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Novel interactions of LDL-receptor 1 (LRP1) with components of CNS myelin reveal LRP1 as a novel regulator of CNS regenerative failure after injury

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Travis L. Stiles

Committee in charge:

Professor Steven Gonias, Chair
Professor Katerina Akassoglou
Professor Eliezer Masliah
Professor Christina Sigurdson
Professor Binhai Zheng

2013

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Chair

University of California, San Diego

2013

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LIST OF ABBREVIATIONS

a_2M, alpha-2-macroglobulin

AD, Alzheimer's disease

AP, alkaline phosphatase

ApoE, apolipoprotein E

CCR, cluster of complement-like repeats

CGN, cerebellar granule neuron

CHO, chinese hamster ovary cell

CNS, central nervous system

COS, African green monkey SV40 transfected kidney fibroblast cell

CR, complement-like repeat

CREB, cyclic AMP response-element binding

CSPG, chondroitin sulfate proteoglycans

EEA, early endosomal antigen

EGF, epidermal growth factor

GPI, glycosyl phosphatidylinositol

GST, glutathione s-transferase

GDP/GTP, guanosine di/tri-phosphate

HSPG, heparin sulfate proteoglycans

HSV, herpes simplex virus

IB, immunoblot (immunodetection)

IF, immunofluorescent

Ig, immunoglobulin

IP, immunoprecipitation

LDL, low density lipoprotein

LDLR, low density lipoprotein receptor

LRP1, low density lipoprotein receptor-related protein

LRR, leucine rich repeat

MAG, myelin-associated glycoprotein

MAI, myelin-associated inhibitor of neurite outgrowth

MAPK, mitogen activated protein kinase

MBP, myelin basic protein

MMP, matrix metalloproteinase

MS, multiple sclerosis

N2a, mouse neuroblastoma cell line

NGF, nerve growth factor

NgR, Nogo receptor

NTC, non-targeted control

OMgp, oligodendrocyte myelin glycoprotein

OPC, oligodendrocyte precursor cell

PC12, rat pheochromocytoma cell

PirB, paired immunoglobulin-like receptor B

PNS, peripheral nervous system

RAP, receptor-associated protein

RBD, receptor binding domain

ROCK, Rho-associated coiled-coil-containing protein kinase

RPTP, receptor protein tyrosine phosphatase

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

SFM, serum-free medium

TGFβ, Transforming growth factor-β

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VITA

2005	Bachelor of Arts in Exercise Science, Willamette University
2005-2007	Graduate Research Assistant, Pacific Fatigue Lab, University of the Pacific.
2005-2007	Teaching Assistant, Exercise Physiology, Kinesiology, Heart Exercise and Nutrition, Department of Sport Sciences, University of the Pacific.
2006-2007	Lecturer/Adjunct Professor, Kinesiology, Department of Sport Sciences, University of the Pacific.
2007	Master of Arts in Sport Sciences – Exercise Physiology, University of the Pacific.
2007-2013	Research Assistant, University of California San Diego.
2013	Doctor of Philosophy in Biomedical Science, University of California San Diego.

PUBLICATIONS

"LDL Receptor-related Protein-1 is a sialic acid-independent receptor for myelin-associated glycoprotein (MAG) and functions in neurite outgrowth inhibition by MAG and CNS myelin". 2012. *J Cell Sci.* 2012, PMID: 23132925

"Identification of the LDL Receptor-Related Protein-1 interactome in central nervous system myelin suggests a role in the clearance of necrotic cell debris". *J Bio Chem.* 2012. PMID: 23264627

"A re-assessment of the effects of treatment with a non-steroidal anti-inflammatory (ibuprofren) on promoting axon regeneration via RhoA inhibition after spinal cord injury". 2013. *Exp Neuro*. (in press).

FIELD OF STUDY

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Studies in Cell Biology

Prof. Steven Gonias. MD, PhD

ABSTRACT OF THE DISSERTATION

Novel interactions of LDL-receptor 1 (LRP1) with components of CNS myelin reveal LRP1 as a novel regulator of CNS regenerative failure after injury

by

Travis L. Stiles

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2013

Professor Steven Gonias, Chair

There are an estimated 12,000 to 20,000 new cases of spinal cord injury (SCI) each year and 1.28 million people in the United States are paralyzed in some form due to SCI. Currently, there are no clinically available treatments that target degraded myelin, one of the primary causes of regenerative failure in the central nervous system

(CNS) after acute SCI. Contrary to conventional wisdom, neurons of the central nervous system (CNS) possess substantial regenerative capacity after trauma such as spinal cord injury (SCI). The failure of these neurons to regenerate is therefore not due to intrinsic inability, but rather to inhibitory cues within the CNS microenvironment, including myelin-associated inhibitors (MAIs) and the chondroitin sulfate proteoglycans within the glial scar. While these potent molecular obstacles have been well characterized, the neuronal receptors mediating their ability to blunt neuroregeneration have remained elusive. In this work, I identify LRP1 as a novel receptor of MAG (and likely other MAIs) that mediates inhibition of neurite outgrowth and the inhibitory signaling in neurons. In response to exposure to MAIs, LRP1 forms a signaling complex with p75, which leads to activation of RhoA. Additionally, treatment of myelin with soluble fragments of LRP1, which contain the sequence necessary for MAI binding, also attenuate neuronal growth inhibition. These effects are due to the ability of LRP1 to facilitate MAI-mediated activation of RhoA, which is the necessary and sufficient cellular signal for cessation of axonal extension. Antagonism of LRP1 also attenuates RhoA activation *in vivo* in rat models of spinal cord injury. LRP1 and p75 also selectively associate from lesion extracts from injured rat spinal cord, and this effect is blocked by infusion of RAP into the lesion site. In total, this work identifies LRP1 as a novel and exciting potential therapeutic target in the regeneration of neurons after damage to the spinal cord.

CHAPTER 1 - INTRODUCTION

Spinal Cord Injury Incidence and Prognosis

SCI is a term commonly used to refer to mechanical damage to the spinal vertebrae which results in neurologic symptomology and paralysis. During development, neuronal cells extend axons tipped with path-finding growth cones which navigate the environment to innervate synaptic targets[1]. In the mature nervous system, mechanical injury severs these axons which results in loss of innervation and function. Unsevered axons can also suffer loss of innervation and function due to secondary damage from inflammation or non-transecting damage such as crush. These types of damage can result in demyelination and axonal degradation, which often results in the same forms of functional loss as observed with axonal transection[2]. Symptomatic consequences of SCI can vary widely depending on the extent and spinal level of injury and may result in combinations of pain, paralysis, incontinence, and infertility. The most comprehensive study of SCI prevalence in the United States was conducted by the Christopher & Dana Reeve Foundation in 2009. *One Degree of Separation: Paralysis and Spinal Cord Injury in the United States* found that approximately 0.4 % of the U.S. population, or 1.28 million people, are paralyzed in some form due to spinal cord injury. Additionally, there are an estimated 12,000 to 20,000 new cases of SCI each year in the U.S. The estimated cost of SCI to the U.S. health care system is $40.5 billion annually, which doesn't take into account the financial toll on the patient in terms of employment and daily complications of living [3].

Regenerative Failure of Neurons in SCI

SCI results in severed and damaged axons, which results in loss of innervation and function. Axonal severing results in the distal aspect of the severed axon which has been separated from the soma undergoing Wallerian degeneration, while the proximal axon undergoes retraction and reformation of the growth cone prior to attempting regeneration[4]. In the peripheral nervous system, nerve cells have a tremendous capacity to regrow or repair following injury. This is not the case for neurons in the CNS[5,6]. Inability of injured neurons to regenerate or repair in the CNS has been attributed to neuronal interactions with specific environmental factors of the CNS, namely myelin debris and chondroitin sulfate proteoglycans (CSPGs) contained within the glial scar[7–11]. In peripheral injury, the rapid recruitment of macrophages to the site of injury results in efficient clearance of the myelin debris that results from damage, which greatly reduces the inhibitory load from inhibitory myelin components[12]. Additionally, schwann cells, the myelinating cells of the PNS, also provide a regenerative advantage after injury. As opposed to their CNS counterparts, oligodendrocytes, schwann cells de-differentiate in response to injury which not only results in down regulation of the expression of myelin proteins which have inhibitory potential, but also results in schwann cell proliferation and assistance with clearance of unwanted debris at the lesion site[13]. The combined result of these PNS responses to neuronal damage is a much smaller inhibitory load from myelin debris in the PNS relative to the CNS, which results in greater regenerative capacity. Additionally, the

marked decrease in inhibitory myelin burden and subsequent robust regenerative response of the PNS relative to the CNS demonstrates just how important the role of myelin is in regenerative failure after SCI.

