Molecular dissection of the myelin-associated glycoprotein receptor complex reveals cell type–specific mechanisms for neurite outgrowth inhibition

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Neuronal Nogo66 receptor-1 (NgR1) binds the myelin inhibitors NogoA, OMgp, and myelin-associated glycoprotein (MAG) and has been proposed to function as the ligand-binding component of a receptor complex that also includes Lingo-1, $p75^{NTR}$, or TROY. In this study, we use *Vibrio cholerae* neuraminidase (VCN) and mouse genetics to probe the molecular composition of the MAG receptor complex in postnatal retinal ganglion cells (RGCs). We find that VCN treatment is not sufficient to release MAG inhibition of RGCs; however, it does attenuate MAG inhibition of cerebellar granule neurons. Furthermore, the loss of $p75^{NTR}$ is not sufficient to release MAG inhibition of RGCs, but $p75^{NTR-/-}$ dorsal root ganglion neurons show enhanced growth on MAG compared to wild-type controls. Interestingly, TROY is not a functional substitute for $p75^{NTR}$ in RGCs. Finally, $NgR1^{-/-}$ RGCs are strongly inhibited by MAG. In the presence of VCN, however, $NgR1^{-/-}$ RGCs exhibit enhanced neurite growth. Collectively, our experiments reveal distinct and cell type–specific mechanisms for MAG-elicited growth inhibition.

Introduction

One well-characterized inhibitor of axonal growth is myelin-associated glycoprotein (MAG), an Ig superfamily member expressed by myelinating glia (Filbin, 2003). In the central nervous system (CNS), MAG is localized to periaxonal oligodendroglial membranes of myelin sheaths and participates in axon–glia interactions (Quarles, 2007). Germline ablation of *MAG* is not sufficient to promote axonal regeneration in vivo (Bartsch et al., 1995), but acute inactivation of MAG in retina-optic nerve cultures leads to enhanced optic nerve regeneration (Wong et al., 2003). In addition to its role as a regulator of neuronal growth, MAG has also been shown to regulate the long-term stability and integrity of axon-myelin associations in the optic nerve (Bartsch et al., 1997) and other fiber systems (Pan et al., 2005). The mechanisms that enable MAG to exert its pleiotropic effects are not well understood and are only now starting to be defined.

MAG is a member of the Siglec family of sialic acid–binding Ig-lectins with an ectodomain comprised of five Ig-like repeats (Crocker, 2002). MAG binds to the neuronal cell surface and inhibits growth in a sialic acid–dependent *Vibrio cholerae* neuraminidase (VCN)–sensitive manner (Kelm et al., 1994; DeBellard et al., 1996). Select gangliosides, including GD1a and GT1b, support MAG binding in a sialic acid–dependent manner, and postnatal cerebellar granule neurons (CGNs) isolated from mice lacking complex gangliosides are substantially less inhibited by MAG, indicating that gangliosides play an important role in MAG inhibitory neuronal responses (Vyas and Schnaar, 2001; Vyas et al., 2002). A soluble fusion protein of MAG comprised of the first three Ig repeats, MAG(1–3)-Fc, binds to neurons in a sialic acid–dependent manner but is not sufficient to bring about inhibition (Tang et al., 1997). This suggests that sialic acid–independent sites located in Ig repeats 4 or 5 of the MAG ectodomain are important for neurite outgrowth inhibition.

More recently, MAG has been found to interact with members of the Nogo receptor family, including neuronal Nogo66 receptor (NgR)-1 and NgR2 (Domeniconi et al., 2002; Liu et al., 2002; Venkatesh et al., 2005). NgR1 has been proposed to function as the ligand-binding component of a tripartite NgR1–$p75^{NTR}$–Lingo-1 receptor complex that signals MAG inhibition (Wang et al., 2002; Yamashita et al., 2002; Mi et al., 2004).

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Abbreviations used in this paper: CGN, cerebellar granule neuron; CNS, central nervous system; DRG, dorsal root ganglion; MAG, myelin-associated glycoprotein; NgR, neuronal Nogo66 receptor; RGC, retinal ganglion cell; VCN, *Vibrio cholerae* neuraminidase.

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Upon MAG binding to the neuronal cell surface, $p75^{NTR}$ undergoes α- and γ-secretase–dependent proteolytic cleavage, and processing of $p75^{NTR}$ is important for RhoA activation and subsequent inhibition of neurite outgrowth (Domeniconi et al., 2005). Similar to $p75^{NTR}$, the structurally related protein TROY associates with NgR1 and Lingo- 1. In the mature CNS, $p75^{NTR}$ expression is restricted, and TROY has been proposed to serve as a functional substitute in neurons that lack $p75^{NTR}$ (Park et al., 2005; Shao et al., 2005). In spite of the growing number of cell surface receptor components implicated in MAG inhibition, their role and relative contribution to growth inhibition in different cell types has not yet been examined. In this study, we provide evidence that MAG uses distinct and cell type–specific mechanisms to signal growth inhibition in different neuronal cell types, a finding that may have important implications for the development of strategies aimed at promoting neural repair after CNS injury.

