a Ganglioside-Dependent Mechanism

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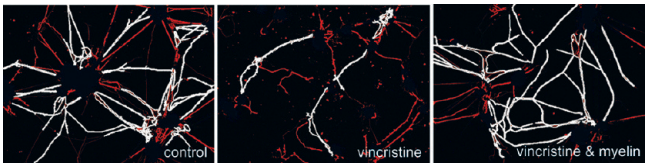
Abstract



Myelin-associated glycoprotein (MAG), a protein expressed on the innermost wrap of myelin, contributes to long-term axon stability as evidenced by progressive axon degeneration in *Mag*-null mice. Recently, MAG was also found to protect axons from acute toxic insults. In the current study, rat dorsal root ganglion neurons were cultured on control substrata and substrata adsorbed with myelin proteins. Neurons on myelin-adsorbed surfaces were resistant to acute degeneration of neurites induced by vincristine, a cancer chemotherapeutic agent with neuropathic side effects. Myelin-mediated protection was reversed by anti-MAG antibody and was absent when cells were cultured on extracts from *Mag*-null mouse myelin, confirming the protective role of MAG. Gangliosides (sialylated glycosphingolipids) are one functional class of axonal receptors for MAG. In the current studies, a direct role for gangliosides in mediating the acute protective effects of MAG was established. Treatment of neurons with sialidase, an enzyme that cleaves the terminal sialic acids required for MAG binding, reversed MAG's protective effect, as did treatment with (1*R*,2*R*)-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol, an inhibitor of glycosphingolipid biosynthesis. In contrast, treatment with phosphatidylinositol-specific phospholipase C, an enzyme that cleaves Nogo receptors (NgR, another class of MAG receptor), or with a peptide inhibitor of an NgR-associated signaling molecule, $p75^{NTR}$, failed to diminish MAG-mediated protection. Inhibiting the Rho-associated protein kinase ROCK reversed protection. We conclude that MAG protects neurites from acute toxic insult via a ganglioside-mediated signaling pathway that involves activation of RhoA. Understanding MAG-mediated protection may provide opportunities to reduce axonal damage and loss.

Keywords: Gangliosides, myelin-associated glycoprotein (MAG), GD1a, GT1b, Siglec-4, vincristine, Nogo receptor

In diseases that primarily target myelin, such as multiple sclerosis, axon degeneration secondary to demyelination is a major factor contributing to disease progression (*1*). This led to the hypothesis, supported by mouse genetic models, that interactions between molecules on the innermost wrap of myelin and complementary receptors on the apposing axon surface are required for long-term axon stability (*2*). Among these molecules is myelin-associated glycoprotein (MAG) (*3*). Mice lacking MAG express abundant myelin but suffer long-term axon degeneration and altered axon cytoskeletal structure (*4*−*6*). Axon degeneration in *Mag*-null mice is long-term, with progressive axon loss only apparent on the time scale of months (*7*, *8*). Mice engineered to lack one potential class of MAG receptors, complex gangliosides (sialylated glycosphingolipids), display the same delayed and progressive axon pathology, leading to the hypothesis that MAG on periaxonal myelin interacts with gangliosides (specifically GD1a and GT1b) on the apposing axolemma to mediate long-term axon stability (*7*, *9*).

A second action of MAG is to inhibit axon regeneration after injury (*10*−*13*). Several axon receptors have been proposed to mediate MAG-induced axon outgrowth inhibition, including gangliosides (*14*), Nogo receptors (*15*−*17*), β1-integrin (*18*), and PirB (*19*). How these different MAG receptors act, independently or interactively, to inhibit axon outgrowth has not been resolved, although a common downstream target is thought to be RhoA (*13*). Recently, different nerve cell types were found to use different MAG receptors to inhibit axon outgrowth (*20*, *21*). For example, MAG inhibition of axon outgrowth from cerebellar granule

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neurons *in vitro* was ganglioside-dependent, whereas inhibition of outgrowth from dorsal root ganglion neurons was primarily NgR-dependent (*21*). The mechanistic relationships, if any, between MAG-mediated axon outgrowth inhibition and long-term axon−myelin stabilization have not been resolved.