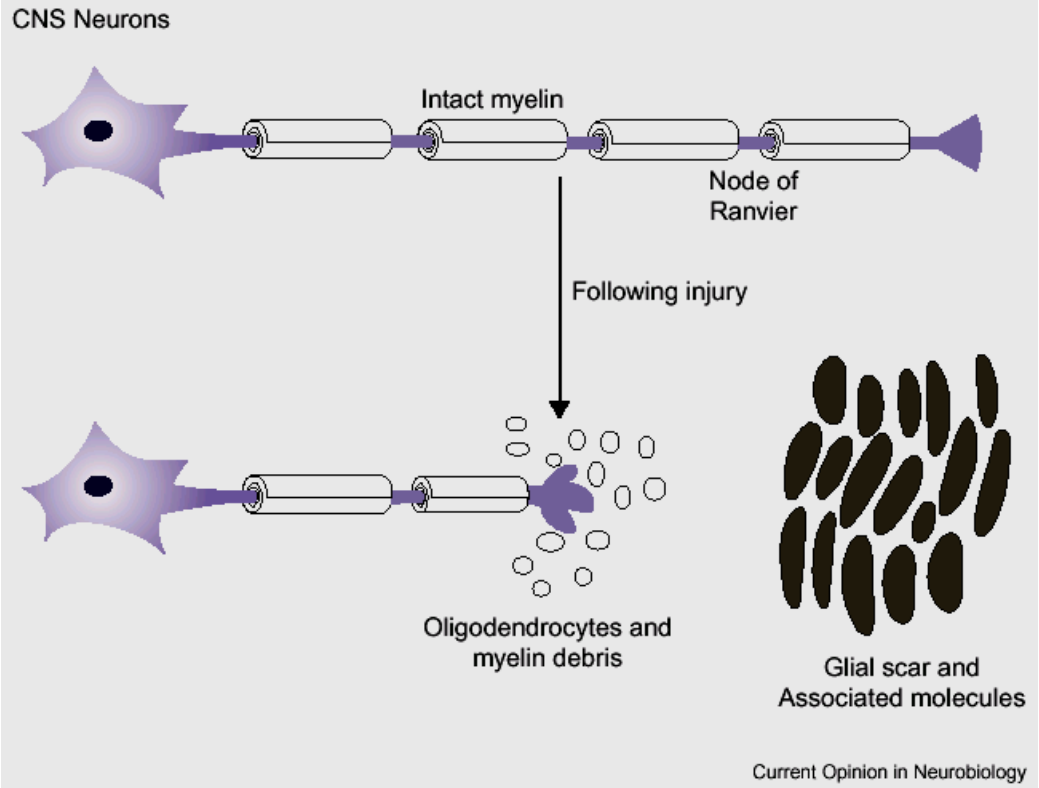


Figure 1-1 Illustration of the environmental components of the CNS that are inhibitory to neuronal regeneration; components of myelin debris and the glial scar.

Myelin-Associated Inhibitors of Regeneration

Several myelin-associated inhibitory proteins (MAIs) have been identified, including members of the reticulon family (Nogo A-C), myelin associated-glyco-protein (MAG), and oligodendrocyte myelin glycolprotein (OMgp)[7,11,14]. MAIs induce growth cone collapse and inhibition of neurite outgrowth, and are one of the major environmental factors of the CNS responsible for regenerative failure of CNS neurons after injury[7,11,15,16].

Nogo/reticulon-4

Nogo was the first identified isolated myelin component capable of inhibiting neuronal regeneration. Over 20 years ago, Martin Schwab and his colleagues isolated two protein-enriched sub-fractions extracted from purified CNS myelin which appeared to be responsible for the vast majority of the inhibitory load of CNS myelin. Scwabb and colleagues characterized these fractions at molecular weights of 35kDa (NI-35) and 250kDa (NI-250)[17]. A monoclonal antibody was subsequently generated against NI-250, which they consequently found to have high affinity for NI-35 as well. They named this antibody as IN-1, and it has persisted for decades as an oft used reagent in the study of myelin-mediated regenerative failure of the CNS[18]. Interestingly, in addition to demonstrating high affinity for both inhibitory fractions of CNS myelin, this IN-1 also demonstrated the ability to restore neurite outgrowth in the presence of myelin debris[19]. Future studies also indicated that IN-1 may have therapeutic regenerative potential *in vivo*, which will be discussed further later[20]. However, it wasn't for many years until Nogo, the now identified antigen for IN-1

antibody, was cloned from a partial sequence of IN-250[19,21]. Nogo is a member of the reticulon family and exists in three different isoforms (-A, -B and -C), created by alternative splicing[22–24]. The variation in isoform expression has been linked to variation in the usage of the promoter[25]. Nogo-A, the largest splice form of the *Nogo/reticulon* 4 gene, is expressed by both oligodendrocytes and neurons, and is localized to the inner and outer loop of myelin and on the surface of oligodendrocytes[19,26,27]. In addition to the reticulon domain, all isoforms of Nogo also share a 66-amino acid extracellular loop called Nogo66, which is potently inhibitory to neurite outgrowth[26]. Nogo-A is also the most inhibitory Nogo isoform, likely due to the fact that it is comprised of at least two distinct growth inhibitory regions: Nogo66 and the potently inhibitory N-terminal sequence referred to as amino-Nogo[28]. Nogo-B is expressed in many tissues and cell types including neurons. Nogo-C is expressed outside the nervous system[29].

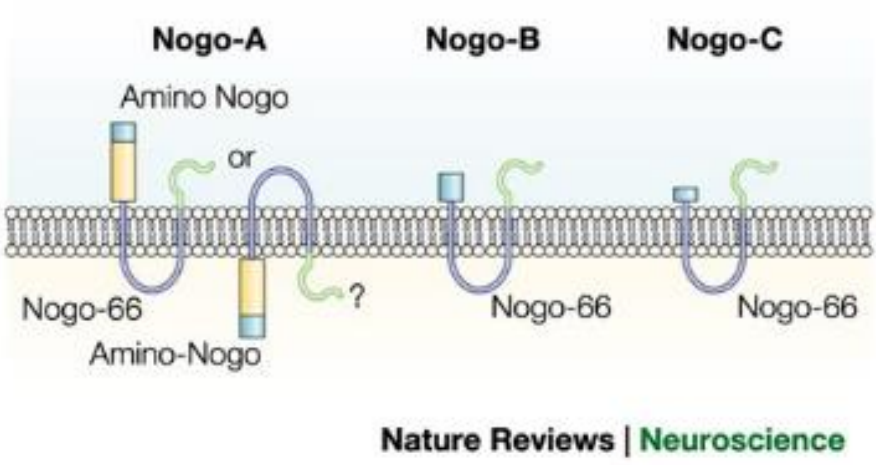


Figure 1-2 Structures of the 3 isoforms of Nogo (A, B, and C).
Nogo-A contains 2 inhibitory domains; amino-Nogo and Nogo-66.

Myelin Associated Glycoprotein

Despite being one of the earliest myelin proteins expressed during development, MAG is a relatively minor component of myelin, representing 1% of the total protein in CNS myelin, and 0.1% in PNS myelin[30]. MAG is expressed in both a long (L-) and short (S-) isoform with the L-MAG isoform being expressed early in myelination, and the S-MAG later[31]. MAG is expressed in the interface between myelinated axons and the periaxonal myelin membrane[32]. It can be primarily found in the first layer, or wrap, around the axon. MAG is also localized to areas of uncompacted myelin such as the paranodal loops[33]. Many myelin proteins expressed early in myelination are important for the initiation, and proper development of, the myelinating process. However, studies from MAG-deficient mice show no delay in myelination nor abnormalities in myelin macrostructure[34,35]. Interestingly, over time MAG-deficient mice display both myelin and axonal degeneration[36], suggesting that MAG interacts with the axon and is important for long-term maintenance and viability. While expressed very early in development, in the intact adult CNS developing growth cones are not normally exposed to MAG because of its localization at the paranodes or periaxonal leaflet of the membrane. This is in contrast to growth cones that form in attempted regeneration after injury to the mature CNS where growth cones come into contact not only with MAG, but also with other inhibitors in myelin.