Results and discussion

Loss of terminal sialic acids attenuates MAG inhibition in a cell type–specific manner

Neurite outgrowth of postnatal retinal ganglion cells (RGCs), a population of myelinated CNS neurons, is strongly inhibited by MAG. On CHO-MAG feeder cells, Thy-1–immunopanned RGCs from postnatal day (P) 7–10 rat retina are strongly inhibited (neurite length = 10.2 ± 0.6 μm) compared with control CHO cocultures (neurite length = 26.0 ± 1.6 μm). To examine whether sialoglycans are important for MAG-mediated inhibition of RGCs, cultures were treated with increasing concentrations of VCN to remove cell surface terminal sialic acids (Fig. 1, A and B). Interestingly, neurite length on CHO-MAG cells is not significantly enhanced in the presence of VCN either at 2.5 (11.6 ± 0.7 μm; P = 0.427) or 5.0 mU/ml (11.5 ± 1.3 μm; P = 0.569) of enzyme when compared with CHO-MAG control cultures not treated with VCN (10.2 ± 0.6 μm). Increasing the VCN dose to 7.5 mU/ml inhibits the growth of RGCs and results in significantly reduced neurite length on control CHO cells (20.9 ± 1.9 μm; P = 0.012). Together, our results suggest that sialoglycan-independent mechanisms are sufficient for MAG inhibition of RGCs.

As an additional cell type to examine whether the loss of terminal sialic acids is important for MAG inhibition, we used rat P7–8 CGNs. Neurite outgrowth of CGNs cultured on CHO-MAG cells (19.0 ± 0.8 μm) is strongly inhibited compared with CGNs grown on CHO cells (38.6 ± 1.3 μm). Consistent with a previous study (Vyas et al., 2002), we found that in the presence of 7.5 mU/ml VCN, neurite length on CHO-MAG is significantly increased (25.0 ± 1.3 μm) compared with untreated CGNs on CHO-MAG (19.0 ± 0.8 μm; P < 0.001). Neurite outgrowth on CHO cells is not significantly altered in the presence (38.9 ± 1.6 μm) or absence of VCN (38.6 ± 1.3 μm; P = 0.838). Increasing the VCN dose to 15 mU/ml did not lead to any further promotion of the neurite growth of CGNs on CHO-MAG (Fig. 1, C and D; and not depicted). To assess VCN activity, we took advantage of a previous observation that NgR1 and NgR2 show a VCN-dependent drop in molecular weight (Venkatesh et al., 2005). Because of the limited yield of immunopanned RGCs, hippocampal neurons were treated for