In addition to its roles in long-term axon−myelin stabilization and inhibition of axon outgrowth, we recently discovered that MAG protects axons from acute toxic insults including exposure to vincristine, acrylamide, and inflammatory mediators (*8*). In the current studies, we use specific enzymes, antibodies, inhibitors, and *Mag*-null mouse myelin to establish that MAG-mediated protection of dorsal root ganglion neurons (DRGN) from an acute insult is ganglioside dependent. These data, together with our prior findings (*8*, *21*), establish that dual receptors on the same cell type mediate independent protective and inhibitory effects of MAG.

Results and Discussion

MAG-Mediated Protection

Postnatal DRGN plated on control substrata extend long neurites that are strongly stained with the anti-β-tubulin antibody TUJ1 (Figure 1). Upon culturing for 24 h in the presence of vincristine, a chemotherapeutic agent that disrupts microtubule dynamics, the neurites appear fragmented and thin. This change was quantified by image analysis as a 65% decrease in neurite integrity, indicating that most (but perhaps not all) classes of DRG neurons extend axons that are susceptible to vincristine toxicity. Plating the same DRGN on substrata precoated with proteins extracted from rat brain myelin had a dual effect. As has been long established (*22*), myelin diminished the extent of neurite outgrowth. At the same time, the neurites that did emerge were fully resistant to the toxic effects of vincristine. Subsequent experiments mechanistically separated myelin protection from outgrowth inhibition.

Prior studies established that treatment of DRGN with the enzyme phosphatidylinositol-specific phospholipase C (PIPLC), which cleaves glycosylphosphatidylinositol (GPI) anchored proteins from cell surfaces (including the MAG receptors NgR1 and NgR2), largely reversed myelin inhibition of neurite outgrowth (*21*). The robust neurites that extended on myelin-adsorbed substrata in the presence of PIPLC were protected from vincristine toxicity, whereas the neurites that extended on control substrata in the presence of PIPLC remained vincristine sensitive (Figure 1). These data established that myelin protects neurons from vincristine sensitivity using a mechanism that does not require GPI-anchored proteins. Since the protection was dependent on MAG (see below), the role of a second class of MAG receptors, gangliosides GD1a and GT1b, was tested.

Starting from the observation that DRGN grown on myelin in the presence of PIPLC extend long neurites resistant to vincristine toxicity, two independent treatments were used to test the role of gangliosides in neuroprotection. Sialidase cleaves the terminal sialic acids from glycolipids and glycoproteins on intact neurons, converting the MAG-binding gangliosides GD1a and GT1b to the nonbinding ganglioside GM1 (*23*). Treatment with sialidase completely reversed myelin's protective effect (Figure 1). Likewise, an inhibitor of glycosphingolipid biosynthesis, (1*R*,2*R*)-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (P4), completely reversed myelin-mediated protection. Ganglioside immunostaining confirmed the efficacy of sialidase and P4 when added to DRGN cultures (Supplemental Figure S1, Supporting Information). These data imply that gangliosides, sialylated glycosphingolipids, are obligatory receptors for myelin-mediated neurite protection.

Based on prior observations (*8*), we inferred that the active protective component in myelin was MAG. This was confirmed by comparing the protective effects of myelin extracts from wild-type and *Mag*-null mice. In the presence of PIPLC to optimize neurite outgrowth, myelin extract from wild-type mice completely protected neurites from vincristine toxicity, whereas myelin extract from *Mag*-null mice had little effect (Figure 2). Under these conditions, addition of anti-MAG antibody, sialidase, or P4 resulted in greatly reduced protection by wild-type mouse myelin extract. *Mag*-null myelin extract did not confer protection under any condition (Figure 3). These data support the conclusion that myelin-mediated protection of neurites from acute toxic insult is dependent on MAG and gangliosides.