MAG is a member of the immunoglobulin (Ig) super-family and contains five Ig-like domains in its extracellular sequences, a single transmembrane domain, and a

short cytoplasmic domain[37–39]. Importantly, MAG is a member of the sialic acid binding Ig-like lectin (Siglec) family of proteins. MAG (Siglec-4) is a sialic acid binding protein and shares 45-50% amino acid sequence similarity in its first four N-terminal domains with the domains of both CD22 and Sialoadhesin[40]. The sialic acid binding site within MAG has been identified in the first Ig –like domain (a conserved sequence in all Siglecs) and is dependent on the residue Arg118[38]. This is of particular importance as the domain of MAG responsible for inhibition of neurite growth is located within the 5th Ig-like domain, making the inhibitory and lectin properties of MAG are independent of one another[37]. Additionally, after damage to the CNS an extracellular, a soluble fragment of MAG is released from damaged white matter and is inhibitory to axonal regeneration[41].

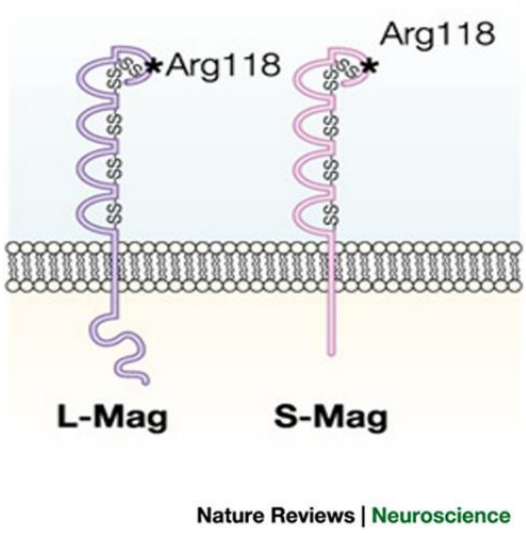


Figure 1-3 Structures of MAG.
MAG exists in a long (L) and short (S) isoform, with the distinction being the length of the cytoplasmic domain. MAG has lectin activity in the first of its 5 immunoglobulin (Ig)-like domains in its extracellular segment which is dependent on the residue Arg118. However, the inhibitory site on MAG is in the 5th domain.

Oligodendrocyte Myelin Glycoprotein

OMgp was originally identified in 1988[42]. OMgp is a GPI anchored membrane protein, which despite the implication of its name is not expressed only in oligodendrocytes, but at high levels in neurons as well[43]. It can also be found in the PNS[44]. OMgp has been implicated to play a role in oligodendrocytes growth, myelination arrest, and myelin compaction and is generally believed to be localized to paranodal loops adjacent to the node of ranvier[11].

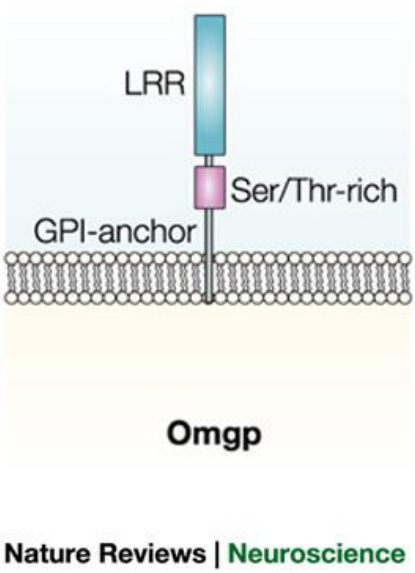


Figure 1-4 Structure of OMgp.
Oligodendrocyte myelin glycoprotein (Omgp) is a glycosyl phosphatidylinositol (GPI)-linked protein that carries a leucine-rich repeat (LRR) region and a serine/threonine (Ser/Thr) rich region.

Mechanisms of Regenerative Failure of the CNS

The failure of neurons of the brain and spinal cord to regenerate was known going back to ancient Egyptian civilization, and multiple case studies of spinal cord patients were documented by the historical Egyptian physician Imhotep[45]. However, for millennia the observed failure of neuronal regeneration was believed to be due to intrinsic inability of CNS neurons to undertake a regenerative phenotype after terminal differentiation. It wasn't until the late 20th century that this concept was challenged, and it was first demonstrated that CNS neurons possess and inherent capacity for regeneration[6]. As we now know that extrinsic variables within the CNS environment are responsible for regenerative failure, today's research has focused on the molecular mechanisms underlying this phenomenon.

Cellular Mechanisms of Regenerative Inhibition

In both myelin and CSPG mediated inhibition of neurite outgrowth, activation of the small GTPase RhoA is a necessary and sufficient signal needed for regenerative failure[46–50]. GTPase activity is a crucial aspect of many aspects of neuronal morphology and behavior. In terms of neuritogenesis and growth cone function, RhoA has a potent reciprocal relationship with Rac1 and Cdc42, with Rac1 and Cdc42 activation having potent promotional effects on growth cone formation, axonogenesis, and other pro-neuritic functions[46]. The antagonist effect of MAIs on both Rac1 and RhoA has been known for over a decade, as MAIs have long been known to cause marked inactivation of Rac1 while readily increasing GTP-bound RhoA levels[51]. Activated RhoA leads to activation of LIM kinase and Slingshot phosphatase, which

in turn leads to activation of cofilin, a potent local destabilizer of the actin cytoskeleton[7]. While a number of the important myelin-associated proteins have been identified, the mechanism by which these molecules signal from the extracellular space to RhoA is incompletely understood and the receptors mediating this process have been elusive.

Receptors of Inhibitory Components of CNS Myelin

The first receptor identified MAI receptor implicated in neurite outgrowth inhibition was the Nogo-66 receptor (NgR)[52]. This receptor is a GPI-anchored protein with no intrinsic signaling capability. Consequently, NgR signaling to RhoA requires the formation of a receptor complex in order to affect intracellular signaling pathways. There have been several proteins identified as being capable of complexing with NgR in response to ligation with MAIs. LINGO-1, a membrane protein selectively expressed in oligodendrocyte precursor cells and neurons, is important for numerous homeostatic CNS processes and complexes with NgR in response to MAI ligation[53]. Additionally, there is some evidence that LINGO-1 can be a viable therapeutic target, and antagonism of LINGO-1 has been shown to restore neurite outgrowth in inhibitory environments[54]. The best studied component of this receptor complex, and the participant which has been shown to mediate signaling to RhoA, is the TNFR-family member p75. It has been reported that the p75 receptor responds to NgR binding of myelin components by associating with NgR and undergoing sequential proteolytic events. This results in the release of the intracellular domain, which then displaces the RhoGEF and leads to activation of RhoA[55–62]. However, not all adult neurons express

p75, yet still respond to MAIs. Troy/Taj, an orphan member of the TNFR family, has been shown to functionally replace p75 in neurons that are p75-deficient[63–65].

While much of the mechanistic elucidation of MAI has centered around NgR as the primary mediating receptor for this process, it has now been shown in multiple labs that NgR is not the primary receptor mediating myelin-induced regenerative failure in CNS injury[66,67]. Interestingly, NgR does appear to effectively modulate growth cone collapse in response to myelin, but does not appear to have any effect on the chronic regenerative failure of CNS neurons in the presence of myelin[66]. While this finding was initially surprising, the decoupling of growth cone collapse and chronic regenerative failure addressed a conceptual limitation in the p75 RIP model of RhoA activation. In that model, intracellular proteolytic fragments of p75 were responsible for the activation of RhoA known to cause growth cone collapse, and were also thought to be responsible for chronic regenerative failure. However, this intracellular fragment is rapidly degraded upon interaction with the RhoGEF, making chronic sustained inhibition of axonal regrowth difficult to address via this process. In recent years, a second model for RhoA activation that seems to be more conducive to sustained signaling and chronic inhibitory effects has arisen. It has now been demonstrated that MAI interaction with neurons results in the formation of MAI-containing endosomes that are efficiently transported to the cell body through the axon. Other ligands have also been demonstrated to induce p75-positive ‘signalosomes’ capable of sustained and directed signal maintenance[59]. MAI endocytosis results in vesicle formation that results in activated RhoA accumulation in

the cell body, which can then cause transcriptional cellular modifications capable of sustaining regenerative failure[14,28]. This new model not only addresses some of the conceptual limitations of the RIP model, but also shares similarities with other neuronal signaling events that have evolved to facilitate communication between neuronal soma and distal projections.

NgR is both the first discovered and best studied MAI receptor. However, genetic deletion studies do not indicate that NgR is capable of significantly impacting regenerative failure of CNS neurons *in vivo.* As such, the search for the elusive mediating receptor has continued. It has been shown that certain gangliosides can bind MAIs and mediate inhibitory signaling[68–71], however those studies have yielded conflicting results and have not held up *in vivo*. The most recently identified putative MAI receptor has been paired immunoglobulin-like receptor B (PirB)[72–74], and it was initially thought that this was likely the missing link in MAI inhibition of neuronal regeneration. However, like its predecessors, PirB failed to stand up to scientific scrutiny and failed to yield therapeutic benefit in genetic deletion models *in vivo* [74,75].

