LDL receptor-related protein-1 is a sialic-acid-independent receptor for myelin-associated glycoprotein that functions in neurite outgrowth inhibition by MAG and CNS myelin

Travis L. Stiles[1,*], Travis L. Dickendesher[2,*], Alban Gaultier[1,3], Anthony Fernandez-Castaneda[1], Elisabetta Mantuano[1], Roman J. Giger[2] and Steven L. Gonias[1,‡]

[1]Department of Pathology, University of California San Diego, La Jolla, CA, 92093, USA
[2]Department of Cell and Developmental Biology and the Neuroscience Program, University of Michigan School of Medicine, Ann Arbor, MI, 48109, USA
[3]Department of Neurosciences, University of Virginia, Charlottesville, VA 22908, USA

*These authors contributed equally to this work.
‡Author for correspondence (sgonias@ucsd.edu).

Accepted 10 October 2012
Journal of Cell Science 126, 209–220

doi: 10.1242/jcs.113191

Summary

In the injured adult mammalian central nervous system (CNS), products are generated that inhibit neuronal sprouting and regeneration. In recent years, most attention has focused on the myelin-associated inhibitory proteins (MAIs) Nogo-A, OMgp, and myelin-associated glycoprotein (MAG). Binding of MAIs to neuronal cell-surface receptors leads to activation of RhoA, growth cone collapse, and neurite outgrowth inhibition. In the present study, we identify low-density lipoprotein (LDL) receptor-related protein-1 (LRP1) as a high-affinity, endocytic receptor for MAG. In contrast with previously identified MAG receptors, binding of MAG to LRP1 occurs independently of terminal sialic acids. In primary neurons, functional inactivation of LRP1 with receptor-associated protein, depletion by RNA interference (RNAi) knock-down, or *LRP1* gene deletion is sufficient to significantly reverse MAG and myelin-mediated inhibition of neurite outgrowth. Similar results are observed when LRP1 is antagonized in PC12 and N2a cells. By contrast, inhibiting LRP1 does not attenuate inhibition of neurite outgrowth caused by chondroitin sulfate proteoglycans. Mechanistic studies in N2a cells showed that LRP1 and p75NTR associate in a MAG-dependent manner and that MAG-mediated activation of RhoA may involve both LRP1 and p75NTR. LRP1 derivatives that include the complement-like repeat clusters CII and CIV bind MAG and other MAIs. When CII and CIV were expressed as Fc-fusion proteins, these proteins, purified full-length LRP1 and shed LRP1 all attenuated the inhibition of neurite outgrowth caused by MAG and CNS myelin in primary neurons. Collectively, our studies identify LRP1 as a novel MAG receptor that functions in neurite outgrowth inhibition.

Key words: LDL receptor-related protein-1, LRP1, Myelin-associated glycoprotein, RhoA, Myelin, Neurite outgrowth, p75NTR

Introduction

Neuronal regeneration in the injured adult mammalian CNS is limited. The inhibitory nature of adult CNS myelin and glial scar tissue contribute to the regenerative failure of severed axons (Schwab et al., 1993; Busch and Silver, 2007; Fawcett, 2009). Several myelin-associated inhibitory proteins (MAIs) have been identified, including members of the reticulon family, myelin associated-glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp) (Filbin, 2003; Yiu and He, 2006; Schwab, 2010). Nogo-A, the largest splice form of the *Nogo/reticulon* 4 gene, is comprised of at least two distinct growth inhibitory regions: amino-Nogo and Nogo66 (Schwab, 2010). MAG is a sialic-acid-recognizing Ig-family lectin (Tang et al., 1997; Vinson et al., 2001; Vyas et al., 2002). Deletion of the lectin activity in MAG disrupts binding to gangliosides and to the Nogo receptor family members, NgR1 and NgR2, yet does not abolish growth inhibition (Cao et al., 2007; Robak et al., 2009).

NgR1 is the ligand-binding portion of a tripartite receptor complex that includes Lingo-1 and p75NTR or TROY (Yiu and He, 2006). This receptor complex participates in growth cone collapse in response to MAG, Nogo66 and OMgp (Kim et al., 2004). Similar to NgR1, paired Ig-like receptor B (PirB) binds Nogo66, MAG and OMgp and participates in growth cone collapse. Loss of PirB, but not NgR1, leads to a significant, yet incomplete release of neurite outgrowth inhibition in response to MAIs (Zheng et al., 2005; Chivatakarn et al., 2007; Atwal et al., 2008). Myelin inhibition also can be released by pre-treating neurons with BDNF or by blocking activation of RhoA (Cai et al., 1999; Schmandke et al., 2007). Nogo and MAG promote association of p75NTR with Rho-GDP Dissociation Inhibitor (RhoGDI), which results in release and activation of RhoA (Yamashita and Tohyama, 2003). Loss of p75NTR in sensory neurons, but not in cerebellar neurons, attenuates MAG and myelin inhibition *in vitro*, suggesting the existence of neuronal cell type-specific signaling mechanisms (Zheng et al., 2005; Mehta et al., 2007; Venkatesh et al., 2007).

LDL receptor-related protein-1 (LRP1) is a type-1 transmembrane receptor that binds over forty structurally and

functionally distinct ligands, mediating their endocytosis and delivery to lysosomes (Strickland et al., 2002). LRP1 also functions in phagocytosis of large particles, including degenerating myelin (Lillis et al., 2008; Gaultier et al., 2009). Neurons in the CNS and PNS express LRP1 (Wolf et al., 1992; Bu et al., 1994; Campana et al., 2006), which is partially localized to axons and neuronal growth cones, in intracellular vesicles and at the cell surface (Steuble et al., 2010).

LRP1 regulates cell-signaling in conjunction with diverse co-receptors, including uPAR, TNFR1, PDGF receptor, Trk receptors, and Frizzled-1 (Webb et al., 2001; Boucher et al., 2003; Zilberberg et al., 2004; Gaultier et al., 2008; Shi et al., 2009). Given the diversity of LRP1 co-receptors, it is reasonable to hypothesize that the activity of LRP1 in cell-signaling may be ligand-specific and cell type-specific. In neurons and neuron-like cell lines, binding of tissue-type plasminogen activator (tPA) or α_2-macroglobulin (α_2M) to LRP1 activates ERK and AKT to promote neurite outgrowth (Qiu et al., 2004; Hayashi et al., 2007; Mantuano et al., 2008; Fuentealba et al., 2009; Shi et al., 2009). This response requires Trk receptor transactivation downstream of phosphorylated c-Src (Shi et al., 2009).

Our previous work demonstrating myelin phagocytosis by LRP1 (Gaultier et al., 2009) prompted us to examine the role of LRP1 in the mechanism by which myelin inhibits axonal regeneration. In this study, we demonstrate that LRP1 is a high-affinity, endocytic MAG receptor, which may be essential for inhibition of neurite outgrowth by MAG and CNS myelin.

Results

MAG binds to LRP1

Full-length LRP1 is a 600-kDa, two-chain transmembrane receptor (Herz et al., 1988). Two of the four clusters of complement-like repeats (CII and CIV) in the extracellular α-chain of LRP1 are responsible for most of the ligand-binding activity of this receptor (Willnow et al., 1994). To screen for proteins in CNS myelin that bind to LRP1, we expressed CII and CIV as Fc-fusion proteins (Fig. 1A). CNS myelin vesicles were purified from rat brain as previously described (Gaultier et al., 2009), solubilized, and incubated with CII-Fc and CIV-Fc. The Fc-fusion proteins were precipitated with Protein A-Sepharose. Associated proteins were identified by LC-MS/MS (Gaultier et al., 2010). In experiments with CII-Fc and CIV-Fc, but not Fc alone, MAG was identified as a candidate binding partner. MAG-derived sequences that were detected by LC-MS/MS, in triplicate studies with CII-Fc and CIV-Fc, are underlined in the complete sequence of MAG (supplementary material Fig. S1).

As a first approach to test whether MAG binds to full-length LRP1, the ectodomain of MAG was expressed as an Fc-fusion protein (MAG-Fc) and incubated with extracts of mouse N2a neuroblastoma cells (Fig. 1B). MAG-Fc and associated proteins were precipitated with Protein A-Sepharose. LRP1 was readily detected in MAG-Fc affinity precipitates by immunoblot analysis using an antibody that detects the 85-kDa β-chain. LRP1 did not co-precipitate with purified Fc, demonstrating specificity in the MAG-LRP1 interaction.

Next, we prepared membrane extracts from rat brain and tested whether endogenous MAG associates with LRP1 in these extracts. Polyclonal rabbit antibodies that are specific for MAG or the LRP1 β-chain were incubated with the brain extracts for 4 h at 4℃ and then precipitated with Protein A/G-Agarose. Non-specific rabbit IgG was added to the brain extracts and precipitated similarly, as a control. Fig. 1C shows that LRP1 co-immunoprecipitated (co-IPed) with MAG from brain extracts and that MAG co-IPed with LRP1. Neither MAG nor LRP1 co-IPed with non-specific IgG.

To test whether MAG binds to LRP1 in a purified system, full-length LRP1 was purified from rat liver (Gorovoy et al., 2010) and incubated for 4 h at 4℃ with MAG-Fc or Fc, which were immobilized on Protein A-Sepharose. Fig. 1D shows that LRP1 was detected in affinity precipitates with MAG-Fc but not Fc, providing evidence for direct association of MAG with full-length LRP1 in a purified system.