Signaling Pathways for MAG-Mediated Protection

MAG interacts with different classes of receptors on axons to initiate transmembrane signals (*18*−*21*). Among these, gangliosides (GD1a and GT1b) and Nogo receptors (NgR1 and NgR2) are lipid-linked and presumably act via transmembrane transducers, whereas β1-integrin and PirB are transmembrane proteins. Signaling downstream of MAG binding is thought to involve activation of the small GTPase RhoA (*12*, *13*) and its effector Rho kinase (ROCK). The neurotrophin receptor $p75^{NTR}$, a transmembrane protein, has been proposed to transduce MAG binding, via NgR, through Rho-GDI to RhoA (*24*−*26*). A cell-permeable peptide, TAT-Pep5, blocks the intracellular association of $p75^{NTR}$ with Rho-GDI, blocking its ability to activate RhoA and reversing MAG's inhibition of neurite outgrowth in some neurons (*21*, *27*). The addition of TAT-Pep5 to DRGN cultured on myelin-extracted proteins partially reverses

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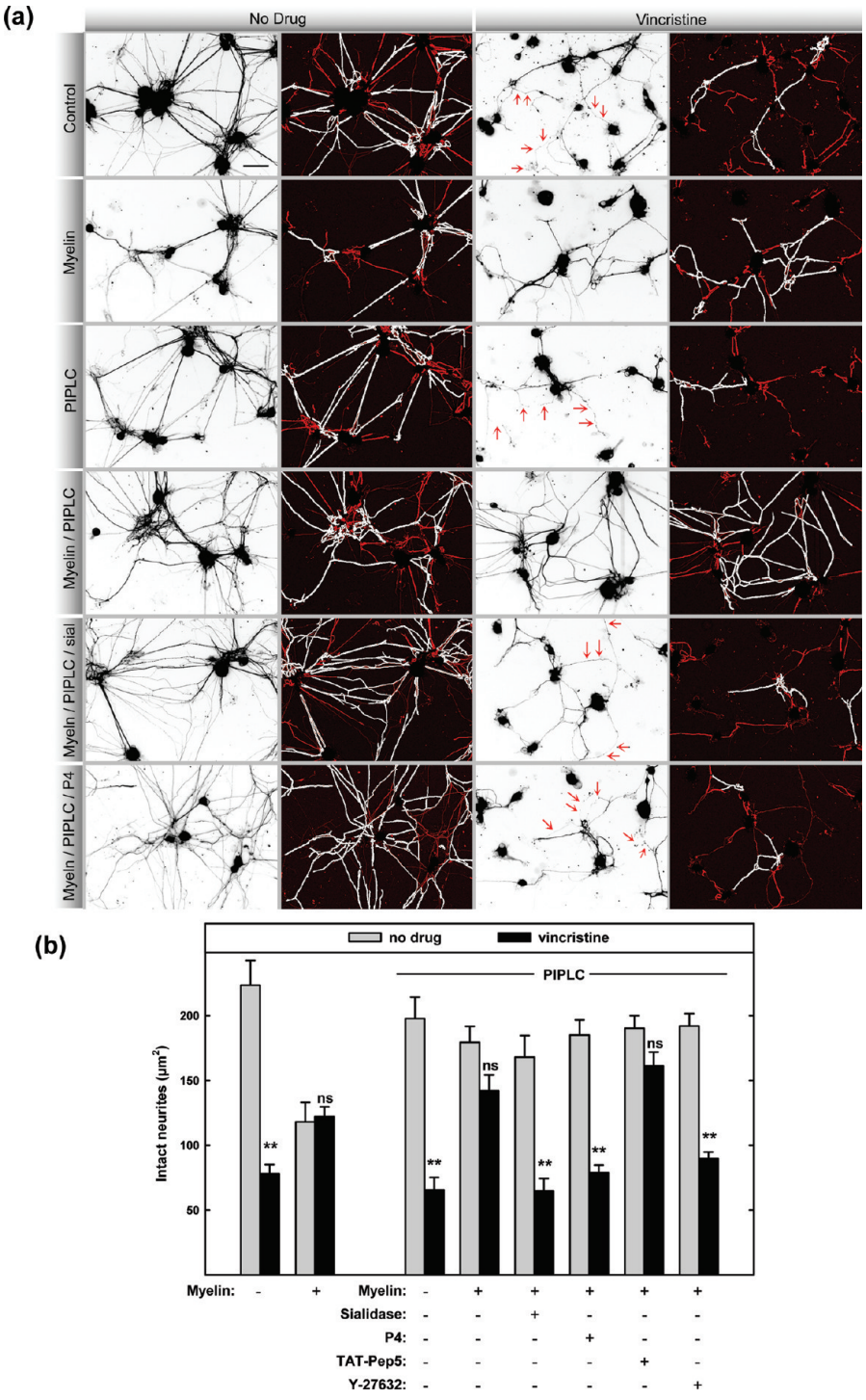