The failure of PirB to provide a potent therapeutic target as a receptor of MAIs left questions as to the validity of MAIs as therapeutically relevant factors of regenerative failure. After several identified receptors capable of binding multiple functionally distinct MAIs not resulting in a regenerative phenotype, it seemed as though the theory of disrupting MAI/neuronal interaction as a therapeutic approach was not a relevant approach to SCI. However, the binding of MAG by these receptors yielded a clue as to why these receptors had failed as therapeutic targets. As

previously discussed, MAG is a lectin protein with sialic acid binding activity distinct from its inhibitory function. As such, receptors of MAG capable of mediating its inhibitory effects must bind in a sialic acid independent manner. Interestingly, all previously identified receptors of MAG, including NgR and PirB, depend on sialic acid for binding. These findings indicate that while several receptors have been previously identified, they do not demonstrate the binding characteristics that would be identifiable in a functional MAI receptor with therapeutically relevant implications.

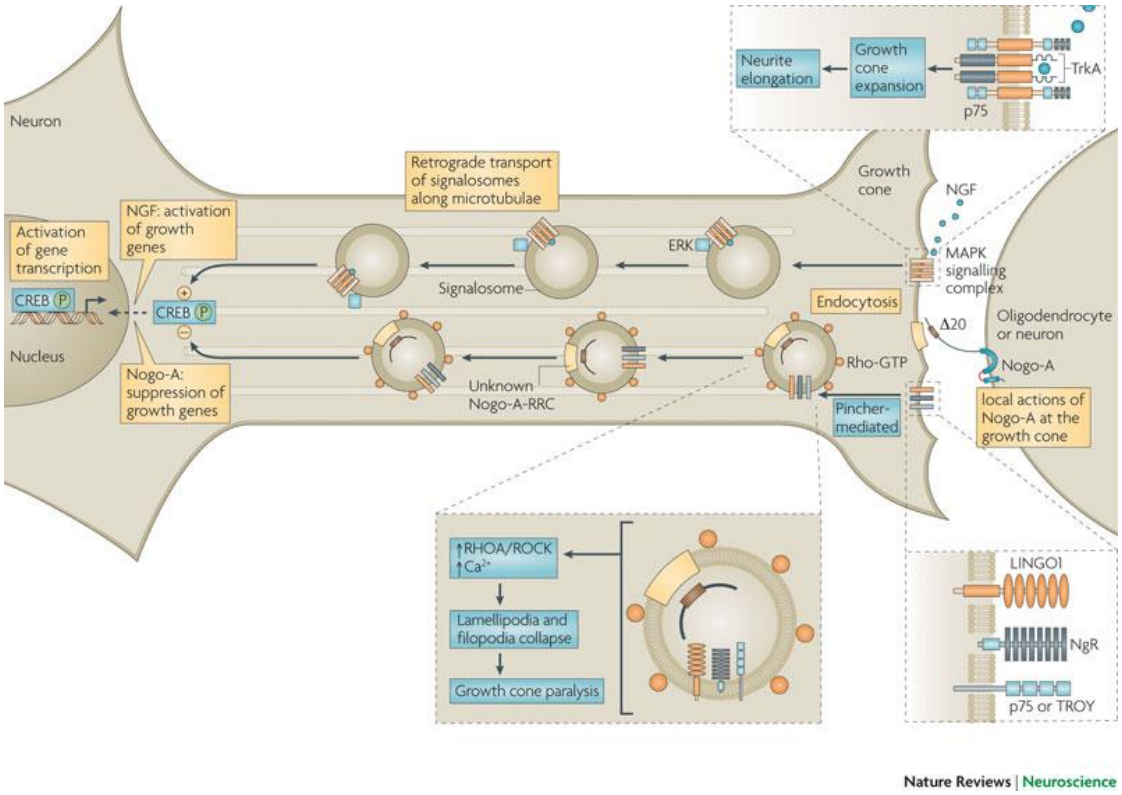


Figure 1-5 Illustration of MAI activity on a regenerating axon.
At the growth cone, binding of Nogo-A (or its active, shed fragments) to the Nogo receptor complex triggers endocytosis. This is followed by collapse of lamellipodia and filopodia, presumably mediated by RHOA activation and an increase in intracellular Ca2+. The Nogo-A–Nogo receptor 1 (NgR1) complex is then retrogradely transported to the cell body in signalosomes (signalling endosomes) carrying Rho-GTP (shown as orange circles) on their membranes. In the cell body they induce a decrease in levels of phosphorylated cyclic AMP response element-binding (CREB) and a downregulation of neurite growth specific proteins and transcription factors. Neurotrophins such as nerve growth factor (NGF) interact at the growth cone with high affinity nerve growth factor receptors (Trks) and p75, leading to internalization of the neurotrophin–receptor complex, growth cone expansion and neurite elongation. Internalized neurotrophin–receptor complexes are transported retrogradely as signalling endosomes with mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) on their surface. Once in the cell body, they increase phosphorylated CREB levels and subsequent activation of the genetic programme for neurite growth. Nogo-A-RRC; unknown Nogo-A receptor.

Current Treatment for Spinal Cord Injury and Drugs in Clinical Trial

Currently there are no neuroregenerative therapies that are considered standard of care for SCI. To date there have been 4 therapeutics that address neuronal regeneration that have made it past phase I clinical trial. One compound (GM-1 (Sygen)) has completed a randomized placebo controlled Phase III study and two other compounds are in pre-Phase III investigations. Additionally, while a comprehensive list of preclinical agents is not contained in this document, a select potential therapeutics that are not yet in clinical trial will be discussed below.

GM-1 (Sygen)

Gangliosides are major components of neuronal cell membranes. In the 1980's a handful of studies showed that isolated versions of these proteins could have regenerative effects preclinically both *in vitro* and *in vivo*. To evaluate the human therapeutic benefit of ganliosides in spinal cord injury, "The Maryland GM-1 Ganglioside Study" was organized and executed from January 1986 – May 1987 and included 34 patients. The results of this study, published in 1991, showed that GM-1 increased neurological function significantly as compared to placebo. A large multi-center study, the "Sygen Acute Spinal Cord Injury Study", sponsored by Fidia Pharmaceuticals, was conducted between April 1992 and January 1997 with the results published in 1998. This large Phase III trial enrolled 797 patients but the results were ultimately disappointing with no significant difference between Sygen and placebo. No further studies have been made public. Subsequent research on the role of

gangliosides in regenerative failure have indicated that these molecules are not a central factor for neuronal regrowth after injury[76].

Cethrin (BioAxone)

Cethrin, the trademark name of the bacteria-derived toxin BA-210, inhibits Rho activation that is a known inhibitor of the neuronal growth. Rho has been shown to be effective at treating SCI in pre-clinical animal studies. A Phase I/IIa open-label clinical trial was conducted from February 2005 to November 2007 and included 48 patients. Results from this small study are encouraging especially for those patients afflicted with cervical injury. BioAxone BioSciences is actively recruiting strategic partners to conduct further clinical trials to establish the efficacy of Cethrin[77,78].

Cethrin is an exciting advancement for spinal cord injury. However, the approach taken with this treatment has 2 primary limitations. First, because Cethrin is directly applied to damaged spinal cord during surgery, you are limited in the quantity, duration and mode of administration. Second, while Rho can limit regeneration of damaged neurons, it is important for proper function and other cell types in the CNS that aid in normal healing after injury. Impairing Rho function in these cell types could exacerbate some of the secondary complications of spinal cord injury.

Anti-Nogo/ATI355 (Novartis, Basel)

Anti-Nogo is a monoclonal antibody that targets Nogo-A, a component of the myelin sheath that encases neurons that has been shown to inhibit the growth of axons following injury. Pre-clinical data is encouraging and early stage clinical investigations have begun. A Phase I trial has been completed with Novartis and

showed no safety issues . A Phase II trial is currently underway. However recent data suggests that the window for anti-Nogo therapy is quite small and the functional benefits have not been confirmed[79].

AC105 (Acorda Therapeutics)

AC105 is a proprietary magnesium formulation licensed by Acorda in 2011. In preclinical studies, AC105 has demonstrated neuroprotective properties leading to improvement of locomotor function in SCI and cognitive function in TBI when therapy was initiated within four hours of injury. AC105 has completed Phase 1 trials in healthy volunteers and Acorda expects to initiate a Phase 2 clinical trial program in SCI, potentially expanding into other central nervous system indications, such as TBI and stroke. The U.S. Food and Drug Administration (FDA) granted Fast Track designation on February 12, 2009 for AC105 to improve functional recovery following treatment of acute SCI patients. Acorda expects to apply for FDA Orphan drug designation for the acute treatment of SCI and will explore Orphan drug designations in Europe and in other parts of the world given its worldwide development and commercialization rights.