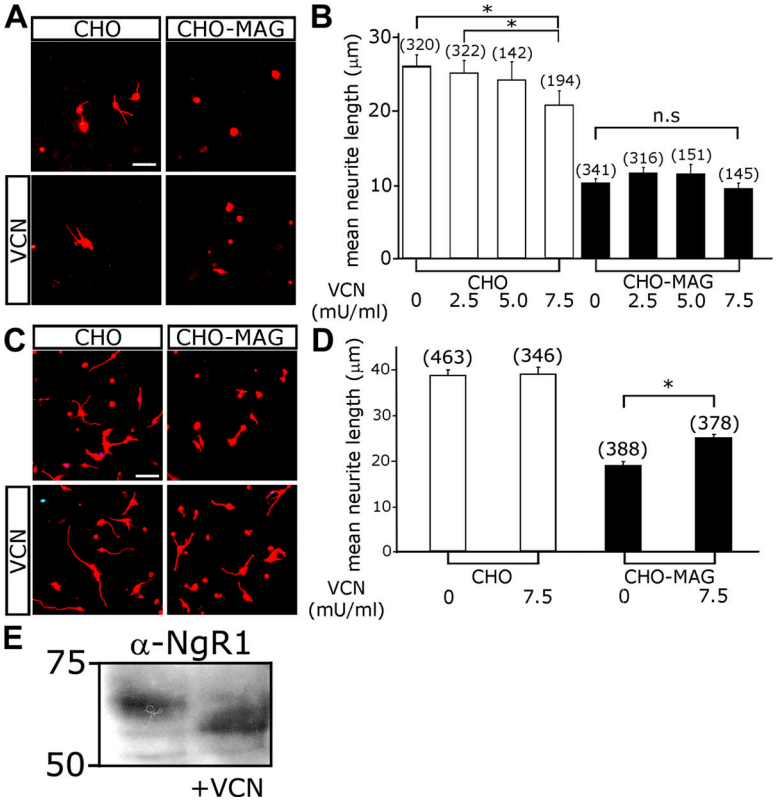

Figure 1. **Cell type–specific requirement of terminal sialic acids for MAG inhibition.** (A–D) P7–10 rat retinal (RGCs; A) and P7–8 rat cerebellar (CGNs; C) neurons were plated on CHO (white bars) or CHO-MAG (black bars) feeder layers. VCN was added to the culture medium at the indicated concentrations. (B and D) Quantification of the neurite length of TuJ1-stained cultures. The number of neurites measured for each condition is indicated in parentheses. Results are presented as mean neurite length ± SEM (error bars). One-way analysis of variance with post-hoc pairwise comparisons (Holm-Sidak method). *, P < 0.001. (E) To assess whether VCN treatment of primary neurons results in a loss of terminal sialic acids, hippocampal neurons treated for 4 h with 2.5 mU/ml VCN were lysed, separated by SDS-PAGE, and subjected to anti-NgR1 immunoblotting. In the absence of VCN, NgR1 runs at an apparent molecular mass of 65 kD. Upon VCN treatment, an ∼3.0-kD drop in the molecular mass of NgR1 is observed, confirming that terminal sialic acids were removed successfully. Bars, 50 μm.

4 h with 2.5 mU/ml VCN. Immunoblotting of lysed cells showed an ~3-kD shift in the molecular mass of NgR1, indicating that terminal sialic acid moieties were removed successfully (Fig. 1 E). In addition, we show that VCN treatment of feeder cells does not change neuronal growth behavior (Fig. S1, A and B; available at http://www.jcb.org/cgi/content/full/jcb.200702102/DC1). Together, our results provide evidence for a neuronal cell type–specific requirement of terminal sialic acids for MAG inhibition.

Neuronal cell type–specific requirement of $p75^{NTR}$ for MAG inhibition

To begin to address whether previously identified protein components of the MAG receptor complex are necessary for neurite outgrowth inhibition, RGCs from wild-type and $p75^{NTR}$-deficient mice were assayed for MAG responsiveness (Fig. 2). On CHO cells, the neurite length of wild-type (33.8 ± 1.8 μm) and $p75^{NTR-/-}$ RGCs (33.1 ± 1.7 μm) is indistinguishable. Furthermore, on CHO-MAG cells, the neurite length of wild-type (15.0 ± 0.9 μm) and $p75^{NTR-/-}$ RGCs (15.2 ± 1.0 μm) is very similar (Fig. 2, A and B). Statistical analysis revealed no significant difference in neurite length between the two genotypes (P = 0.929).

In a parallel approach, we examined MAG inhibition of wild-type and $p75^{NTR-/-}$ dorsal root ganglion (DRG) neurons. On CHO control cells, the neurite length of wild-type (79.9 ± 2.5 μm) and $p75^{NTR-/-}$ (78.6 ± 2.8 μm) DRG neurons is comparable. Consistent with previous studies (Wang et al., 2002; Yamashita et al., 2002), we found that postnatal DRG neurons from $p75^{NTR}$ mutants (64.8 ± 1.8 μm) are disinhibited on MAG compared with wild-type controls (53.4 ± 1.7 μm; Fig. 2, C and D). The disinhibition of $p75^{NTR-/-}$ neurons is statistically significant (P < 0.001) but still incomplete, and fiber length is decreased compared with CHO control cultures. Previously, it was reported that CGNs of $p75^{NTR-/-}$ and wild-type mice show strong and comparable inhibition on crude CNS myelin (Zheng et al., 2005). To expand on this study, we examined the importance of $p75^{NTR}$ in the MAG-mediated inhibition of CGNs. On CHO-MAG cells, wild-type (14.4 ± 0.7 μm) and $p75^{NTR}$-deficient (12.0 ± 0.4 μm) CGNs are both strongly inhibited (Fig. S2, A and B; available at http://www.jcb.org/cgi/content/full/jcb.200702102/DC1). On CHO cells, the neurite length of wild-type (28.3 ± 1.0 μm) and $p75^{NTR-/-}$ (30.7 ± 0.8 μm) CGNs is comparable. Importantly, in the presence of the Rho kinase inhibitor Y27632 or dibutyryl-cAMP, the MAG inhibition of $p75^{NTR}$-deficient CGNs is completely released (Fig. S2 B). Together, our findings suggest that MAG inhibits the growth of RGCs and CGNs through activation of the RhoA pathway in a $p75^{NTR}$-independent manner.