Figure 1. Myelin protects DRGN neurites from vincristine toxicity via a ganglioside-dependent mechanism. DRGN were plated on control surfaces or the same surfaces adsorbed with proteins extracted from rat myelin. After 1 h, the cultures (as indicated) were treated with 1 U/mL PIPLC, 8 mU/mL sialidase (sial), 1 μM P4, 200 nM TAT-Pep5, or 10 μM Y-27632. After 24 h, cultures were treated with vincristine (10 nM) or vehicle (no drug). After an additional 24 h, cultures were fixed and immunostained with antitubulin mAb to detect neurites. (a) Fluorescent images are presented as reversed grayscale to enhance clarity. For each condition, representative fluorescent micrographs are shown (bar = 100 μm). Red arrows mark examples of vincristine-induced neurite degeneration. To the right of each fluorescent image, software-analyzed micrographs are presented showing quantification of neurite integrity, with white representing intact uninterrupted neurites and red indicating segmented or atrophic neurites. (b) Image analysis was used to quantify intact neurites, normalized for the number of cell bodies in the field. For each condition, four or five random images from each of four to ten independent wells from an average of three independent experiments were analyzed. Data are presented as the mean ± SEM. Vincristine toxicity was compared with the matched control in the absence of drug: **, $p < 0.001$; ns, no significant vincristine toxicity.

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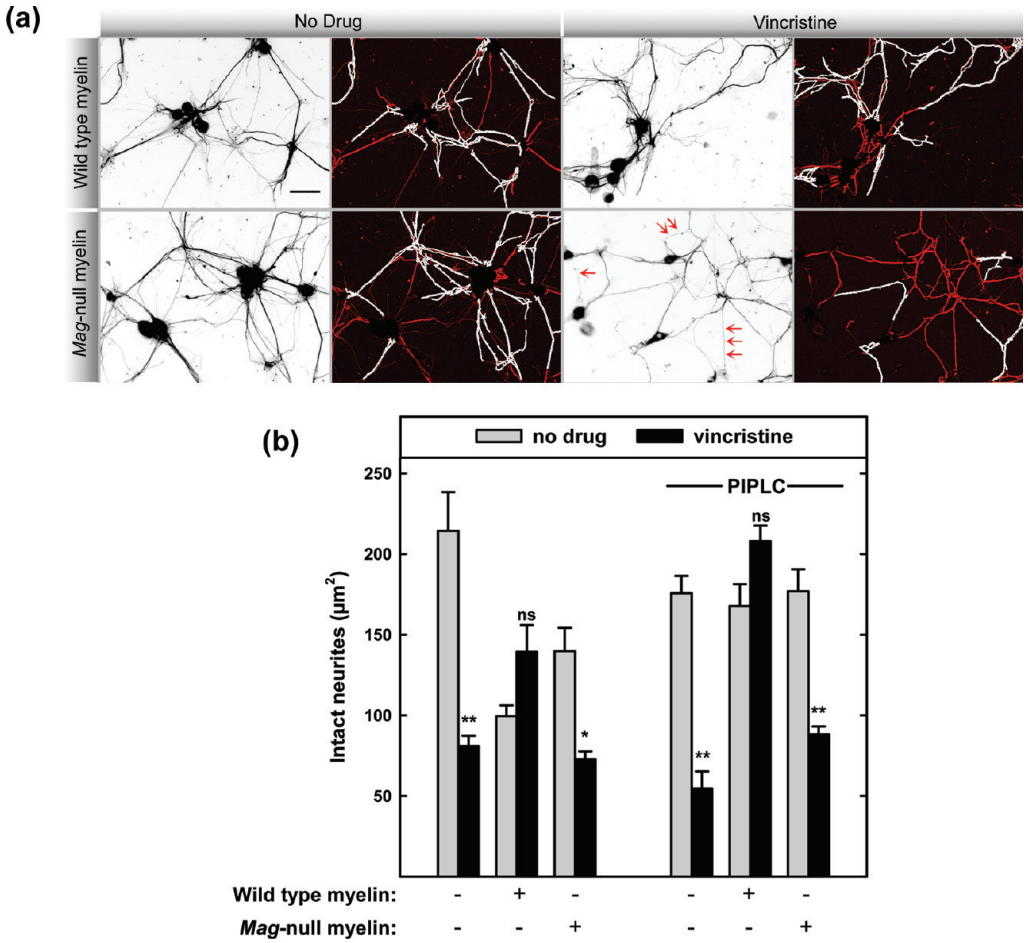