Umbilical Cord Blood and Lithium (Stemcyte)

Spearheaded by work performed by Dr. Wise Young at Rutgers University, it has been demonstrated that lithium treatment caused proliferation of neural precursors that stimulate recovery after SCI[80]. Stemcyte has developed a lithium-based therapeutic that combines with umbilical cord blood to deliver stem cells to the site of injury with a source of lithium that leads to neuronal cell development. Stemcyte is

preparing for multinational clinical trials coordinated by the SCI network. These clinical trials are being planned to be the first that are controlled for both surgical intervention and placebo conditions.

Stem Cell Therapy

In addition to chemical compounds that seek to regrow damaged neurons after injury, researchers are investigating stem cells as a potential regenerative therapy. As of now, no study has demonstrated that this treatment option in efficacious, although there are a number of preclinical studies showing beneficial effects. However, many beneficial effects of stem cells therapies have now been linked to secondary trophic benefits and not to the ability of these cells to replace damaged neuronal populations. Stem cell therapy is purely an investigational line of research at this point. However, very recently scientists have had success with neuronal stem cells being implanted and differentiated into mature neurons, effectively bridging the lesion site and improving neuronal function[81]. These studies have since been scaled up to non-human primate models and work is being done to prepare this for human clinical application.

Low density Lipoprotein Leceptor-Related Protein-1

LRP1 is a type-1 transmembrane receptor that binds over forty structurally and functionally distinct ligands, mediating their endocytosis and trafficking[82]. LRP1 also functions in phagocytosis of large particles, including degenerating myelin[83,84] and apoptotic cell debris[85]. Neurons in the CNS and PNS express LRP1[13,86,87], and LRP1 expression in the CNS is among the highest expression amongst tissues as indicated by the Human Protein Atlas. Neuronal LRP1 is upregulated during development, as

well as after injury, and is at least partially localized to axons and neuronal growth cones, both in intracellular vesicles and at the cell surface during attempted regeneration[88,89].

LRP1 is also an important regulator of the plasma-membrane proteome as has been demonstrated by its ability to mediate the localization, maturation and signaling of integrins[90–92], regulate the composition of extracellular matrices[93], as well as regulating other cell-surface receptors such as TNFR[94]. LRP1 has also been shown to function in cell-signaling both directly via binding of signaling adapter proteins and indirectly as a result of its ability to regulate levels of other surface receptors such as TNFR.

LRP1 also plays an important role in the regulation of cell-signaling, often in -conjunction with a diverse array of ligand-specific co-receptors. Examples include: uPAR, TNFR1, PDGF receptor, Trk receptors, and Frizzled-1[94–98]. The multifunctional effects of LRP1 on cell-signaling are then likely due to this diversity of LRP1 co-receptors, which seem to be distinct in both a ligand-specific and a cell type-specific manner.

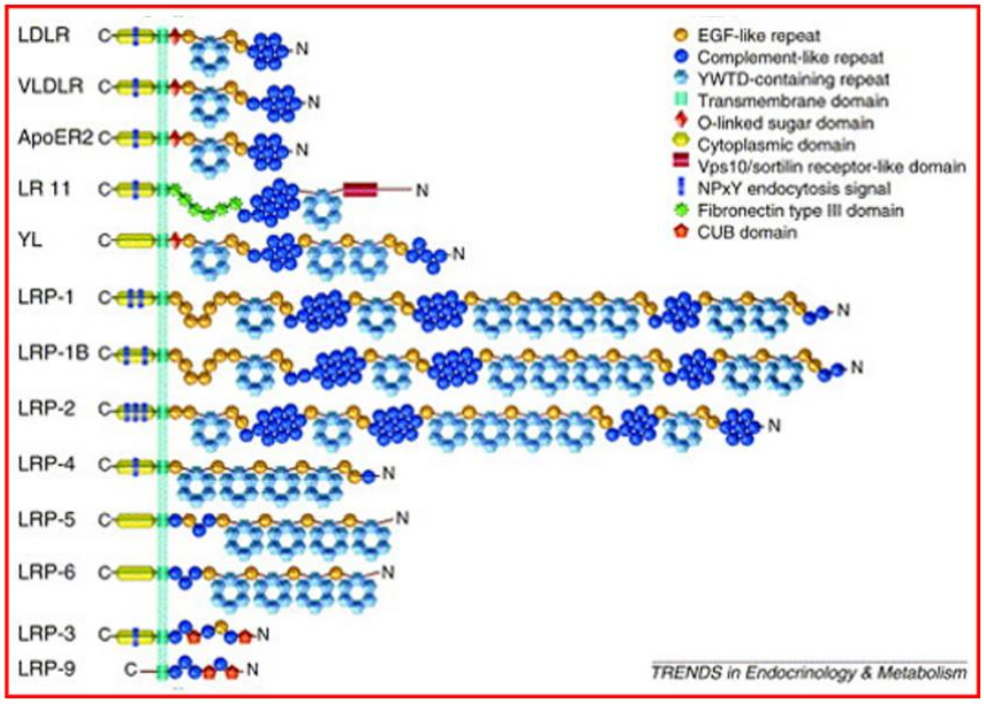


Figure 1-6 The domain structure of members of the LDLR family.
Abbreviations: ApoER2, apolipoprotein E receptor 2; CUB domain, domains found in complement subcomponents C1r/C1s, Uegf and bone morphogenetic protein-1; EGF, epidermal growth factor; LDL, low-density lipoprotein; LDLR, LDL receptor; LRP, LDL-related protein; NPxY, asparigine-proline-x-tyrosine, where x is any amino acid; VLDLR, very low-density lipoprotein receptor; YL, yolkless; YWTD, tyrosine-tryptophan-threonine-aspartic acid.

LRP1 in the CNS

Several studies have demonstrated a variety of ways LRP1 is important in neuronal function such as; endocytosis and trafficking of prion and amyloid proteins[99,100], promotion/inhibition of neurite outgrowth via ligation[101], and association with post-synaptic proteins that is necessary for normal motor function[102]. LRP1 has also been implicated in the pathogenesis of several neurodegenerative diseases such as Alzheimers disease[103,104], multiple sclerosis[84,85], and prion disease[99]. LRP1 is known to be expressed in neurons and has been shown to be important in maintaining normal motor function[102].

Statement of Hypothesis

Our previous work demonstrating myelin phagocytosis by LRP1[84] prompted us to examine the specific components of myelin that interact with LRP1. As the receptor mediating MAI-mediated regenerative failure is currently unknown, I hypothesized that LRP1 was the novel receptor responsible for myelin-mediated inhibition of neuronal regeneration after damage to the CNS.

CHAPTER 2 - LRP1 IS A NOVEL RECEPTOR FOR MAG

ABSTRACT

We have previously reported that LRP1 is a novel endocytic receptor for myelin debris. To identify the specific interacting components of myelin with LRP1, we performed mass spectrometric analysis of proteins pulled-down from purified CNS myelin with recombinant LRP1 binding domains. Among the binding partners indicated we identified MAG as a putative abundant binding partner. Follow up mass spectrometric studies using purified recombinant MAG incubated and pulled down from neuronal protein extracts also indicated an abundant LRP1/MAG interaction. We then confirmed this interaction through numerous biochemical and cell-binding assays. Additionally, in this study we demonstrate that binding of MAG to neuronal LRP1 results in efficient endocytosis, which is lost when LRP1 is antagonized or genetically silenced. MAG is one of the three most commonly studied myelin-associated inhibitors (MAI) to neuronal regeneration present in myelin. Along with OMgp and Nogo-(A-C), MAG binding to neuronal surfaces results in activation of RhoA and inhibition of neurite outgrowth. Here we demonstrate that LRP1 is a novel receptor of MAG that binds with high affinity and is necessary for neuronal MAG endocytosis.

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[8,105,106]. Several myelin-associated inhibitory

proteins (MAIs) have been identified, including members of the reticulon family (Nogo A-C), myelin associated-glycoprotein (MAG), and oligodendrocyte myelin glycolprotein (OMgp)[7,11,28]. However, the neuronal receptors facilitating inhibitory signaling by MAIs have remained elusive. 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. MAG is a sialic acid-recognizing Ig-family lectin[38,68,70]. Deletion of the lectin activity in MAG disrupts binding to gangliosides, PirB and to the Nogo receptor family members, NgR1 and NgR2, yet does not abolish growth inhibition[37,107].