Receptor-associated protein (RAP) is an LRP1 chaperone that binds to LRP1 and inhibits binding of other known LRP1 ligands (Williams et al., 1992; Strickland et al., 2002). To test whether RAP inhibits binding of MAG to LRP1, we incubated MAG-Fc with extracts from a second cell line, N20.1, which is an abundant source of LRP1, in the presence and absence of 200 nM RAP, which is expressed as a GST-fusion protein. As control, MAG-Fc was incubated with N20.1 cell extracts in the presence of 200 nM GST. Fig. 1E shows that MAG-Fc bound to LRP1 in the cell extracts and that RAP greatly reduced this interaction.

The affinity-precipitation and IP studies provided qualitative evidence that MAG binds to LRP1. To develop a model system that would allow determination of binding affinities, we performed additional experiments with CII-Fc and CIV-Fc. First, we tested whether CII-Fc and CIV-Fc bind to MAG in rat CNS myelin. Fig. 1F shows that MAG in CNS myelin affinity-precipitated with CII-Fc and CIV-Fc, but not with Fc. To test whether MAG binds to CII-Fc and CIV-Fc in a purified system, we immobilized the fusion proteins on nitrocelluose. Fig. 1G shows that MAG-Fc bound to immobilized CII-Fc and CIV-Fc, but not Fc. These results validated our LC-MS/MS findings, demonstrating that the ligand-binding sequences of LRP1 bind to MAG in a similar fashion as full-length LRP1.

To examine binding of CII-Fc and CIV-Fc to membrane-anchored MAG, we utilized an established model system in which full-length MAG is expressed in COS-7 cells (Dickendesher et al., 2012). CII-Fc and CIV-Fc bound to cell-surface MAG and the level of binding was similar to that observed with NgR^{OMNI}-Fc (Fig. 1H). In NgR^{OMNI}-Fc, Fc is fused to a chimeric form of NgR1 and NgR2 that binds MAG with higher affinity than wild-type NgR1 (Robak et al., 2009). Purified Fc did not bind to MAG-expressing COS-7 cells, indicating that binding of CII-Fc and CIV-Fc is specific. As a control, we incubated CII-Fc, CIV-Fc and NgR^{OMNI}-Fc with COS-7 cells that were transfected to express GFP. Binding was not detected with any of the Fc-fusion proteins. As a second control, we examined binding of CII-Fc and CIV-Fc to COS-7 cells that were transfected to express PirB, which, like MAG, is a member of the Ig superfamily. Neither CII-Fc nor CIV-Fc bound to the PirB-expressing cells (supplementary material Fig. S2A).

Next, we examined the ability of RAP (200 nM) to inhibit binding of CII-Fc and CIV-Fc to MAG-expressing COS-7 cells. GST (200 nM) was added to control cultures. Fig. 1I shows that RAP almost completely inhibited binding of CII-Fc and CIV-Fc to the cells. Importantly, RAP did not affect binding of NgR^{OMNI}-Fc.

MAG binds to LRP1 with high affinity and in a sialic-acid-independent manner

To calculate binding affinities, we incubated MAG-expressing COS-7 cells with increasing concentrations of CII-Fc, CIV-Fc,

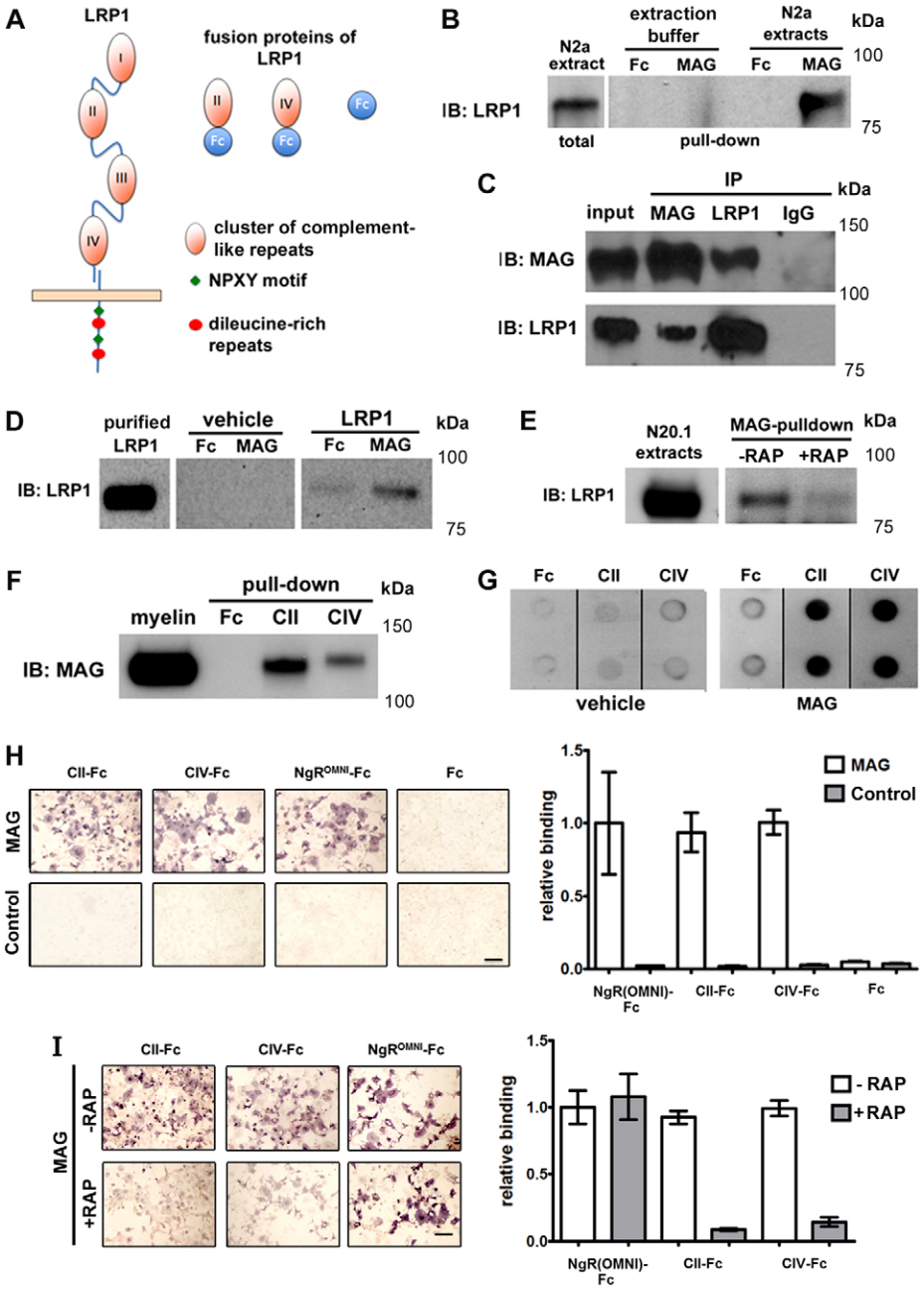

Fig. 1. LRP1 is a MAG receptor. (**A**) Schematic diagram showing the derivation of CII-Fc and CIV-Fc. (**B**) N2a cell extracts were incubated with MAG-Fc or Fc, immobilized on Protein A-Sepharose beads. Precipitated proteins were subjected to immunoblot (IB) analysis for LRP1. (**C**) Endogenous MAG and LRP1 were IPed from membrane preparations of adult rat brain. Non-specific IgG was added as a control. Precipitated proteins were immunoblotted for MAG and LRP1. (**D**) MAG-Fc and Fc were immobilized on Protein A-Sepharose and incubated with purified LRP1 or vehicle. Affinity-precipitated proteins were subjected to immunoblot analysis for LRP1. (**E**) N20.1 cell protein extracts were treated with 200 nM RAP or GST and affinity precipitated with MAG-Fc. Affinity-precipitates were subjected to immunoblot analysis for LRP1. (**F**) CII-Fc, CIV-Fc, and Fc were incubated with purified myelin and precipitated with Protein A-Sepharose. Immunoblot analysis was performed to detect MAG. (**G**) CII-Fc, CIV-Fc and Fc were immobilized in duplicate on nitrocellulose membranes and incubated with MAG-Fc (10 μg/ml) or vehicle. MAG-binding was detected using MAG-specific antibody. (**H**) MAG-expressing and control COS-7 cells were incubated with CII-Fc, CIV-Fc, NgR^{OMNI}-Fc or Fc. Bound fusion proteins were detected by developing the AP reaction. The graph shows binding of each fusion protein relative to NgR^{OMNI}-Fc (means±s.e.m., n=5). (**I**) MAG-expressing COS-7 cells were incubated with CII-Fc, CIV-Fc or NgR^{OMNI}-Fc in the presence of 200 nM RAP or GST (means±s.e.m., n=4). Scale bars: 20 μm.

and NgR^{OMNI}-Fc. In our initial studies, the fusion proteins were pre-clustered with alkaline phosphatase (AP)-conjugated anti-Fc antibody, allowing direct analysis of binding. The results were most accurately fit by non-linear regression to sigmoidal curves, suggesting possible complexity in the binding interactions, beyond a simple single-site model (Fig. 2A). To estimate binding constants, we determined the concentration of ligand that yielded half-maximal saturation. The K_D for NgR^{OMNI}-Fc was 5.7±0.1 nM (n=3). The K_D for CII-Fc was 8.1±0.2 nM (n=3) and the K_D for CIV-Fc was 7.4±0.2 nM (n=3). The equivalent data also were fit to rectangular hyperbolae, assuming a simple, single-site model. The K_D values were largely unchanged, ranging from 5–15 nM for all three fusion proteins, with r values that exceeded 0.9.