Figure 2. Myelin extract from *Mag*-null mice does not protect DRGN neurites from vincristine toxicity. Rat DRGN were plated on surfaces adsorbed with detergent-extracts of myelin isolated from wild-type or *Mag*-null mice as indicated. Cultures were treated 1 h after plating with 1 U/mL PIPLC, then after 24 h with 10 nM vincristine or vehicle (no drug) as indicated. After an additional 24 h, cultures were fixed, immunostained, and analyzed as described in the legend to Figure 1. (a) Representative fluorescent micrographs (reversed grayscale) are shown for each condition (bar = 100 μm). Red arrows mark examples of vincristine-induced neurite degeneration. To the right of each fluorescent image, software-analyzed micrographs are presented (white, intact uninterrupted neurites; red, segmented or atrophic neurites). (b) Image analysis was used to quantify intact neurites normalized for the number of cell bodies in the field. Data are presented as the mean ± SEM. Vincristine toxicity was compared with the matched control in the absence of drug: **, $p < 0.001$; *, $p < 0.005$; ns, no significant vincristine toxicity.

neurite outgrowth inhibition. However, TAT-Pep5 had no effect on MAG's neuroprotective effect in the same cells (Figure 1b). In contrast, Y-27632 (*28*), which blocks ROCK, reversed MAG's neuroprotective effect. These data imply that MAG−ganglioside binding results in protective signals via a pathway that involves activation of RhoA.

The results reported here identify gangliosides as the functional receptor class for MAG protection of DRGN from acute toxic insult. Furthermore, they demonstrate that two independent MAG-mediated pathways, with two independent functional outcomes, coexist in the same cells: MAG−NgR binding mediates neurite outgrowth inhibition, whereas MAG−ganglioside binding mediates neurite protection.

Myelin-associated glycoprotein is multifunctional (*29*). It supports long-term axon−myelin stability, modulates the axon cytoskeleton, supports the proper molecular distributions at nodes of Ranvier, inhibits axon outgrowth at sites of injury, and protects axons from acute toxic insults. To initiate its various biological effects, MAG binds to complementary receptors on the axon surface. Multiple MAG receptors have emerged as potential functional candidates: gangliosides GD1a and GT1b (*14*), Nogo receptors NgR1 and NgR2 (*12*−*14*, *17*), β1-integrin (*18*), and PirB (*19*). In the case of MAG-mediated axon outgrowth inhibition, different nerve cell types use different MAG receptors (*20*, *21*). Other studies suggest that different MAG receptors are involved in different types of growth cone control, with NgRs mediating short-term growth cone collapse but not longer term inhibition of axon extension (*30*). Whether the different MAG receptors interact to generate downstream signals has yet to be fully determined. For