NgR1 is the ligand-binding portion of a tripartite receptor complex that includes Lingo-1 and p75NTR or TROY[7]. This receptor complex participates in growth cone collapse in response to MAG, Nogo66 and OMgp[108]. Nogo and MAG promote association of p75NTR with Rho-GDP Dissociation Inhibitor (RhoGDI), which results in release and activation of RhoA[109]. 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[67,69,110]. Myelin inhibition of neuroregeneration can be released by a variety of approaches; preconditioning neurons via BDNF treatment[111], inhibition of RhoA activation[50], artificial increases in cAMP[8,16], or in the case of dorsal root ganglia, by performing a preconditioning lesion on the peripheral branch[11,16]. However, these approaches do not represent clinically viable options for therapeutics aimed at overcoming the adverse effects of myelin debris.

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 , has been reported to lead to a significant, yet incomplete release of neurite outgrowth inhibition in response to MAIs[66,67,72]. Unfortunately, loss of PirB has not held up as a viable therapeutic target as genetic deletion has not demonstrated significant therapeutic benefit *in vivo*[75].

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[82]. LRP1 also functions in phagocytosis of large particles, including degenerated/disorganized myelin[83,84]. Neurons in the CNS and PNS express LRP1[13,86,87], which is partially localized to axons and neuronal growth cones, both in intracellular vesicles and at the cell surface[88].

LRP1 regulates cell-signaling in conjunction with diverse co-receptors, including uPAR, TNFR1, PDGF receptor, Trk receptors, and Frizzled-1[94–98]. 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[98,112–116]. This response requires Trk receptor transactivation downstream of phosphorylated c-Src[98].

Our previous work demonstrating myelin phagocytosis by LRP1[84] prompted us to examine the role of LRP1 in the mechanism by which myelin inhibit axonal re-

generation. 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.

MATERIALS AND METHODS

Recombinant and purified proteins

CCRII, which includes amino acids 804-1185 of mature LRP1; CCRIV, 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[72,107,117]. 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[84]. Shed LRP1 and full-length LRP1 were purified from human plasma and rat liver, respectively, by RAP-affinity chromatography and molecular exclusion chromatography[118]. OMgp-AP, AP-Nogo66, and AP-Fc were expressed in HEK293T cells as previously described[117].

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[72,107]. 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[117]. N20.1 cells were a gift from Dr. Anthony Campagnoni (University of California Los Angeles) and were cultured as previously described[119].

CNS myelin purification

Myelin vesicles were purified from rat brain, as described by Norton and Poduslo[120]. The purity of the preparation was determined by Coomassie Blue staining and by immunoblot analysis for myelin basic protein, as previously described[84].

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 CaCl2, and protease inhibitor cocktail). Protein extracts (2 mg) were incubated with 1 μM CCRII-Fc, CCRIV-Fc, or Fc overnight at 4° C. 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 CCRII-Fc or CCRIV-Fc, but not Fc, were identified by LC-MS/MS as previously described[121].

For studies utilizing protein extracts from N2a cells incubated and pulled down with purified MAG-Fc, 1 mg of N2a extract was prepared as described above and incubated with 10 µg MAG-Fc, or Fc alone, for 1 hour at room temperature. The fusion proteins and associated proteins were recovered by incubation with Protein A-Sepharose. Pulled down proteins were then assessed by SDS-PAGE and commassie staining. Protein bands unique to the MAG-Fc pulldown were then digested with trypsin in the presence of ProteaseMAX surfactant (Promega, Madison, WI, USA). Proteins within each unique band were then identified by LC-MS/MS as previously described[121].

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 CCRII-Fc, CCRIV-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 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, 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 CCRII-Fc, CCRIV-Fc, or Fc was 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^{o}C. Bound proteins were detected using appropriate antibodies.

Binding of CCRII-Fc and CCRIV-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°C 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 quantitated 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±SEM are presented.

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[94]. 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).

Immunofluorescent 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°C and then with 25 nM MAG-Fc, RPTPσ-Fc, or Fc for 60 min at 4°C. The cells were then

warmed to 37° C 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).

Immunoblot analysis of MAG endocytosis

For immunoblot studies assessing LRP1 mediated endocytosis of MAG, LRP1 positive and deficient cells were treated with Fc-receptor blocking antibody (1 μg/ml) for 30 min at 4°C and then with 25 nM MAG-Fc, RPTPσ-Fc, or Fc for 60 min at 4°C. The cells were then warmed to 37° C for 30 min. Surface-associated fusion protein was dissociated by treatment with acetic acid/sodium acetate pH 3.0 for 4 min on ice. Cells were then washed in cold PBS and 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 and actin as a loading control. Cellular protein concentration was determined by a bicinchoninic acid assay (Pierce, Rockford, IL) and used to standardize sample concentrations.

Radiolabeled MAG Internalization

MAG-Fc was radioiodinated with 1 mCi of Na125-I using Iodobeads (Pierce, Rockford, IL, USA) and separated from free Na125-I by molecular exclusion chromatography. - cells were plated in 12-well plates and equilibrated in DMEM with 25 mM HEPES, pH 7.4, 0.1% BSA and Fc-Blocker (BD Biosciences, San Jose, CA, USA). 125I-MAG-Fc (25 nM) was incubated with cells for 2 h at 37°C. Unlabeled

MAG (1.25 μM) was added to some wells. At the end of the incubation, cells were washed and treated with 0.25% Pronase (Roche, Pleasanton, CA, USA) 22 for 15 min to eliminate surface-associated 125I-MAG-Fc. Cell extracts were prepared in 0.1 M NaOH and 1% SDS. Cell-associated radioactivity was determined using a Wallac 1470 Wizard Gamma Counter (Perkin Elmer, Waltham, MA, USA). Cellular protein was determined by bicinchoninic acid assay (Pierce, Rockford, IL, USA). Specific MAG-Fc uptake was calculated as the fraction of total uptake that was inhibited by excess unlabeled MAG.

qPCR analysis

To ensure efficient silencing in experiments total RNA was isolated using the RNeasy kit (Qiagen). cDNA was synthesized using the iScript kit (Bio-Rad). qPCR was performed using an Applied Biosystems instrument and a one-step program: 95°C, 10 minutes; 95°C, 30 seconds; and 60°C, 1 minutes for 40 cycles. HPRT-1 gene expression was measured as a normalizer. Results were analyzed by the relative quantity (ΔΔCt) method. All experiments were performed in triplicate with internal duplicate determinations.

Immunofluorescence microscopy

Cells were plated on glass coverslips, fixed in 4% paraformaldehyde, and as indicated, permeabilized in 0.2% Triton X-100, and incubated with antibodies specific for the specified antigens followed by secondary detection with antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594. Preparations were mounted on slides using

ProLong Gold with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) and examined using a Leica DMIRE2 fluorescence microscope.

RESULTS

LRP1 associates with MAG in mass spectrometric studies

MAIs have potent morphologic and cell signaling effects on regenerating neurons. While numerous putative receptors have been identified as mediators of these MAI effects, none have demonstrated the desired binding characteristics and have subsequently shown little to no therapeutic relevance in *in vivo* models. Consequently, the receptor mediating the critical inhibitory functions of MAIs has yet to be discovered, and would represent a potent therapeutic target for regeneration of CNS neurons after injury.

As our previous work had demonstrated a novel interaction between LRP1 and myelin debris, we sought to assess the specific components of CNS myelin that bind to LRP1. Because LRP1 is a very large protein, it is difficult to obtain large enough amounts of purified protein to conduct certain biochemical binding studies. However, it has been shown that the vast majority of ligand binding within LRP1 takes place within the 2nd and 4th clusters of complement repeats (CCRII and CCRIV respectively) [94,122,123]. Recombinant versions of CCRII and CCRIV have been commonly used in LRP1 research and are much easier to obtain and utilize in significant quantity. As such, our initial studies to uncover LRP1 binding partners within myelin were conducted using Fc-conjugated recombinant CCRII and CCRIV, which we incubated with protein extracts from CNS myelin. Associated proteins were pulled down and

digested for mass spectrometric analysis. Figure 2-1 shows a selection of binding candidates identified by the mass spec screen. Hits were distinguished as proteins detected at a minimum of 10-fold abundance as detected by spectral counts. Myelin basic protein was the most abundant binding partner, but as the figure shows, both MAG and OMgp were identified as well.