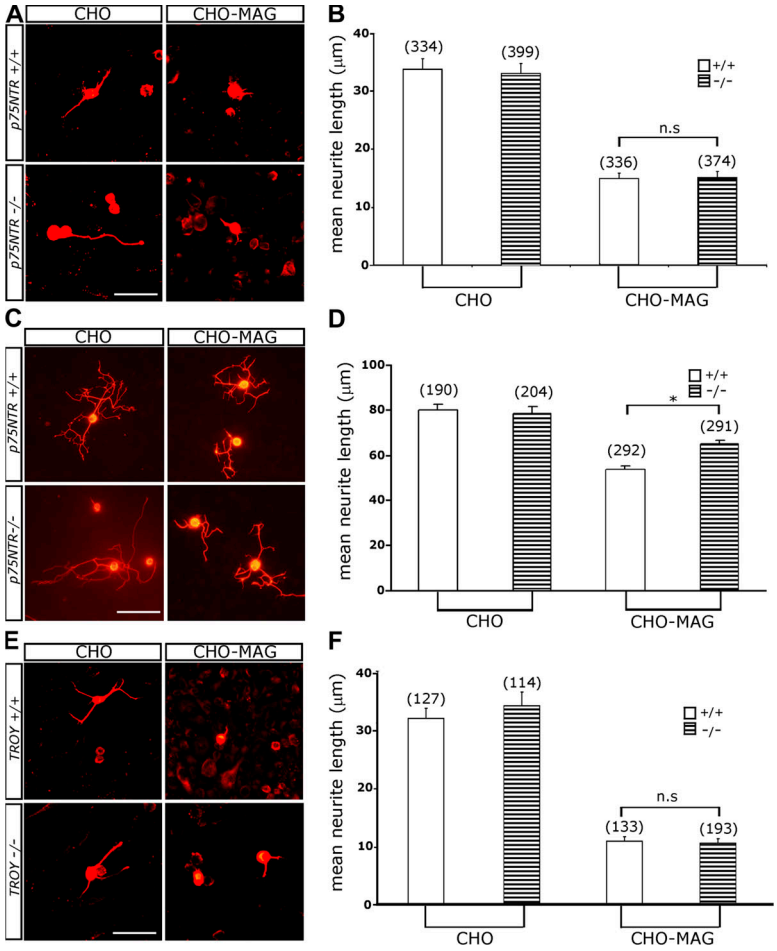

Figure 2. **Limited role for the TNF receptor family members $p75^{NTR}$ and *TROY* in MAG inhibition.** (A–D) P7–10 RGCs (A) and P14 DRGs (C) from wild-type ($p75^{NTR+/+}$; white bars) and mutant ($p75^{NTR-/-}$; striped bars) littermates were plated on CHO or CHO-MAG feeder layers. (E and F) P8 wild-type ($TROY^{+/+}$; white bars) or mutant ($TROY^{-/-}$; striped bars) RGCs plated on CHO or CHO-MAG cells. (B, D, and F) Quantification of the neurite length of TuJ1-stained cultures. The number of neurites measured for each condition is indicated in parentheses. Results are presented as mean neurite length ± SEM (error bars). One-way analysis of variance with post-hoc pairwise comparisons (Dunn's method). *, P < 0.001. Bars (A and E), 25 μm; (C), 50 μm.

In RGCs, TROY is not a functional substitute for $p75^{NTR}$

Although $p75^{NTR}$ is strongly expressed in postnatal CGNs, there is little, if any, $p75^{NTR}$ present in postnatal and adult RGCs either in steady state (Hu et al., 1998) or after optic nerve injury (Hu et al., 1999; Hirsch et al., 2000). In neurons that lack $p75^{NTR}$, the TNF receptor family member TROY has been proposed to serve as a functional substitute (Park et al., 2005; Shao et al., 2005). Because TROY is expressed in RGCs (Park et al., 2005), we asked whether *TROY*-deficient RGCs are less responsive to MAG inhibition. As shown in Fig. 2 (E and F), neurite outgrowth of RGCs isolated from P8 *TROY* mutant (10.4 ± 0.8 μm) and wild-type (10.8 ± 0.8 μm) pups is strongly inhibited by MAG. No significant difference in neurite length was observed between $TROY^{-/-}$ (34.4 ± 2.4 μm) and wild-type (32.2 ± 1.8 μm) RGCs plated on CHO cells (P = 0.305). In a parallel experiment, we examined the MAG responsiveness of CGNs isolated from $TROY^{-/-}$ mice and found no significant release of growth inhibition (P = 0.689; Fig. S2, C and D). Together, these findings suggest that TROY does not serve as a functional substitute for $p75^{NTR}$ in RGCs and is not necessary to bring about MAG inhibition in RGCs or CGNs.