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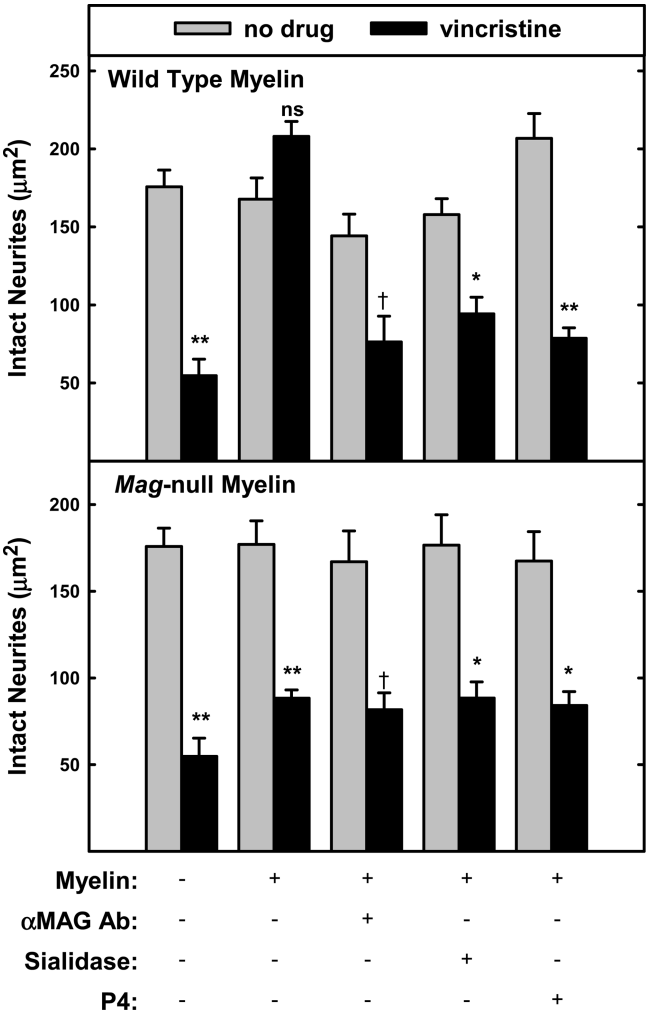

Figure 3. Wild-type mouse MAG protection of DRGN neurites from vincristine toxicity is ganglioside dependent. Experiments were performed as described in the legend to Figure 2, with 1 U/mL PIPLC added to all cultures 1 h after plating, along with (as indicated) 10 μg/mL anti-MAG antibody mAb 513, 8 mU/mL sialidase, or 1 μM P4. Image analysis was used to quantify intact neurites normalized for the number of cell bodies in the field. Data are presented as the mean ± SEM. Vincristine toxicity was compared with the matched control in the absence of drug: **, $p < 0.001$; *, $p < 0.005$; †, $p < 0.02$; ns, no significant vincristine toxicity.