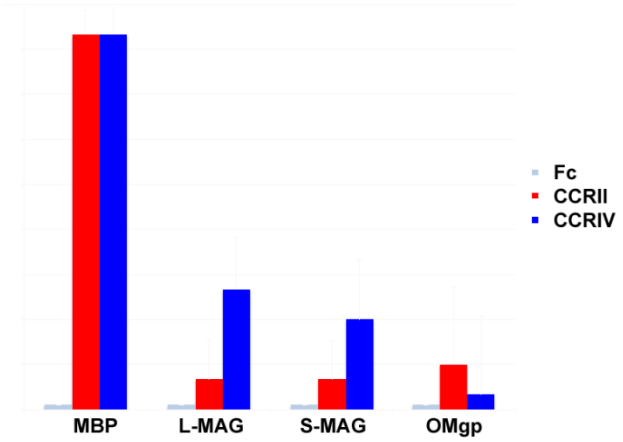
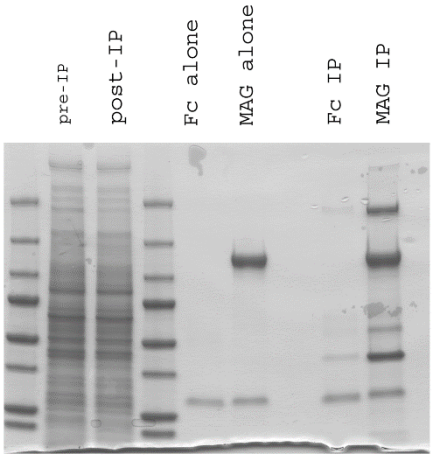


Figure 2-1 The 2nd and 4th cluster of complement repeats of LRP1 associate with various components of CNS myelin, including the MAI's MAG and OMgp, as detected by mass spectrometric analysis.
Recombinant Fc-conjugated CCRII and CCRIV, as well as Fc control, were incubated with CNS myelin and associated proteins were pulled down and analyzed by mass spectrometry. Both CCRII and CCRIV show selective association with both the long and short (L and S) isoforms of MAG, as well as OMgp. Bars represent spectral counts relative to the Fc control.

To further ascertain the specificity and validity of this interaction, we performed the reciprocal study, incubating recombinant MAG with neuronal extracts and performing mass spec analysis of the associated proteins. Figure 2-2 shows that in these studies, MAG pulled down 7 unique protein bands relative to Fc control. Interestingly, 5 of the 7 unique bands contained peptide fragments of LRP1, which validated the results of our first mass spec study.

A



B

protein	PepCount	UniquePepCount	CoverPercent	ProteinProb	Mw	pI	Length	GroupIdx	CrossProCount	IsTarget?
IgE binding prot	30	11	25.85%		62746.6	9.38	557	3917	4	1
RAP	16	8	20%		42214.55	7.35	360	2737	1	1
lrp1	11	6	1.06%		504738	5.14	4545	473	1	1
lsp1	6	5	24.84%		35762.72	5.07	322	611	1	1
cingulin	11	4	2.35%		136544.5	5.72	1192	965	1	1

Figure 2-2 MAG-Fc pull-downs from N2a protein extracts results in unique protein bands by SDS-PAGE and coomassie stain. Mass spectrometric analysis of MAG-specific bands identify LRP1 and RAP as the 2nd and 3rd most abundant associated proteins.

A. Neuronal N2a extracts were incubated with MAG-Fc or Fc, which were then pulled down and analyzed by SDS-PAGE and coomassie staining. Complete protein extracts pre and post incubation were also analyzed, and as a control, Fc and MAG-Fc alone were also ran. B. MAG-specific proteins pulled down from N2a extracts were digested and analyzed by mass spectrometry.

As mass spec studies provided strong evidence for the binding of MAG to LRP1, we sought to confirm this interaction biochemically. To accomplish this we employed the same recombinant LRP1 binding domains and experimental paradigm used in our mass spec studies. Figure 2-3A illustrates the structure of LRP1 and the domains which are represented in the CCRII and CCRIV Fc-fusion proteins. In these studies, proteins bound to CCRII, CCRIV and Fc alone were pulled down and analyzed by SDS-PAGE and immunoblot analysis. Figure 2-3B illustrates that pull-down analysis confirmed the association of CCRII and CCRIV with MAG from CNS myelin. While this validated the results of our mass spec study, it did not address the nature of the LRP1/MAG relationship. The association of CCRII and CCRIV with MAG does not prove direct binding. As such, we wished to assess the directness of this association in a purified system. To accomplish this, we immobilized Fc, CCRII, and CCRIV on a nitrocellulose membrane in dot-blot format. We then incubated immobilized proteins with soluble purified MAG, or vehicle control. As figure 2-3C demonstrates, MAG selectively associates with CCRII and CCRIV, and no MAG is detected in vehicle control studies. As a result, these studies support the hypothesis that CCRII and CCRIV are capable of direct binding to MAG.

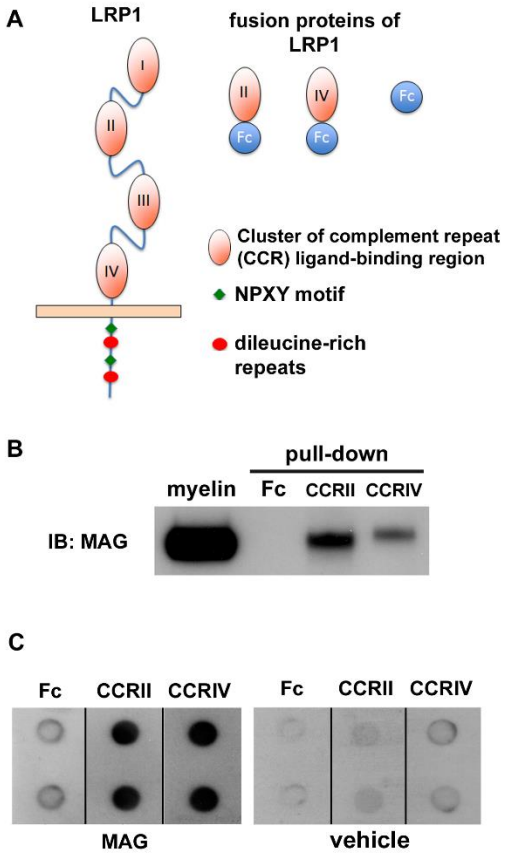


Figure 2-3 MAG selectively pulls down from CNS myelin extracts with CCRII and CCRIV.
A. Schematic representation of LRP1 and the recombinant CCRII and CCRIV fused to Fc. B. CCRII and CCRIV were incubated with myelin extracts and associated proteins were pulled down and analyzed by SDS-PAGE and western blot immunodetection. MAG selectively associated with both CCRII and CCRIV, but not Fc control. C. CCRII, CCRIV, and Fc (40 pmol) 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.

We next sought to confirm that CCRII and CCRIV binding of MAG could be recapitulated by full-length LRP1. 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. 2-4). Similar to pull-down studies with CCRII and CCRIV, MAG-Fc and associated proteins were precipitated with Protein A-Sepharose. Precipitated proteins were analyzed by SDS-PAGE and immunodetection of the 85-kDa β-chain LRP1. LRP1 did not co-precipitate with purified Fc, demonstrating specificity in the MAG-LRP1 interaction. These studies indicate that binding of CCRII and CCRIV with MAG are mimics of the full-length LRP1 binding.

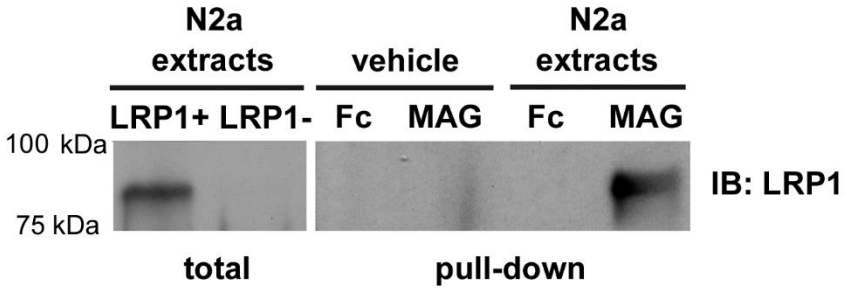


Figure 2-4 MAG-Fc pulls down LRP1 from N2a protein extracts.
A. Neuronal N2a extracts were incubated with MAG-Fc or Fc. Fc and MAG were then pulled down and analyzed by SDS-PAGE and immunodetection of LRP1. LRP1 was specifically detected in pull-downs with MAG-Fc. No LRP1 was detected in lanes with MAG-Fc only. Specificity of LRP1 immunodetection was validated by assessment of extracts from N2a cells in which LRP1 was present or stably silenced.