Next, we analyzed binding of monomeric fusion proteins, which were not pre-clustered. Once again, the binding data were most accurately fit to sigmoidal curves (supplementary material Fig. S2B). The K_D values were 9.6±0.3 nM for CII-Fc (n=3) and 17±3 nM for CIV-Fc (n=3).

MAG is an Ig-family lectin, which binds to gangliosides and Nogo receptors in a sialic-acid-dependent manner (Vyas et al., 2002; Robak et al., 2009). To test whether the interaction of MAG with LRP1 is sialic-acid dependent, we treated CII-Fc, CIV-Fc, and NgR^{OMNI}-Fc with *V. cholera* neuraminidase (VCN) (Venkatesh et al., 2007; Robak et al., 2009). Fig. 2B shows that treatment of NgR^{OMNI}-Fc with VCN abolished binding to MAG-expressing cells. Treatment of CII-Fc and CIV-Fc with VCN did not inhibit MAG binding.

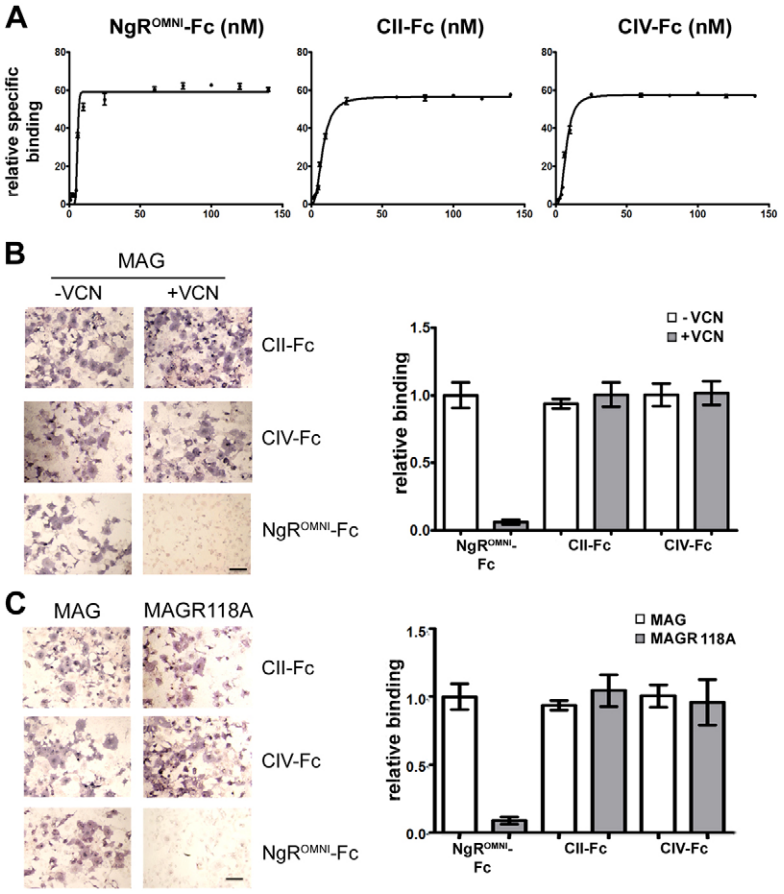

Fig. 2. Comparison of binding of MAG to CII, CIV, and NgR^{OMNI}. (**A**) MAG-expressing COS-7 cells were incubated with increasing concentrations of CII-Fc, CIV-Fc, or NgR^{OMNI}-Fc. The fusion proteins were pre-coupled to AP-conjugated anti-Fc antibody. Binding was detected by developing the AP reaction. The presented results summarize three independent experiments (means±s.e.m.). (**B**) MAG-expressing COS-7 cells were incubated with CII-Fc, CIV-Fc or NgR^{OMNI}-Fc. The ligands were pre-treated with VCN (1 mU/ml) for 1 h or vehicle. Fusion protein binding was assessed and expressed relative to the level observed with NgR^{OMNI}-Fc (means±s.e.m., n=4). (**C**) COS-7 cells that express MAG or MAG^{R118A} were incubated with CII-Fc, CIV-Fc or NgR^{OMNI}-Fc. Binding was assessed (means±s.e.m., n=3). Scale bars: 20 μm.

Next, we examined binding of CII-Fc, CIV-Fc, and NgR^{OMNI}-Fc to COS-7 cells that express MAG^{R118A}. This point mutation in MAG greatly reduces lectin activity (Tang et al., 1997). MAG^{R118A} failed to bind NgR^{OMNI}-Fc, as previously demonstrated (Robak et al., 2009); however, robust binding was still observed with CII-Fc and CIV-Fc (Fig. 2C). These results indicate that the interaction of MAG with LRP1 is not sialic acid dependent. In control experiments, we compared binding of CII-Fc and CIV-Fc to MAG and MAG^{R118A}, using fusion proteins that were not pre-clustered. CII-Fc and CIV-Fc still bound comparably to both variants of MAG (supplementary material Fig. S2C).

LRP1 mediates the endocytosis of MAG

To study endocytosis of MAG, MAG-Fc (25 nM) was incubated for 1 h at 4℃ with N2a cells in the presence of 200 nM RAP or GST (control). The cells were then washed and warmed to 37℃ for 30 min. A mild acid wash was performed so that only internalized MAG-Fc remained cell-associated. By immunofluorescence microscopy, MAG-Fc was internalized and the degree of internalization was substantially inhibited when RAP was added (Fig. 3A). To show that the interaction of MAG-Fc with LRP1 is specific, we expressed receptor protein tyrosine-phosphatase-σ as an Fc-fusion protein (RPTP-Fc) and studied uptake of this fusion protein by N2a cells. Although RPTP-Fc was internalized by N2a cells, the extent of internalization was not inhibited by RAP. In additional control experiments, we incubated MAG-Fc with N2a cells at 4℃, but did not increase the temperature to 37℃ before performing the mild acid wash. MAG-Fc binding was not detected, confirming that the mild acid wash is effective and this assay reports endocytosis.

To further assess the role of LRP1 in MAG-Fc endocytosis, we developed a stable, cloned cell line in which LRP1 was silenced with shRNA in N2a cells. By immunoblot analysis, LRP1 was undetectable in these cells (supplementary material Fig. S3). MAG-Fc (25 nM) was incubated with N2a cells in which LRP1 was silenced and with cells that had been transfected with empty vector. Immunofluorescence microscopy was then performed. MAG-Fc internalization was much greater in the LRP1-expressing cells, compared with cells in which LRP1 was silenced (Fig. 3B). LRP1 gene-silencing did not inhibit internalization of RPTP-Fc.

To independently confirm the results of our immunofluorescence microscopy studies, we analyzed endocytosis by immunoblotting. MAG-Fc was incubated with LRP1-positive and gene-silenced N2a cells, using a protocol that was identical to that applied in our microscopy studies. Fig. 3C shows that LRP1-expressing N2a cells internalized substantially more MAG-Fc, compared with cells in which LRP1 was silenced. RPTP-Fc was internalized by N2a cells, but the extent of internalization was not inhibited by LRP1 gene-silencing.

Finally, we used radioiolabeled MAG-Fc to study endocytosis in N2a cells. Specific endocytosis was defined as the fraction of internalized ^{125}I-MAG-Fc that was inhibited by a 50-fold molar excess of unlabeled MAG-Fc (>80% of total endocytosis in our studies). N2a cells that were treated with 25 nM ^{125}I-MAG-Fc specifically internalized 44±4 fmol ^{125}I-MAG-Fc/mg cell

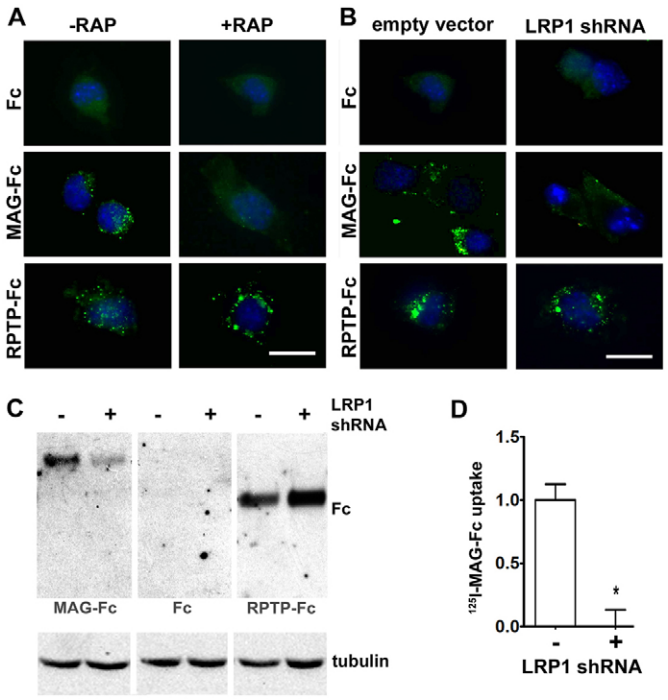

Fig. 3. LRP1 mediates the endocytosis of MAG. (**A**) N2a cells were treated with RAP or GST (200 nM) and then with 25 nM MAG-Fc, RPTP-Fc or Fc. Internalized proteins were visualized by immunofluorescence microscopy. (**B**) N2a cells in which LRP1 was silenced with shRNA and cells transfected with empty vector were incubated with 25 nM MAG-Fc, RPTP-Fc or Fc. Internalization was assessed. (**C**) N2a cells in which LRP1 was silenced and control cells were incubated with 25 nM MAG-Fc, RPTP-Fc or Fc. A mild acid wash was used to remove surface-associated proteins. Proteins extracts were immunoblotted for Fc. (**D**) N2a cells in which LRP1 was silenced and control cells were incubated with 25 nM ^{125}I-MAG-Fc, in the presence or absence of unlabeled MAG-Fc. Specific MAG-Fc internalization was determined (n=3, *P<0.01). Scale bars: 20 μm.

protein/h (n=3). LRP1 gene-silencing completely abolished specific internalization of ^{125}I-MAG-Fc (Fig. 3D).