Combined loss of *NgR1* and terminal sialic acids attenuates MAG inhibition of RGCs

Next, we assessed the role of *NgR1* in the MAG-mediated inhibition of RGCs (Fig. 3). When plated on CHO cells, neurite outgrowth of wild-type RGCs (29.5 ± 1.8 μm) and $NgR1^{-/-}$ RGCs (24.7 ± 1.4 μm) is robust, but $NgR1^{-/-}$ RGC neurite length is decreased by 16.3% compared with wild-type littermate controls. Although there was a statistically significant difference between wild-type and $NgR1^{-/-}$ RGC neurite outgrowth on control CHO cells (P = 0.006), the percent outgrowth inhibition of wild-type (57%) and $NgR1^{-/-}$ RGCs (60%) on CHO-MAG cells is very similar (Fig. 3 B). On CHO-MAG cells, the neurite length of $NgR1^{-/-}$ RGCs (9.8 ± 0.7 μm) is decreased compared with wild-type RGCs (12.0 ± 0.8 μm; Fig. 3 C), suggesting that the reduced growth of $NgR1^{-/-}$ RGCs is substrate independent. Thus, compared with wild-type RGCs, $NgR1^{-/-}$ RGCs do not display enhanced fiber growth in the presence of MAG.

To expand on this observation, we considered the possibility that MAG uses multiple and perhaps independent pathways to signal growth inhibition. Consistent with this idea, we found that the combined loss of terminal sialic acids and *NgR1* in RGCs leads to a significant increase in neurite length on CHO-MAG cells compared with untreated controls (Fig. 3 C). On CHO-MAG cells, the neurite length of *NgR1*-null RGCs increases significantly from 9.8 ± 0.7 to 14.1 ± 1.2 μm after VCN treatment (P = 0.036). MAG inhibition of wild-type RGCs treated with VCN is not significantly attenuated (P = 0.108). Importantly, the release of MAG inhibition of *NgR1*-deficient neurons treated with VCN is significant but not complete. This suggests the existence of additional mechanisms for MAG inhibition in RGCs. Importantly, in the presence of the Rho kinase inhibitor Y27632 or dibutyryl-cAMP, MAG inhibition of RGCs is fully released (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200702102/DC1). Together, our data imply that there are multiple and independent pathways for MAG to bring about neurite outgrowth inhibition in RGCs.

Cell type–specific mechanisms for MAG inhibition

In this study, we used a mouse genetic approach combined with the enzymatic removal of terminal sialic acid moieties to dissect the molecular composition of the neuronal receptor complex that signals MAG inhibition. The principal findings of our study