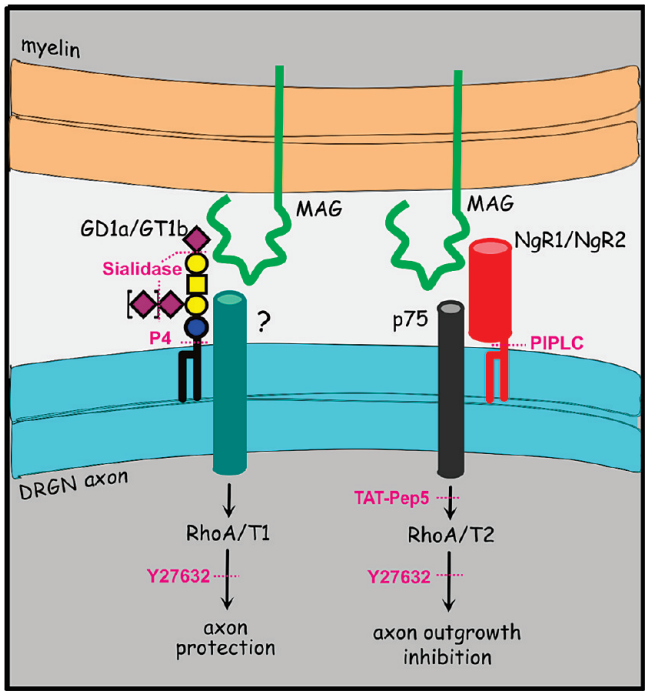

Figure 4. Model of MAG function. MAG in the myelin membrane engages multiple distinct receptor classes, including gangliosides and NgRs, on the DRGN surface. Engaging NgR (inhibited by PIPLC) results in axon outgrowth inhibition via a pathway that includes $p75^{NTR}$ (inhibited by TAT-Pep5) and RhoA effector Rho kinase (ROCK, inhibited by Y-27632). MAG binding to gangliosides GD1a or GT1b (inhibited by sialidase and P4) results in protection from acute toxicity via a pathway that is independent of $p75^{NTR}$ and NgR but requires ROCK (inhibited by Y-27632). The transmembrane transduction system activated by MAG−ganglioside binding has not been established and may include additional identified MAG receptors such as β1-integrin or PirB (*18*, *19*). Since outgrowth inhibition and protection are via separate pathways that both require ROCK, we conclude that RhoA activation is necessary but not sufficient for each independent pathway. This is represented by the inclusion of additional transduction factors (T_1, T_2) that are unique to (and independently required for) each pathway. Pathway inhibitors used in this study are shown in red font and their sites of action by red dotted lines. Gangliosides are represented using standard glycan symbol nomenclature, in which sialic acids are indicated by purple diamonds (*44*).

example, in some studies gangliosides and NgRs appear to act independently (*21*), whereas in other studies they have been found to associate (*31*).

MAG is a member of the Siglec family of sialic acid binding proteins and binds with high selectively to gangliosides GD1a and GT1b (*32*). Immunohistochemical studies (*33*) demonstrated that GD1a is selectively expressed on subpopulations of adult rodent DRGN (depending on their size), whereas GT1b is expressed on a large majority of all DRGN. An antibody that binds to both GD1a and GT1b (as well as to minor gangliosides with the same glycan terminus that binds MAG) labels nearly every cell in the dorsal root ganglion.

Mice engineered to lack the MAG-binding glycan terminus on gangliosides (*B4galnt1*-null mice) display long-term axon degeneration, altered axonal neurofilament spacing, altered molecular distributions at nodes of Ranvier, and motor behavioral deficits that are similar to those in *Mag*-null mice, consistent with the conclusion that gangliosides mediate these MAG functions (*7*−*9*, *34*). In contrast, MAG engages various receptors to inhibit axon (neurite) outgrowth (*20*, *21*). For example, in similar in vitro systems, MAG inhibition of axon outgrowth from cerebellar granule neurons requires gangliosides but not NgR, whereas inhibition of outgrowth from DRGN is primarily via NgRs independent of gangliosides, despite the fact that both cell types express both receptor classes (*15*, *20*, *21*, *35*, *36*).

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NgR is primarily responsible for MAG-mediated neurite outgrowth inhibition in DRGN (ref *21* and Figure 1). However, it is dispensable for MAG-mediated acute protection in the same cells. In contrast, the ganglioside signaling pathway is largely dispensable for MAG-mediated neurite outgrowth inhibition (*21*) but is essential to MAG-mediated acute protection (Figures 1 and 3). This implies that two independent MAG-mediated pathways coexist in DRGN (Figure 4). Although mediated by different upstream receptors in these cells, MAG-mediated inhibition of axon outgrowth (*21*, *37*) and protection (this paper) are both reversed by Y-27632, an inhibitor of the RhoA effector ROCK. Together, these observations imply that RhoA activation is a necessary but not sufficient component of both signaling pathways and that other signal transducers coordinate with RhoA activation to generate downstream signaling networks that either inhibit axon outgrowth or protect axons from acute toxicity (Figure 4).

The roles of the newly described MAG receptors β1-integrin (*18*) and PirB (*19*) in axon protection have yet to be determined. In this light, it is intriguing to note that integrins and gangliosides can form lateral associations in what has been called a "glycosynapse" (*38*) and that PirB modulates integrin signaling (*39*). Although MAG binds directly to gangliosides GD1a and GT1b independent of proteins (*40*), a functional multicomponent complex is worthy of further investigation.

Regardless of the intricacies of the signaling pathways, the results reported here identify MAG and gangliosides as components involved in the protection of axons from toxic insult and may inform strategies to reduce axon damage and loss.

Methods

Materials

Phosphatidylinositol-specific phospholipase C (from *Bacillus cereus*), vincristine, and Y-27632 (Rho kinase inhibitor) were from Sigma-Aldrich, St. Louis, MO. TAT-Pep5, a cell-permeable $p75^{NTR}$ signaling inhibitor (*27*) was from EMD Biosciences, La Jolla, CA. Sialidase (from *Vibrio cholerae*) was overexpressed in *Escherichia coli* using an expression plasmid kindly provided by Dr. G. Taylor, University of St. Andrews, Fife, Scotland, and was purified as described (*41*). Anti-MAG antibody 513 was prepared as described (*42*). The glycosphingolipid biosynthesis inhibitor P4 was synthesized by Dr. D. Meyers, Synthetic Core Facility, The Johns Hopkins School of Medicine, Baltimore, MD.