LRP1 is required for inhibition of neurite outgrowth by MAG

To test whether LRP1 is involved in the pathway by which MAG is known to inhibit neurite outgrowth, first we cultured rat PC12 pheochromocytoma cells and N2a cells on monolayers of CHO cells that express MAG (MAG-CHO) and on control (R2-CHO) cells, which do not express MAG (Mukhopadhyay et al., 1994). RAP (200 nM) was added to antagonize the ligand-binding activity of LRP1. GST (200 nM) was added as a control. As shown in Fig. 4A, MAG-CHO cells strongly inhibited neurite outgrowth by both cell types and RAP significantly reversed this inhibition. As a control, MAG-CHO cells were treated with 200 nM RAP for 48 h in the absence of other cells. MAG expression was not affected by RAP, as determined by immunoblot analysis (supplementary material Fig. S4).

Next, we transiently silenced LRP1 gene expression in PC12 and N2a cells. Control cells were transfected with non-targeting control (NTC) siRNA. LRP1 gene-silencing was 80% and 85% effective in PC12 cells and N2a cells, respectively, as determined by RT-PCR. Similar extents of gene-silencing were evident by

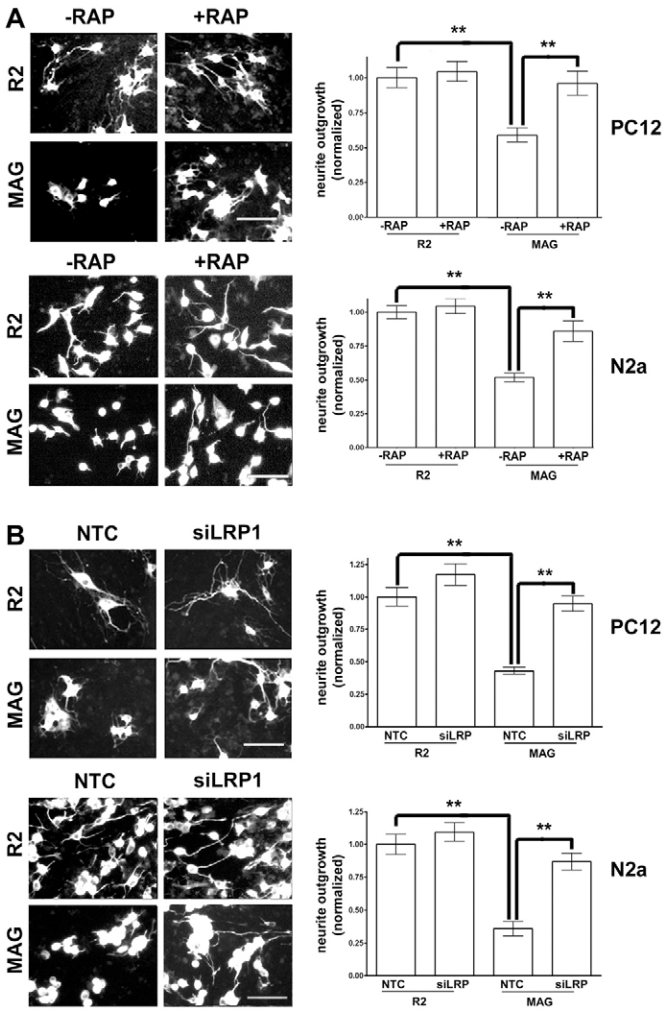

Fig. 4. LRP1 antagonism or gene-silencing attenuates the effects of MAG-CHO cells on neurite outgrowth in PC12 and N2a cells. (**A**) PC12 and N2a cells were plated on R2-CHO or MAG-CHO cells and cultured for 48 h in the presence of RAP or GST (200 nM). Neurite outgrowth was determined. Results were normalized against those obtained when cells were plated on R2-CHO cells with GST (means±s.e.m., n=3, **P<0.01). (**B**) PC12 and N2a cells were transfected with NTC or LRP1-specific siRNA (siLRP1) prior to plating on R2-CHO or MAG-CHO cells for 48 h. Neurite outgrowth was determined (means±s.e.m., n=3, **P<0.01). Scale bars: 100 μm.

immunoblot analysis (supplementary material Fig. S5A,B). No change in cell viability was detected by CCK8 assay (supplementary material Fig. S5B). Fig. 4B shows that LRP1 gene-silencing significantly reversed the inhibition of neurite outgrowth observed in co-cultures with MAG-CHO cells. These results suggest that LRP1 expression in neuron-like cells is important for MAG-mediated neurite outgrowth inhibition.

To further explore this hypothesis, we conducted neurite outgrowth experiments with primary rat cerebellar granule neurons (CGNs). The CGNs were co-cultured with MAG-CHO or R2-CHO cells, in the presence of 200 nM RAP or GST. Fig. 5A shows that significant inhibition of neurite outgrowth was observed in CGNs plated on MAG-CHO cells and that RAP significantly reversed this inhibition.

Next, we silenced LRP1 expression in CGNs using siRNA. At the mRNA level, silencing was ~60% effective; however,

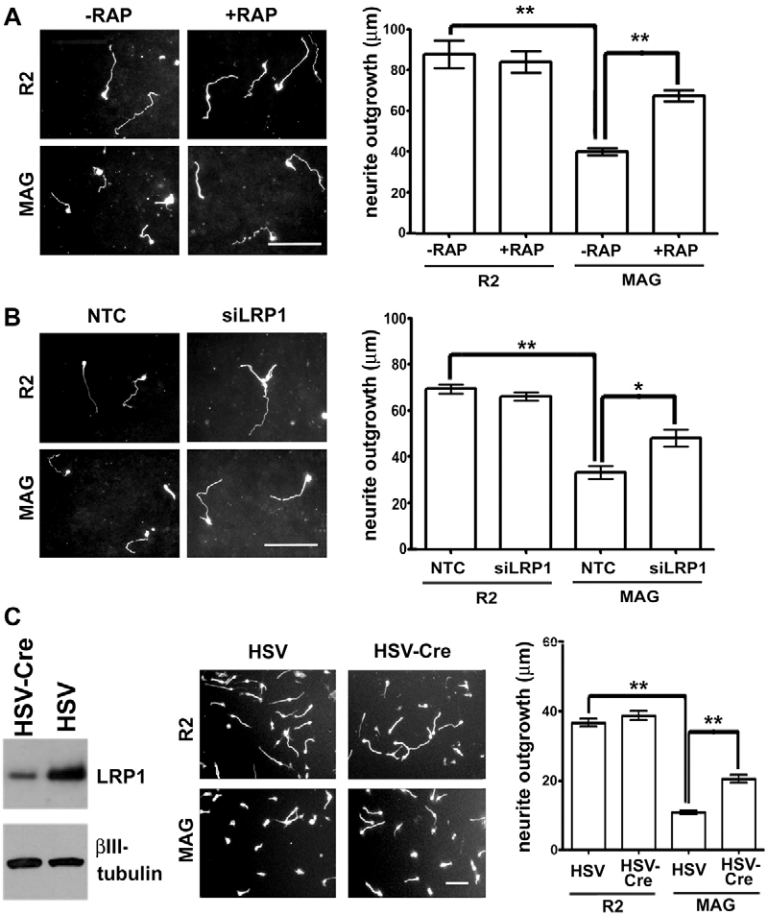

Fig. 5. LRP1 inactivation attenuates the effects of MAG-CHO cells on neurite outgrowth in CGNs. (**A**) CGNs pre-treated with RAP or GST were plated on MAG-CHO or R2-CHO cells and cultured for 48 h. Neurite outgrowth was measured (means±s.e.m., $n=3$, $^{**}P<0.01$). (**B**) CGNs transfected with NTC or LRP1-specific siRNA (siLRP1) were plated on MAG-CHO or R2-CHO cells and cultured for 48 h. Neurite outgrowth was determined (means±s.e.m., $n=3$, $^{*}P<0.05$, $^{**}P<0.01$). (**C**) Mouse CGNs, in which both LRP1 genes were floxed, were infected with HSV1-Cre or control HSV1 and cultured for 24 h. LRP1 expression was determined by immunoblot analysis. Neurite outgrowth on MAG-CHO and R2-CHO cells was determined (means±s.e.m., $n=3$, $^{**}P<0.01$). Scale bars: 100 μm.

immunoblot analysis indicated that the level of LRP1 protein was substantially decreased by gene-silencing (supplementary material Fig. S5A). LRP1 gene-silencing did not compromise CGN viability (supplementary material Fig. S5B). As shown in Fig. 5B, LRP1 gene-silencing caused a significant reversal of neurite outgrowth inhibition in CGNs plated on MAG-CHO cells.