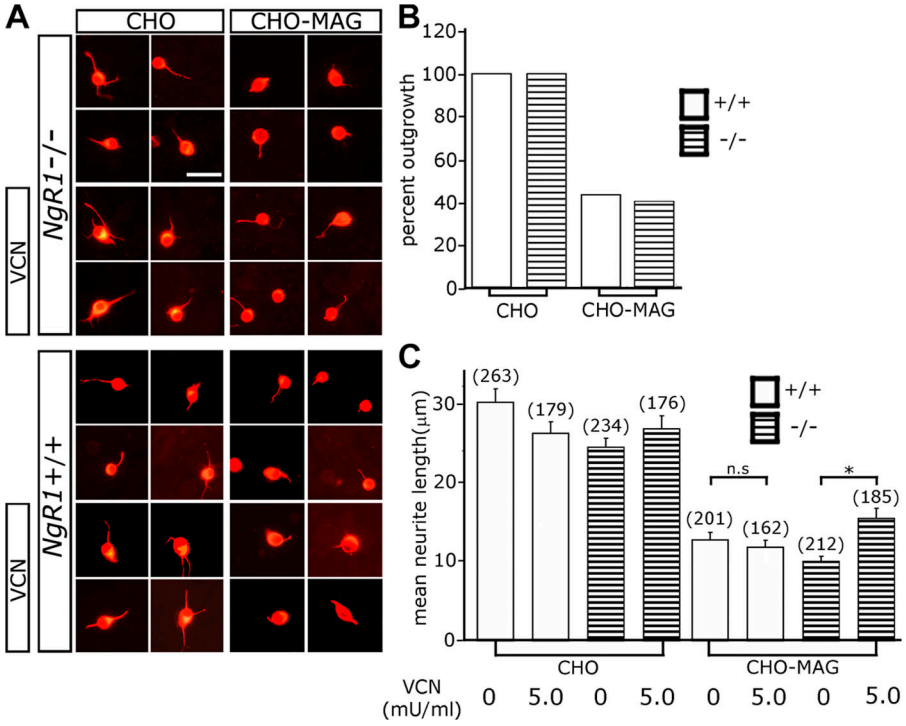

Figure 3. **Loss of terminal sialic acids unmasks a role for *NgR1* in MAG inhibition.** (A) P7–10 RGCs from wild-type ($NgR1^{+/+}$; white bars) or mutant ($NgR1^{-/-}$; striped bars) mice were cultured on CHO or CHO-MAG cells in the presence or absence of VCN and stained with TuJ1. (B) Quantification of the neurite length of cultures not treated with VCN revealed no difference in MAG inhibition between $NgR1^{-/-}$ and wild-type RGCs. (C) Quantification of the neurite length of VCN-treated cultures revealed an additive effect of VCN and the loss of $NgR1^{-/-}$ in overcoming MAG inhibition. The number of neurites measured for each condition is indicated in parentheses. Results are presented as mean neurite length ± SEM (error bars). One-way analysis of variance with post-hoc pairwise comparisons (Holm-Sidak method). *, P < 0.05. Bar, 25 μm.

are as follows: (1) loss of terminal sialic acids in CGNs but not RGCs is sufficient to attenuate MAG inhibition; (2) in DRG neurons but not in RGCs or CGNs, *p75^{NTR}* is important for MAG-elicited inhibition; (3) *TROY*-deficient RGCs and CGNs are strongly inhibited by MAG; and (4) loss of *NgR1* in RGCs is not sufficient to attenuate MAG inhibition, but the combined loss of *NgR1* and terminal sialic acids in RGCs attenuates MAG inhibition. Based on these observations, we conclude that MAG uses distinct and cell type–specific mechanisms to signal neurite outgrowth inhibition.

The neuronal distribution of receptor components implicated in MAG inhibitory signaling is distinct and only partially overlapping (Pioro and Cuello, 1990a,b; Lauren et al., 2003; Park et al., 2005; Venkatesh et al., 2005; Barrette et al., 2007; Satoh et al., 2007). When coupled with previous findings that MAG inhibits a broad spectrum of postnatal neurons (DeBellard et al., 1996), this implies the existence of distinct and perhaps cell type–specific mechanisms for MAG inhibition. Initially, our studies focused on RGCs, a population of heavily myelinated neurons. Remarkably, neither the loss of sialoglycans nor individual components of the NgR1 receptor complex alone is sufficient to overcome MAG inhibition in RGCs. Interestingly, when combined with VCN treatment, the loss of *NgR1* in RGCs leads to a significant disinhibition on MAG substrate ($P = 0.036$). The release of inhibition in VCN-treated *NgR1*$^{-/-}$ RGCs is still incomplete when compared with CHO control cultures. We conclude that neither the selective loss of *NgR1* nor the enzymatic cleavage of sialoglycans alone is sufficient to overcome MAG inhibition of RGCs. When combined, however, enzymatic removal of terminal sialic acids unmasks a contribution of *NgR1* in MAG inhibition. This suggests that NgR1 and sialoglycans function independently and in redundant signaling pathways to bring about MAG inhibition.

Nogo receptors and terminal sialic acids participate in MAG inhibition

Select gangliosides and the neuronal glycoproteins NgR1 and NgR2 have been shown to support MAG binding in a sialic acid–dependent manner. Functional studies revealed that CGNs treated with VCN or CGNs isolated from mice deficient for complex gangliosides are more resistant to MAG inhibition (DeBellard et al., 1996; Vyas et al., 2002). Previously, it has been found that the first three Ig repeats of MAG bind to neurons in a sialic acid–dependent manner but are not sufficient to inhibit growth (Tang et al., 1997).

In spite of the broad distribution of MAG-binding gangliosides, we find that enzymatic removal of terminal sialic acids is not sufficient to attenuate the MAG inhibition of RGCs. This is in marked contrast to CGNs, a neuronal cell type in which the enzymatic removal of terminal sialic acids substantially attenuates MAG inhibition (Vyas et al., 2002). The differences in MAG inhibition observed between VCN-treated CGNs and RGCs is unexpected and suggests that MAG uses cell type–specific mechanisms to bring about inhibition. When compared with RGCs, CGNs are a population of largely unmyelinated neurons that express low levels of NgR1 and lack NgR2 protein expression (Venkatesh et al., 2005; Zheng et al., 2005). This suggests that Nogo receptors play a minor role, if any, in the MAG-mediated inhibition of CGNs. In RGCs, however, NgR1 and NgR2 are more abundantly expressed, providing a potential explanation for why the selective loss of terminal sialic acids in RGCs is not sufficient to attenuate MAG inhibition. In this model, NgR1 and NgR2 would have to participate in MAG inhibition in the absence of terminal sialic acids. Consistent with this idea, NgR1 and NgR2 both harbor multiple MAG docking sites, including sialic acid–dependent as well as sialic acid–independent sites (Venkatesh et al., 2005). We propose that in the presence of VCN, sialic acid–independent mechanisms of NgR1 and NgR2 are sufficient to bring about MAG inhibition. Furthermore, the incomplete release of MAG inhibition in VCN-treated *NgR1*-null RGCs may be a reflection of NgR2-dependent mechanisms. Consistent with the finding that *p75$^{NTR-/-}$* and *TROY*$^{-/-}$ RGCs are still inhibited by MAG, NgR2 does not support the binding of p75^{NTR} or TROY (unpublished data), suggesting novel, as yet unidentified signal transduction mechanisms for NgR2. Future studies are needed to determine whether the loss of MAG-binding gangliosides combined with the functional ablation of NgR1 and NgR2 is sufficient to fully overcome MAG inhibition in RGCs and other neuronal cell types.