Myelin-Absorbed Surfaces

Microwell (96-well) tissue culture plate wells were precoated with poly-D-lysine (125 μg/mL in water, MW >300 000, Sigma) for 1 h, then were washed with water. MAG was extracted from purified myelin membranes using mild detergent and was adsorbed to culture surfaces as described (*14*, *21*). Briefly, myelin was purified (*43*) from brains freshly dissected from adult Sprague−Dawley rats or adult wild-type or *Mag*-null mice (*7*) and stored at −70 °C prior to use. Myelin membranes were suspended at 1 mg of protein/mL in extraction buffer (0.2 M sodium phosphate buffer (pH 6.8), 0.1 M Na_2SO_4, 1 mM EDTA, 1 mM DTT, protease inhibitor mixture (Sigma), and 1% octylglucoside), incubated at 4 °C for 16 h with gentle agitation, then centrifuged at 100 000 × *g* for 1 h at 4 °C. The supernatant was collected and diluted with an equal volume of detergent-free buffer, and an aliquot (50 μL) was added to each well of the poly-D-lysine coated plate. After 4 h at ambient temperature, the plate was washed with Dulbecco's phosphate-buffered saline (PBS) and then with the culture medium (see below) prior to plating freshly prepared cells.

DRGN Neurite Integrity Assay

DRGN were isolated by protease treatment of ganglia dissected from 5−6 day old Sprague−Dawley rats and plated at 10 000 cells/well onto precoated 96-well plates (see above) in 100 μL of growth medium (Neurobasal medium, Invitrogen) containing 50 ng/mL of murine nerve growth factor. Cells were cultured for a total of 48 h at 37 °C in a 5% CO_2 atmosphere. When enzymes, antibodies, or pharmacological inhibitors were used, they were added 1 h after plating and remained in the growth medium for the remainder of the culture period. Vincristine (10 nM final; 25 μL of a 50 nM stock in culture medium) or vehicle (25 μL culture medium, control) was added to the growth medium 24 h after cell plating.

After 48 h of culture (with or without vincristine during the final 24 h), wells were washed with Dulbecco's phosphate-buffered saline (PBS), fixed overnight with 2% paraformaldehyde in PBS, then permeabilized using 0.1% Triton X-100 in PBS. Cells were immunostained with antineuronal class III β-tubulin monoclonal antibody (TUJ1, 1:2000, Covance, Berkeley, CA) followed by Cy3-conjugated antimouse IgG (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA). After washing, images of multiple random fields were captured for analysis using a Nikon TE300 epifluorescent microscope fitted with a Photometrics CoolSNAP HQ2 camera (Roper Scientific, Duluth, GA).

Intact healthy neurites were quantified in images collected from TUJ1-stained cultures using a custom image analysis protocol (NIS-Elements, Nikon, Melville, NY) that captured neurite extent and continuity. Cell bodies were counted based on their shape and high staining intensity, then were deleted from the image. Next, neurites >150 μm long (uninterrupted length) were selected, and their total length was summed. A small number of highly fasciculated neurites with staining intensity equivalent to cell bodies were excluded from quantification. Intact neurite length in each image was divided by the number of DRGN cell bodies in the same image to provide a value of average intact neurite length per neuron. For each experimental condition, four or five random images from each of four to ten independent wells from an average of three independent experiments were analyzed. Data are presented as the mean ± SEM. Pairwise statistical analyses were by Student's *t* test.

Supporting Information Available

Sialidase and P4 efficacy data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Abbreviations

DRGN, dorsal root ganglion neurons; MAG, myelin-associated glycoprotein; NgR, Nogo receptor; P4, (1*R*,2*R*)-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol; PIPLC, phosphatidylinositol-specific protein kinase C; PBS, Dulbecco's phosphate-buffered saline; ROCK, RhoA-associated protein kinase.

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