Finally, we isolated CGNs from mice in which LoxP sites flank part of the *LRP1* promoter and the first two exons ($LRP1^{LoxP/LoxP}$), allowing *Cre*-mediated *LRP1* gene deletion (Rohlmann et al., 1996). These CGNs were transduced with a herpes simplex virus-1 vector that encodes Cre (HSV1-Cre) or GFP (HSV1-eGFP), as a control. Immunoblot analysis showed that this HSV1-Cre caused *LRP1* gene deletion in a fraction of the primary CGNs (Fig. 5C). Incomplete deletion reflected a ~70% transduction efficiency. Importantly, CGNs from $LRP1^{LoxP/LoxP}$ mice that were transduced with HSV1-Cre and cultured on CHO-MAG cells demonstrated a significant increase in neurite length compared with cells treated with HSV1-eGFP. Collectively, three different approaches show that functional ablation of LRP1 in primary neurons is sufficient to significantly attenuate MAG inhibition.

Binding of MAG to LRP1 recruits p75NTR and activates RhoA

RhoA activation is critical in the pathway by which MAIs inhibit neurite outgrowth in neurons and neuron-like cells (Kozma et al., 1997; Kuhn et al., 1999; Yamashita et al., 2002; Madura et al., 2004). Blocking RhoA activation promotes neurite outgrowth (Jalink et al., 1994; Jeon et al., 2012), even in cells plated on inhibitory substrata (Niederöst et al., 2002; Fu et al., 2007; Tan et al., 2007). To test the role of LRP1 in MAG-induced RhoA activation, N2a cells in which LRP1 was silenced with shRNA and control cells were treated with MAG-Fc or Fc for 10 min. MAG-Fc significantly increased GTP-loaded RhoA ($P<0.05$, $n=7$) in LRP1-expressing N2a cells (Fig. 6A). When LRP1 was silenced, MAG-Fc failed to increase GTP-loaded RhoA.

RhoA activation by MAIs may require p75NTR or TROY (Wang et al., 2002; Yamashita et al., 2002; Yamashita and Tohyama, 2003; Park et al., 2005). To test whether p75NTR is required in our model system, we treated N2a cells with TAT-pep5, a TAT-fusion peptide that binds to p75NTR and blocks RhoGDI-binding to p75NTR and p75NTR-dependent RhoA activation (Yamashita and Tohyama, 2003). Fig. 6B shows that TAT-pep5 blocked RhoA activation in response to MAG-Fc, suggesting that p75NTR and LRP1 may both contribute to RhoA activation in N2a cells.

It has been reported that p75NTR is recruited into complex with NgR1 or PirB when these receptors bind MAIs (Wang et al., 2002; Shao et al., 2005; Fujita et al., 2011). To determine whether p75NTR forms a complex with LRP1, we performed co-IP experiments. When N2a cells were treated with purified Fc or with vehicle, p75NTR did not co-IP with LRP1 at detectable levels (Fig. 6C). By contrast, when N2a cells were treated with MAG-Fc, p75NTR was found to co-IP with LRP1. Densitometry analysis of p75NTR in cell extracts that were subjected to co-IP suggested that ~2–5% of the total cellular p75NTR associated

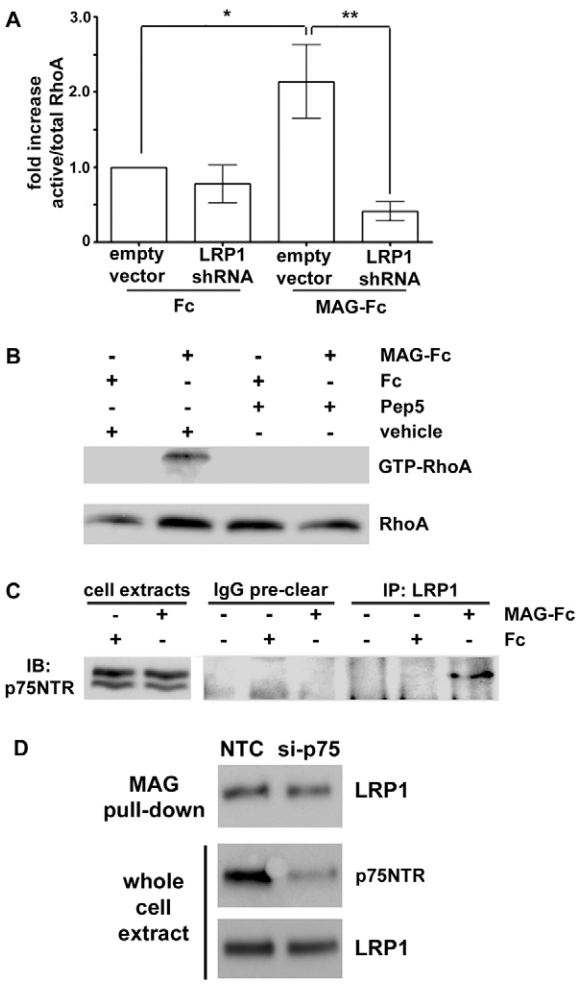

Fig. 6. LRP1 and p75NTR promote MAG-mediated RhoA activation. (**A**) N2a cells in which LRP1 was silenced with shRNA and control cells were treated with MAG-Fc or Fc (20 nM). The ratio of GTP-loaded RhoA to total RhoA was determined. Results were standardized against the ratio observed with Fc-treated control cells (means±s.e.m., $n=7$). (**B**) N2a cells were pre-treated with TAT-pep5 (0.5 μM) or vehicle for 30 min and then with MAG-Fc or Fc (20 nM). GTP-loaded and total RhoA were determined. (**C**) N2a cells were treated with 20 nM MAG-Fc or Fc, extracted, and subject to sequential IP with control IgG and an LRP1-specific antibody. Precipitated proteins were subjected to immunoblot (IB) analysis to detect p75NTR. Whole cell extracts are shown in the left-hand lane. (**D**) N20.1 cells were transfected with p75NTR-specific or NTC siRNA and analyzed 48 h later. MAG-Fc binding to LRP1 in the N20.1 cell extracts was determined by affinity precipitation. The extracts also were subjected to immunoblot analysis to detect total p75NTR and LRP1.

with LRP1 in the presence of MAG-Fc ($n=3$). Nevertheless, complex formation between p75NTR and LRP1 was induced specifically by MAG, reminiscent of the ability of MAG to induce association of p75NTR with NgR1 and PirB.

To test whether p75NTR regulates MAG-binding to LRP1, we achieved partial p75NTR gene-silencing in N20.1 cells (Fig. 6D). Total cellular LRP1 was not affected by p75NTR gene-silencing. MAG-Fc binding to LRP1 was examined by co-IP, 72 h after introducing siRNA. Association of MAG-Fc with LRP1 was not significantly affected by p75NTR gene-silencing. These results suggest that the binding of MAG to LRP1 does not require p75NTR, consistent with the results presented in Fig. 1.

LRP1 inhibits neurite outgrowth in cells plated on purified CNS myelin

Next, we examined neurite outgrowth inhibition in cells plated on purified rat CNS myelin. Neurite outgrowth in CGNs was inhibited by myelin, as anticipated, and RAP significantly reversed the extent of inhibition (Fig. 7A). LRP1 gene-silencing also significantly reversed the effects of myelin on neurite outgrowth in CGNs (Fig. 7B). When PC12 cells were plated on myelin, inhibition of neurite outgrowth inhibition was observed. Adding RAP or silencing LRP1 significantly reversed the inhibition of neurite outgrowth (supplementary material Fig. S6).

As a specificity control, we examined the effects of LRP1 antagonism on neurite outgrowth in CGNs plated on substrate-bound chondroitin sulfate proteoglycans (CSPGs). The CSPGs strongly inhibited neurite outgrowth; however, RAP failed to reverse the inhibition caused by CSPGs (supplementary material Fig. S7).

LRP1 supports binding of Nogo66 and OMgp

Purified CNS myelin contains MAIs in addition to MAG. Initially, we tested whether Nogo-A co-IPs with LRP1 from rat brain extracts. Reproducing the protocol utilized in Fig. 1, we showed that Nogo-A co-IPs with LRP1 and vice versa (Fig. 7C). Furthermore, Nogo-A and OMgp in purified rat CNS myelin co-IPed with CII-Fc and CIV-Fc, but not Fc (Fig. 7D,E).

As an additional confirmation, OMgp and Nogo66 were expressed as fusion proteins with alkaline phosphatase (OMgp-AP and AP-Nogo66). These fusion proteins were incubated with CII-Fc, CIV-Fc, and Fc, which were pre-immobilized on nitrocellulose. Fig. 7F shows that OMgp-AP and AP-Nogo66 bound to CII-Fc and CIV-Fc but not to Fc, suggesting that these interactions occur in purified systems. Overall, these qualitative binding studies demonstrate that LRP1 may interact with MAIs other than MAG. Additional studies will be needed to understand the significance of these interactions.