Limited role of the TNF receptor family members p75^{NTR} and TROY in MAG inhibition

The transmembrane-spanning proteins Lingo-1, p75^{NTR}, and TROY have been identified as the signal transducing components in the NgR1 receptor complex. Upon MAG binding to the neuronal cell surface, p75^{NTR} undergoes regulated intramembrane proteolysis, and release of the intracellular domain of p75^{NTR} is important for RhoA activation and inhibition of neurite outgrowth (Domeniconi et al., 2005). Our functional studies with *p75^{NTR}*-deficient neurons revealed that p75^{NTR} is important in DRG neurons but not in RGCs or CGNs for MAG inhibition (Figs. 2 and S2). This suggests a more limited and cell type–specific requirement for p75^{NTR}. Commensurate with this observation, an independent study (Zheng et al., 2005) found that *p75^{NTR}*-deficient DRG neurons are more resistant to CNS myelin inhibition than wild-type littermate controls. Under similar experimental conditions, no difference in inhibition between CGNs of wild-type and *p75^{NTR}*-deficient mice was observed (Zheng et al., 2005). In the present study, we expand on these findings by showing that *p75^{NTR}* in DRG neurons but not in CGNs participates in MAG inhibition. It is of interest that *p75$^{NTR-/-}$* CGNs are disinhibited on Nogo66 but not on MAG substrate (Zheng et al., 2005), This suggests that Nogo66 and MAG use distinct receptor systems to bring about growth inhibition. Because CGNs do not express the MAG-specific receptor NgR2, the mechanism used by MAG to inhibit the growth of *p75$^{NTR-/-}$* CGNs is unknown.

RGCs express low levels of p75^{NTR}, and, after injury to the optic nerve, p75^{NTR} is not up-regulated in RGCs (Hu et al., 1998, 1999; Hirsch et al., 2000). Consistent with this, RGCs from *p75^{NTR}* mice are strongly inhibited by MAG. TROY is expressed in RGCs and has been proposed to serve as a functional substitute in neurons that lack *p75^{NTR}* (Park et al., 2005; Shao et al., 2005).

However, similar to wild-type controls, *TROY*$^{-/-}$ RGCs are strongly inhibited by MAG. This suggests that in RGCs, TROY does not serve as a functional substitute for p75^{NTR}, a cell type that normally lacks p75^{NTR} expression. Consistent with our functional studies, it was recently reported that TROY has a restricted neuronal expression in the adult mouse CNS (Barrette et al., 2007).

Implications for axonal regeneration after CNS injury

Commensurate with its pleiotropic effects, a growing number of MAG receptors has been identified. However, their relative contribution to MAG-elicited outgrowth inhibition has not yet been examined systematically. In this study, we provide evidence for cell type–specific mechanisms for MAG-elicited growth inhibition, an observation that may have important implications for the development of strategies aimed at overcoming the growth inhibitory barrier of adult CNS myelin. We propose that depending on the CNS fiber tracts injured, cell type–specific strategies may be needed to promote axonal growth and regeneration. It has not yet been examined whether other myelin inhibitors, including Nogo-A and OMgp, use cell type–specific mechanisms to inhibit neuronal growth. The lack of robust regenerative growth in spinal cord–injured mice with germline ablation of individual receptor components (Kim et al., 2004; Song et al., 2004; Zheng et al., 2005; Cafferty and Strittmatter, 2006) or myelin inhibitory proteins (Bartsch et al., 1995; Kim et al., 2003; Simonen et al., 2003; Zheng et al., 2003) suggests that multiple and at least partially redundant mechanisms exist to bring about CNS myelin inhibition. Our studies also provide evidence that within a specific neuronal cell type, multiple and partially redundant pathways contribute to MAG inhibition. Based on these findings, the existence of one major cell surface receptor system that serves as a convergence point for multiple myelin inhibitors appears unlikely. Additional studies are needed to determine whether the combined loss of multiple receptors or the ablation of multiple growth inhibitors leads to a more robust neuronal growth response in vitro and after CNS injury in vivo.