Soluble forms of LRP1 reverse the effects of MAIs on neurite outgrowth on primary neurons

We hypothesized that purified LRP1 and LRP1 derivatives that retain ligand-binding activity may compete for MAI-binding with membrane-anchored LRP1 and other MAI receptors and thereby reverse the activity of MAIs. To test this hypothesis, first we examined the effects of purified full-length LRP1 on neurite outgrowth in CGNs. Purified LRP1 did not affect neurite outgrowth when it was added to CGNs that were cultured on control R2-CHO cells (Fig. 8A). When CGNs were cultured on MAG-CHO cells, purified LRP1 significantly reversed the inhibition of neurite outgrowth observed. Purified LRP1 also significantly reversed the inhibition of neurite outgrowth observed when CGNs were plated on purified rat CNS myelin (Fig. 8B).

LRP1 is released from cells as a 'shed' product by α-secretase and accumulates in blood and cerebrospinal fluid (Liu et al., 2009). Shed LRP1 retains the entire α-chain and ligand-binding activity (Quinn et al., 1999). We purified shed LRP1 from human plasma (Gorovoy et al., 2010). When CGNs were plated on purified rat CNS myelin, shed LRP1 reversed the inhibitory effects of myelin, restoring neurite outgrowth to nearly the level observed in the absence of myelin (Fig. 8C).

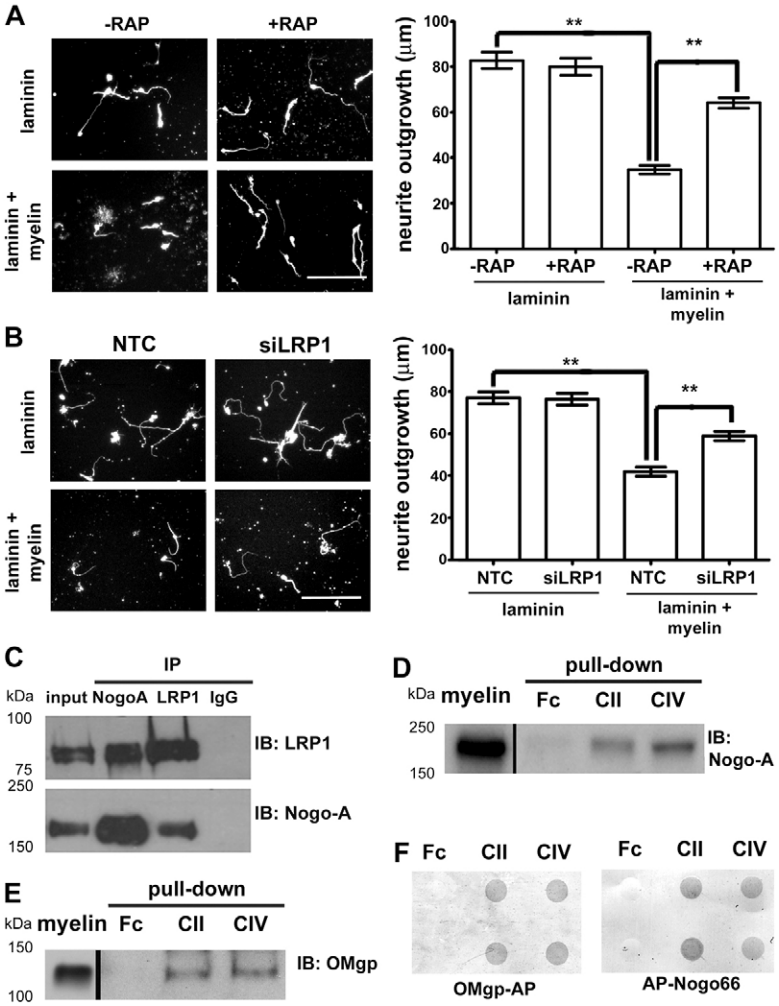

Fig. 7. LRP1 antagonism or gene-silencing attenuates the effects of myelin on neurite outgrowth in CGNs. (**A**) CGNs were pre-treated with RAP or GST and plated on laminin or laminin+myelin for 48 h. Neurite outgrowth was determined (means±s.e.m., $n=3$, $^{**}P<0.01$). (**B**) CGNs transfected with NTC or LRP1-specific siRNA (siLRP1) were plated on laminin or laminin+myelin and cultured for 48 h. Neurite outgrowth was determined (means±s.e.m., $n=3$, $^{**}P<0.01$). (**C**) Endogenous Nogo-A and LRP1 were IPed from adult rat brain membranes. Non-specific IgG was added as a control. IPed proteins were subjected to immunoblot (IB) analysis for Nogo-A and LRP1. (**D**,**E**) CII-Fc, CIV-Fc and Fc were incubated with purified myelin extracts and precipitated with Protein A-Sepharose. Immunoblot analysis was performed to detect Nogo-A in D and OMgp in E. (**F**) OMgp-AP and AP-Nogo66 were expressed in HEK293T cells and conditioned medium was recovered as a source of these proteins. CII-Fc, CIV-Fc and Fc were immobilized in duplicate on nitrocellulose membranes and incubated with equivalent amounts of conditioned medium. Binding was determined by AP detection. In control experiments, medium that was not conditioned with OMgp-AP or AP-Nogo66 was incubated with the membranes. Scale bars: 100 μm.

Finally, we examined the activity of CII-Fc and CIV-Fc. Both fusion proteins reversed the inhibition of neurite outgrowth observed when CGNs were plated on MAG-CHO cells or purified myelin, as shown in representative images (supplementary material Fig. S8) and in summary form in Fig. 8D,E.

Discussion

A detailed understanding of the mechanisms by which MAIs inhibit neuronal growth is of considerable interest, both biologically and clinically. Previously, NgR1 was reported to bind Nogo66, MAG, OMgp, and CSPGs; however, *NgR1* null neurons are not dis-inhibited when plated on substrate-bound ligands, suggesting some degree of mechanistic redundancy by which MAIs signal growth inhibition (Zheng et al., 2005; Chivatakarn et al., 2007; Dickendesher et al., 2012). PirB is a promiscuous receptor for Nogo66, MAG, and OMgp. Antagonism of PirB leads to a significant, yet incomplete release of neurite outgrowth inhibition in the presence of substrate-bound MAIs or crude CNS myelin (Atwal et al., 2008). The combined functional ablation of NgR1 and PirB is not sufficient to fully release Nogo66, MAG, or OMpg inhibition, suggesting the existence of additional receptor mechanisms. In addition to NgR1 and PirB, MAG has been shown to bind to brain gangliosides, NgR2, and β1-integrins, and depending on the neuronal cell type examined, these interactions contribute to various degrees in growth inhibition (Mehta et al., 2007; Venkatesh et al., 2007; Goh et al., 2008; Wörter et al., 2009).

Herein, we report the identification of LRP1 as a novel receptor for MAG. We initially identified MAG as an LRP1-binding partner in LC-MS/MS screening experiments. We subsequently determined that MAG binds LRP1 directly and independently of its lectin activity. This is important because the inhibitory effects of membrane-associated MAG on neurite outgrowth are known to occur independently of sialic acid binding (Tang et al., 1997; Cao et al., 2007; Robak et al., 2009). To our knowledge, LRP1 is the first receptor to demonstrate this anticipated characteristic of an inhibitory MAG receptor. Binding of MAG to LRP1 recruits p75NTR into a receptor complex and both LRP1 and p75NTR are necessary for RhoA activation in N2a cells. In CGNs and neuritogenic cell lines, functional ablation of LRP1 by RNAi knock-down, treatment with RAP, or gene deletion attenuated MAG-induced inhibition of neurite outgrowth. Additional studies will be needed to determine whether LRP1 functions alone or, more likely, in concert with other MAI receptors to mediate the effects of MAG.

Functional depletion of LRP1 attenuated the inhibitory activity of purified CNS myelin but not CSPGs in neurite outgrowth experiments with primary neurons. This observation prompted us to test whether LRP1 binds MAIs in addition to MAG. Similar to NgR1 and PirB, LRP1 supported interactions with Nogo66 and

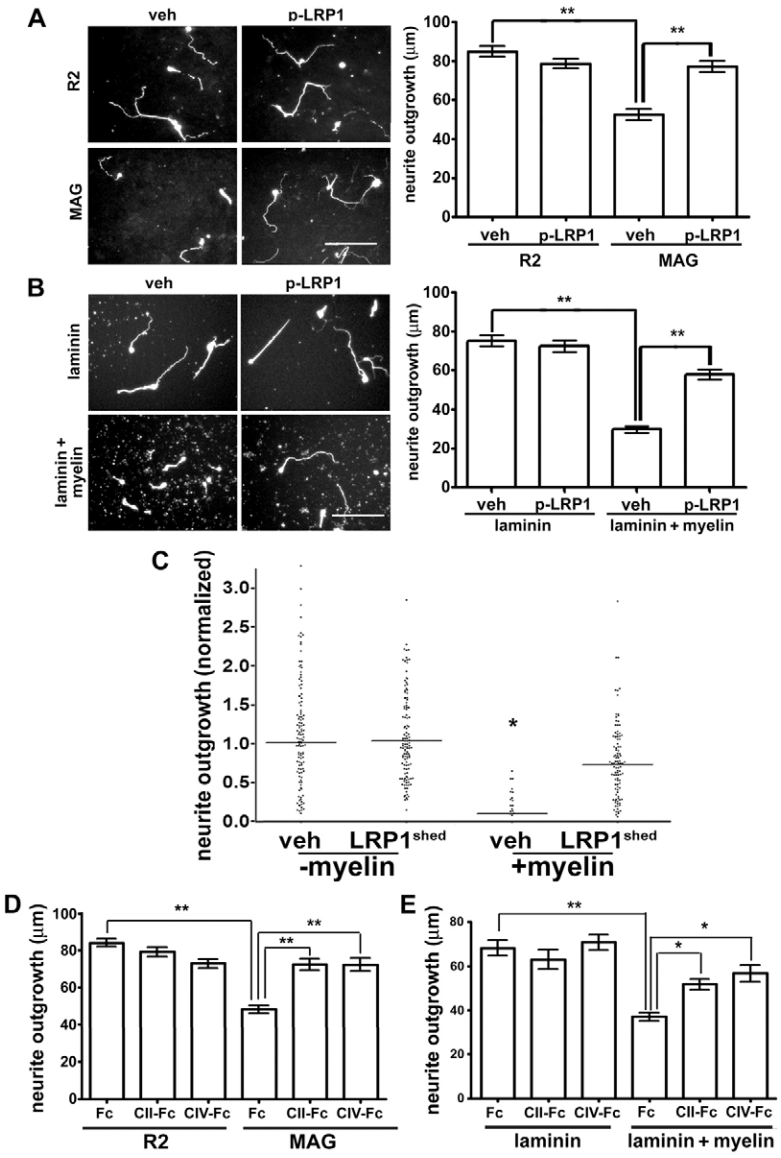

Fig. 8. Soluble LRP1 derivatives attenuate the effects of MAG and myelin on neurite outgrowth in CGNs. (**A**) Monolayers of MAG-CHO and R2-CHO cells were incubated for 15 min with purified LRP1 (0.5 μM) or vehicle (veh) prior to adding CGNs. Cultures were maintained for 48 h. Neurite outgrowth was determined (means±s.e.m., $n=3$, $^{**}P<0.01$). (**B**) Surfaces coated with laminin or laminin+myelin were treated for 15 min with purified LRP1 (0.5 μM) or vehicle. CGNs were then cultured for 48 h. Neurite outgrowth was determined (means±s.e.m., $n=3$, $^{**}P<0.01$). (**C**) Culture wells coated with myelin and control wells were pre-treated for 15 min with shed LRP1 (0.5 μM) or vehicle. CGNs were cultured for 48 h. Neurite outgrowth was determined. The scatter plots shows neurite outgrowth in individual cells ($^{*}P<0.05$). (**D**) Monolayers of MAG-CHO and R2-CHO cells were incubated for 15 min with 1 μM CII-Fc, CIV-Fc or Fc prior to adding CGNs for 48 h. Neurite outgrowth was determined (means±s.e.m., $n=3$, $^{**}P<0.01$). (**E**) Surfaces coated with laminin or laminin+myelin were pre-treated with 1 μM purified CII-Fc, CIV-Fc or Fc for 15 min. CGNs were then added for 48 h. Neurite outgrowth was determined (means±s.e.m., $n=3$, $^{*}P<0.05$, $^{**}P<0.01$). Scale bars: 100 μm.

OMgp. Although our studies suggest that LRP1 binds MAIs in addition to MAG, more detailed work, including binding affinity determinations and functional studies, will be necessary to elucidate whether the interactions of LRP1 with Nogo-A and OMgp are important for myelin-mediated neurite outgrowth inhibition.

NgR1 and NgR2 are GPI-anchored proteins and depend on interaction with membrane-spanning receptors for cell-signaling. Lingo-1 and the TNF receptor family members, p75NTR and TROY, form complexes with NgR1. More recent studies showed that PirB associates with p75NTR to signal growth inhibition (Fujita et al., 2011). NgR2 interacts with Troy (Wills et al., 2012). Similar to NgR1 and PirB, we show that LRP1 associates with p75NTR in the presence of MAG. This interaction may be important for activation of RhoA. Because of the limited distribution of p75NTR in the mature CNS and the strong inhibition observed in MAI-treated primary neurons null for *p75NTR*, additional signal transducing components remain to be discovered that participate in MAI inhibition (Zheng et al., 2005; Venkatesh et al., 2007). In addition to p75NTR, LRP1 may also associate with TROY or Lingo-1 and it will be interesting to examine the functional relationship of PirB, NgRs and LRP1.

Binding of tPA and α_2M to neuronal LRP1, in the absence of MAIs, results in neurite outgrowth and neuronal survival (Qiu et al., 2004; Hayashi et al., 2007; Mantuano et al., 2008; Shi et al., 2009). These LRP1 ligands activate Src, transactivate Trk receptors, and activate ERK and AKT in a Trk-dependent manner (Shi et al., 2009). NMDA receptors also may be involved (May et al., 2004; Rebeck, 2009). The neuronal response to tPA and α_2M is thus, very different from that observed with MAIs. We propose a model in which the activity of LRP1 in cell-signaling is dependent on the co-receptors that are recruited to LRP1 by specific ligands. We further propose that whether p75NTR or Trk receptors are recruited to LRP1 may represent an important checkpoint in neuronal LRP1 signaling.

Joset et al. (Joset et al., 2010) demonstrated that Nogo-A activates RhoA by a mechanism that requires Pincher-dependent macro-endocytosis. Although this pathway occurs independently of clathrin-coated pits, formation of the signalosome and vesicular transport of Nogo-A within the cell was pivotal for growth cone

collapse. Endocytosis of MAG by LRP1, possibly in combination with p75NTR and other MAI receptors, may provide a related pathway for intracellular trafficking of myelin products and RhoA activation. Interestingly, Steuble et al. (Steuble et al., 2010) co-localized Nogo with LRP1 in early endosomes when they analyzed growth cone vesicles isolated from mouse brain.

Purified LRP1 and shed LRP1 attenuated the inhibitory effects of MAG and purified myelin in neurite outgrowth experiments with CGNs. We interpret these results to reflect competition for MAI-binding with membrane-anchored LRP1 and possibly, other MAI receptors. This pre-emptive binding model was supported by experiments with CII-Fc and CIV-Fc, which also partially reversed the inhibition of neurite outgrowth observed when CGNs were plated on MAG-CHO cells or myelin. We propose that the activity of MAG and other MAIs may be neutralized by any soluble LRP1 derivative that retains ligand-binding activity. An alternative approach involves direct targeting of the CII/CIV domains of LRP1. In the injured CNS, proteins that bind to LRP1, such as tPA, may inhibit binding of MAIs to LRP1, similarly to RAP. The activity of any candidate ligand, in displacing MAIs from LRP1, will depend on the concentration of that ligand and its relative affinity for CII and CIV.

Shed LRP1 is generated by the α-secretase, ADAM17 (Gorovoy et al., 2010). Inflammation increases LRP1 shedding and promotes the accumulation of shed LRP1 in the plasma (Gorovoy et al., 2010). In CNS ischemia, shedding of LRP1 from perivascular astrocytes is significantly increased (Polavarapu et al., 2007). Our results suggest that shed LRP1, which is generated in the brain, may serve as an endogenous antagonist of the growth inhibitory activity of MAIs. As such, LRP1 shedding in the brain may represent a previously unappreciated mechanism by which the body promotes neuronal sprouting after CNS insult.

In the normal human brain, LRP1 is expressed by almost all neuronal populations (Wolf et al., 1992; Bu et al., 1994; Lopes et al., 1994). In CNS injury, LRP1 expression significantly increases in reactive astrocytes (Lopes et al., 1994). Our previous studies suggest that LRP1-dependent phagocytosis of myelin debris occurs across diverse cell types (Gaultier et al., 2009). The increase in LRP1 expression by reactive astrocytes in the injured CNS may limit the burden of MAIs presented to neurons and thus, play a protective role. Overall, we propose that a balance between neuronal LRP1, astrocytic LRP1 and shed LRP1 may be critical in determining the effects of MAIs on neuronal repair in the CNS, following injuries of diverse magnitudes.

Materials and Methods

Recombinant and purified proteins

CII, which includes amino acids 804–1185 of mature LRP1; CIV, which includes amino acids 3331–3778; and full-length rat MAG were cloned into pFuse-rFC2 (Invivogen, San Diego, CA) and expressed as Fc-fusion proteins in CHO-K1 cells. NgR^{OMNI}-Fc and RPTPσ-Fc are previously described (Atwal et al., 2008; Robak et al., 2009; Dickendesher et al., 2012). Fc-fusion proteins were purified by affinity chromatography on Protein A-Sepharose (GE Healthcare, Pittsburgh, PA). GST-RAP and GST were expressed in bacteria and purified as previously described (Gaultier et al., 2009). Shed LRP1 and full-length LRP1 were purified from human plasma and rat liver, respectively, by RAP-affinity chromatography and molecular exclusion chromatography (Gorovoy et al., 2010). OMgp-AP, AP-Nogo66 and AP-Fc were expressed in HEK293T cells as previously described (Dickendesher et al., 2012). All animal experiments were performed according to approved guidelines.