Materials and methods

Mice and neurite outgrowth assays

Mice mutant for *NgR1* (provided by M. Tessier-Lavigne, Genentech, South San Francisco, CA; Zheng et al., 2005), *p75^{NTR}* (provided by H. Federoff, Aab Institute for Biomedical Research, Rochester, NY; Lee et al., 1992), and *TROY* (Shao et al., 2005) have been described previously. To assay MAG-mediated growth inhibition, primary neurons were cultured on confluent monolayers of CHO cells that either express recombinant long MAG on their surface or on CHO cells lacking long MAG (Mukhopadhyay et al., 1994). P7–8 rat and mouse CGNs were purified in a discontinuous Percoll gradient (Hatten, 1985). P14 mouse DRG neurons were dissected and incubated in 0.05% trypsin and 0.1% collagenase, triturated, and cultured in SATO^{+} medium for 18–20 h (Venkatesh et al., 2005). RGCs were isolated by anti-Thy1 immunopanning (Barres et al., 1988). In brief, P7–10 rat or mouse retina was dissected in DME (Cellgro). Retinae were digested in 2 ml DME and 30 μl papain solution (10 U/ml; MP Biomedicals) for 30 min at 37°C. The papain was removed by three washes in DME/10% FBS. The cell suspension was then plated onto 60-mm nontissue culture-coated Petri dishes that were precoated with goat anti–mouse IgG (Jackson ImmunoResearch Laboratories) and anti-Thy1.1 (Serotec, Inc.) for rat or goat anti–mouse IgM (Chemicon) and with anti-Thy1.2 (Sigma-Aldrich) for mouse RGCs. After a 1-h incubation at room temperature, unbound cells were removed by several rinses in PBS. Bound cells were lifted using 0.125% trypsin/EDTA and were washed three times in DME/10% FBS. Cells were resuspended in SATO^{+} medium, plated on CHO feeder layers, and cultured for 20–24 h before fixation (Venkatesh et al., 2005). Rho kinase inhibitor (Y27632; Calbiochem), dibutyryl-cAMP (Sigma-Aldrich), and VCN (Calbiochem) were used at the concentrations indicated and directly added to the culture medium at the time of plating. VCN preferentially cleaves α2-3–, α2-6–, and, to a lesser extent, α2-8–linked terminal sialic acids. The loss of sialic acids in VCN-treated neurons results in a molecular weight shift of NgR1 that was assessed by immunoblotting as described previously (Venkatesh et al., 2005).

Quantitative analysis of neurite length

Quantification of neurite outgrowth was performed as described previously (Venkatesh et al., 2005). In brief, cultures were fixed in 4% PFA and blocked in 1% horse serum and 0.1% Triton X-100 in PBS, and neurons were stained with anti–class III β-tubulin antibody (TuJ1; Promega). Images were taken using an inverted microscope (IX71; Olympus) attached to a digital camera (DP70; Olympus), and neurite length was quantified using UTHSCSA ImageTool for Windows. For quantification of neurite outgrowth, processes equal or longer to approximately one cell body diameter were measured. For all experiments, the mean and SEM of neurite length was determined from multiple independent experiments. Several independent experiments were performed for quantification of the neurite length of primary neurons isolated from the following mice: *p75^{NTR}* mice (DRGs, $n = 3$ [+/+] and $n = 3$ (−/−); CGNs, $n = 5$ [+/+] and $n = 5$ [−/−]; and RGCs, $n = 6$ [+/+] and $n = 8$ [−/−]), *TROY* mice (RGCs, $n = 3$ [+/+] and $n = 3$ [−/−]; and CGNs, $n = 2$ [+/+] and $n = 3$ [−/−]), and *NgR1* mice (RGCs, $n = 5$ [+/+] and $n = 4$ [−/−]). For quantification of the neurite length of mouse RGCs treated with 5 mU VCN, several independent experiments were performed: $n = 5$ (*NgR1*$^{+/+}$) and $n = 4$ (*NgR1*$^{-/-}$). RGCs from *NgR1* wild-type (+/+) and heterozygous (+/−) mice behave similarly in neurite outgrowth assays and were included as controls in some experiments. All data were analyzed using one-way analysis of variance followed by Dunn's or Holm-Sidak post-hoc comparisons. All statistics were performed using SigmaStat 3.0 for Windows (Systat Software).

Online supplemental material

Fig. S1 shows that the loss of cell surface sialic acid moieties on feeder cells does not alter neuronal growth behavior. Fig. S2 shows that *p75^{NTR}* and *TROY* are not necessary for MAG-mediated inhibition of CGNs. Fig. S3 shows that MAG inhibition of RGCs is released in the presence of Rho kinase inhibitor or dibutyryl-cAMP. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200702102/DC1.

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