Cell culture

CHO-K1 cells were cultured in high glucose DMEM with 10% fetal bovine serum (FBS) (Thermo Scientific Hyclone, Logan, UT), 10 mg/L L-glutamine, and 10 mg/L non-essential amino acids (Gibco, Carlsbad, CA). For expression of recombinant proteins, transfected CHO-K1 cells were cultured in Power-CHO CD medium (Lonza, Anaheim, CA). MAG-CHO and R2-CHO cells, a gift from Dr Mark Tuszynski (University of California San Diego), were cultured in DMEM with 10% FBS, 2 mM glutamine, 40 mg/L proline, 0.73 mg/L thymidine, 1 μM methotrexate, 7.5 mg/L glycine and 50 μg/ml G418 (Gibco, Carlsbad, CA). COS-7 cells were transfected to express full-length MAG, MAG^{R118A}, PirB, or GFP as previously described (Atwal et al., 2008; Robak et al., 2009). PC12 cells were cultured in DMEM with 10% FBS, 5% horse serum, and penicillin/streptomycin (P/S, Thermo Scientific Hyclone, Logan, UT). For neurite outgrowth experiments, PC12 cells were treated with 50 μg/ml NGF-β (R&D Systems, Minneapolis, MN). N2a cells were a gift from Dr Katerina Akassoglou (University of California San Francisco). N2a cells were cultured in DMEM with 10% FBS and P/S. Primary cultures of rat CGNs were isolated as previously described (Oberdoerster, 2001) and cultured in DMEM with 50 mM glucose, 10% FBS, 25 mM KCl, and P/S. Mouse CGNs were isolated, purified in a discontinuous Percoll gradient, and cultured as previously described (Dickendesher et al., 2012). N20.1 cells were a gift from Dr Anthony Campagnoni (University of California Los Angeles) and were cultured as previously described (Wight and Dobretsova, 1997).

CNS myelin purification

Myelin vesicles were purified from rat brain, as described by Norton and Poduslo (Norton and Poduslo, 1973). The purity of the preparation was determined by Coomassie Blue staining and by immunoblot analysis for myelin basic protein, as previously described (Gaultier et al., 2009).

Mass spectrometry

Purified rat CNS myelin was solubilized in RIPA buffer (100 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM $CaCl_2$, and protease inhibitor cocktail). Protein extracts (2 mg) were incubated with 1 μM CII-Fc, CIV-Fc, or Fc overnight at 4℃. The fusion proteins and associated proteins were recovered by incubation with Protein A-Sepharose. After extensive washing, proteins were digested with trypsin in the presence of ProteaseMAX surfactant (Promega, Madison, WI, USA). Proteins that were associated with CII-Fc or CIV-Fc, but not Fc, were identified by LC-MS/MS as previously described (Gaultier et al., 2010).

Solution-phase protein-binding experiments

Protein extracts from cells were prepared in 50 mM HEPES pH 7.4, 1% Triton X-100, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, and protease inhibitor cocktail. These extracts and solubilized myelin were incubated with CII-Fc, CIV-Fc, MAG-Fc or Fc, immobilized on Protein A-Sepharose. MAG, OMgp and Nogo-A were identified in affinity precipitates by immunoblot analysis. In some studies, RAP or GST (200 nM) was added with cell extracts. LRP1 was detected in affinity precipitates using an antibody that detects the 85-kDa subunit (Sigma, St Louis, MO). p75NTR was detected using an antibody that recognizes the intracellular domain (Millipore, Temecula, CA).

Membrane preparations from adult rat brain were prepared using sucrose gradient centrifugation (Winters et al., 2011) and extracted in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, and protease inhibitor cocktail. Specific antibodies were used to IP LRP1, MAG (Winters et al., 2011), or Nogo (R&D Systems, Minneapolis, MN). Following precipitation with Protein G Plus/Protein A-Agarose, samples were rinsed six times and bound proteins were eluted with SDS sample buffer. Precipitates were analyzed by immunoblotting.

In dot blotting studies, 40 pmol of CII-Fc, CIV-Fc, or Fc were immobilized on nitrocellulose. Membranes were rinsed and then, blocked with 5% nonfat dry milk or BSA. Incubations with immobilized proteins were conducted for 1 h at 22℃. Bound proteins were detected using appropriate antibodies.

Binding of CII-Fc and CIV-Fc to cell-associated MAG

Fc-fusion proteins were pre-coupled to AP-conjugated anti-Fc antibody and incubated with COS-7 cells that express MAG, MAG^{R118A}, PirB, or GFP (negative control) for 75 min. Unbound fusion protein was removed by extensive rinsing with OptiMEM. Cells were fixed with formaldehyde (1%). Endogenous phosphatases were heat-inactivated by incubation at 65℃ for 90 min. Binding of fusion proteins was visualized by developing the AP reaction with nitro-blue tetrazolium and 5-bromo-4-chloro-3′-indolyphosphate. Binding was quantified with ImageJ software and analyzed using GraphPad Prism software.

In some experiments, monomeric fusion proteins were incubated with cells. Binding was detected by adding AP-conjugated anti-Fc antibody in a second incubation. To determine binding isotherms, increasing concentrations of each Fc-fusion protein were incubated with MAG-expressing COS-7 cells. K_D values were determined by analyzing three replicate binding curves with GraphPad Prism. Mean K_Ds±s.e.m. are presented.

RhoA activation

N2a cells were serum-starved for 1 h. MAG-Fc or Fc (20 nM) were pre-incubated with Fc-specific antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a 2:1 molar ratio and added to N2a cells for 10 min. Cell extracts were prepared and GTP-loaded RhoA was affinity precipitated using the Rho binding domain of

rhotekin, expressed as a GST-fusion protein (Millipore, Temecula, CA). Affinity-precipitated active RhoA and total RhoA were determined by immunoblot analysis (Cell Signaling, Danvers, MA). The ratio of active/total RhoA was determined by densitometry.

Neurite outgrowth experiments

MAG-CHO and R2-CHO cells were cultured on glass slides until confluent (Domeniconi et al., 2002). CGNs, PC12 cells, or N2a cells were then added and cultured for 48 h. CGNs from mice in which both LRP1 genes are floxed (JAX®, Bar Harbor, ME) were transduced with HSV1-GFP or HSV1-GFP-Cre (Viral Gene Transfer Core, McGovern Institute for Brain Research, MIT, Cambridge, MA). 72 h later, the transduced CGNs were extracted for immunoblot analysis or gently dislodged using Cellstripper™ non-enzymatic cell dissociation solution (Corning, Corning, NY) and re-plated on CHO cells or purified myelin to assess neurite outgrowth. Control substrate included CSPGs (10 μg/ml) (Millipore, Temecula, CA) and laminin (2 μg/ml) which were adsorbed to culture wells for 2 h at 22℃. When RAP or GST was added, these proteins were pre-incubated with CGNs or neurite-generating cell lines in suspension for 15 min. Shed LRP1, purified LRP1, CII-Fc, or CIV-Fc also were pre-incubated with cells. Neurite outgrowth was assessed by immunofluorescence microscopy, after immunostaining to detect βIII-tubulin (Promega, Madison, WI) and quantified using ImageJ or Metamorph software.

Gene silencing

PC12 cells were transfected with the previously described rat LRP1-specific siRNA (CGAGCGACCUCCUAUCUUUUU) or with NTC siRNA using the Amaxa rat neuron nucleofector kit. LRP1 was silenced in rat CGNs and mouse N2a cells using ON-TARGET plus, smart-pool LRP1-specific siRNA (Thermo Scientific, Lafayette, CO) and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Stable LRP1 gene silencing was achieved in N2a cells using our previously described LRP1-specific shRNA, cloned into pSUPER (Gaultier et al., 2008). LRP1 gene-silencing was confirmed by RT-PCR and by immunoblot analysis. Silencing of p75NTR was performed using ON-TARGET plus, smart-pool mouse p75NTR-specific siRNA (Thermo Scientific, Lafayette, CO).

Analysis of MAG endocytosis

LRP1-expressing N2a cells and N2a cells in which LRP1 was silenced with shRNA were differentiated for 4 h in SFM and treated with RAP or GST. The cells were treated with Fc-receptor blocking antibody (1 μg/ml) for 30 min at 4℃ and then with 25 nM MAG-Fc, RPTPσ-Fc or Fc for 60 min at 4℃. The cells were then warmed to 37℃ for 30 min. Surface-associated fusion protein was dissociated by treatment with acetic acid/sodium acetate pH 3.0 for 4 min. The cells were then washed, fixed with 4% paraformaldehyde, and permeabilized with Triton X-100 in 5% goat serum. Internalized MAG was detected with MAG-specific antibody and fluorophore-coupled secondary antibody (Invitrogen, Carlsbad, CA).

In immunoblotting experiments, proteins were extracted in RIPA buffer. An equivalent amount of cellular protein (30 μg) was subjected to SDS-PAGE and immunoblot analysis to detect Fc-tag. MAG-Fc was radioiodinated with 1 mCi of $Na^{125}I$ using Iodobeads (Pierce, Rockford, IL). 1×10^5 cells were equilibrated in DMEM with 25 mM HEPES pH 7.4, 0.1% BSA and Fc blocker. ^{125}I-MAG-Fc (25 nM) was incubated with cells for 2 h at 37℃. Unlabeled MAG (1.25 μM) was added to inhibit specific endocytosis. To dissociate surface-associated ^{125}I-MAG-Fc, cells were treated with 0.25% Pronase (Roche, Pleasanton, CA) for 15 min. Cell-associated radioactivity was recovered in 0.1 M NaOH and 1% SDS and determined using a gamma counter. Cellular protein was determined by a bicinchoninic acid assay (Pierce, Rockford, IL).

Acknowledgements

We would like to thank Dr Katerina Akassoglou for insightful discussions.

Funding

This work was supported by the National Institutes of Health [grant numbers R01 NS054671 and R01 HL60551 to S.L.G. and R21 NS071347 to A.G.], the Veterans Administration Research Foundation and the Adelson Medical Foundation in Neural Repair (to R.J.G.). Deposited in PMC for release after 12 months.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.113191/-/DC1

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