An important characteristic of ligand/receptor interactions are the reversibility of the association. As we had previously demonstrated that LRP1 and MAG are interacting directly, we next sought to assess the reversibility of the interaction using the LRP1 antagonist RAP. To accomplish this, we used the N20.1 cell line as an abundant source of LRP1. Protein extracts of N20.1 cells were then incubated with RAP or GST control prior to pull-down analysis with purified MAG-Fc. Similar to experiments above, MAG readily pulled down LRP1 from N20.1 extracts (Fig. 2-5A). RAP blocked the binding of MAG to LRP1. Total protein was ran in lane 1 as a positive control for LRP1 immunodetection. Finally, to confirm that full-length LRP1 and MAG interact in a purified protein-protein system, we assessed the ability of MAG-Fc to pull down purified full-length LRP1 from solution. Full-length LRP1 was purified from rat liver as previously described[118] and incubated with Fc or MAG. Vehicle was also used as an additional control to purified soluble LRP1. As anticipated, purified LRP1 pulled-down with MAG, but not Fc, indicating that the MAG/LRP1 interaction is direct, specific and reversible (Fig. 2-5B).

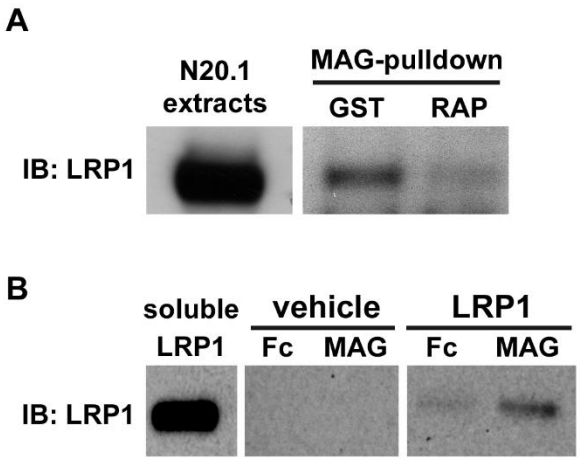


Figure 2-5 MAG interaction with LRP1 is reversible and direct.
A. Protein extracts from N20.1 cells were incubated with RAP or GST control for 20 minutes prior to incubation and pull-down with MAG. LRP1 pull-down with MAG was blocked in extracts treated with RAP. B. MAG or Fc was incubated with vehicle or purified, soluble LRP1 and then pulled down for SDS-PAGE and immunodetection of LRP1. LRP1 was detectable from MAG, but not Fc, pull-downs indicating a direct interaction.

We next sought to assess whether this LRP1/MAG was detectable endogenously. To accomplish this, we prepared protein 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^{o}C and then precipitated with Protein A/G-Argarose. Non-specific rabbit IgG was added to the brain extracts and precipitated similarly, as a control. Figure 2-6 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.

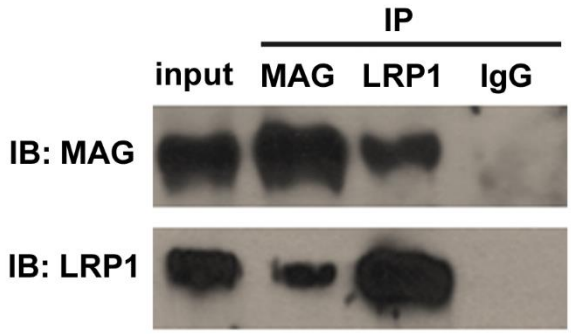


Figure 2-6 LRP1 and MAG associate endogenously from extracts of rat brain.
Antibodies against LRP1 and MAG, as well as control non-specific IgG, were incubated with protein extracts prepared from rat brains and immunoprecipitated for SDS-PAGE analysis. In the case of LRP1 and MAG antibodies, both LRP1 and MAG were immunoprecipitated confirming that LRP1/MAG association can occur in brain tissue. Conversely, neither protein was detectable in control IgG immunoprecipitates confirming the specificity of the IP studies.

As the final assessment of the MAG LRP1 interaction, we sought to analyze the CCRII/CCRIV binding kinetics in a well-established cell-based binding in which full-length MAG is expressed in COS-7 cells[117]. As a control we chose to compare the binding affinities of CCRII-Fc and CCRIV-Fc with an Fc fused chimeric form of NgR1 and NgR2 that binds MAG with higher affinity than wild-type NgR1, referred to as NgR^{OMNI}-Fc[107]. In this system CCRII-Fc and CCRIV-Fc incubation with cell-surface MAG exhibited a level of binding similar to that observed with NgR^{OMNI}-Fc (Fig. 2-7A). Purified Fc did not bind to MAG-expressing COS-7 cells, indicating that the binding of our recombinant Fc-fusion proteins is specific. As a control, we incubated CCRII-Fc, CCRIV-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 CCRII-Fc and CCRIV-Fc to COS-7 cells that were transfected to express PirB, which, like MAG, is a member of the Ig superfamily. Neither CCRII-Fc nor CCRIV-Fc bound to the PirB-expressing cells (data not shown).

As previously discussed, an important aspect of ligand/receptor interactions is reversibility. As we did in our biochemical experiments, we again sought to examine the ability of RAP (200 nM) to inhibit binding of CCRII-Fc and CCRIV-Fc to MAG-expressing COS-7 cells. GST (200 nM) was added in control wells. Fig. 2-7B shows that RAP almost completely inhibited binding of CCRII-Fc and CCRIV-Fc to the cells. Importantly, RAP did not affect binding of NgR^{OMNI}-Fc confirming both the reversibility of the LRP1/MAG interaction and the specificity of the activity of RAP.

Finally, using our cell-based binding model we sought to calculate the binding affinities of CCRII and CCRIV. To accomplish this, we incubated MAG-expressing COS-7 cells with increasing concentrations of CCRII-Fc, CCRIV-Fc, 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. 2-7C). 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 CCRII-Fc was 8.1 ± 0.2 nM (n=3) and the K_D for CCRIV-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. As a control we also analyzed binding of monomeric fusion proteins, which were not pre-clustered. Once again, the binding data were most accurately fit to sigmoidal curves (data not shown). The K_D values were 9.6 ± 0.3 nM for CCRII-Fc (n=3) and 17 ± 3 nM for CCRIV-Fc (n=3).

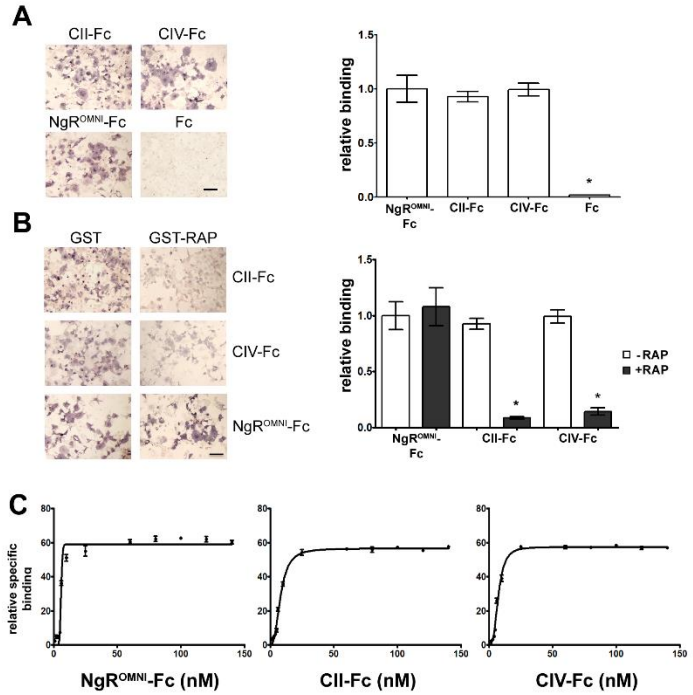


Figure 2-7 CCRII and CCRIV bind MAG with high affinity in RAP-reversible manner.
A. COS-7 cells were transfected with either membrane-bound MAG or control (GFP), and then incubated with CCRII-Fc, CCRIV-Fc, NgR^{OMNI}-Fc, or Fc control. All fusion proteins were conjugated to alkaline phosphatase (AP), and bound proteins were detected by developing the AP reaction with NBT/BCIP. The graph represents the degree of binding relative to NgR^{OMNI}-Fc to MAG-expressing COS-7 cells (+/- SEM, n=5 independent experiments). B. COS-7 cells were transfected with membrane-bound MAG, and then incubated with CCRII-Fc, CCRIV-Fc, or NgR^{OMNI}-Fc in the presence of either GST-RAP or GST (200 nM). The graph represents the degree of binding relative to NgR^{OMNI}-Fc to MAG-expressing COS-7 cells (+/- SEM, n=4 independent experiments). Scale bars, 20 µm. C. COS-7 cells were transfected with membrane-bound MAG, and then incubated (at increasing concentrations) with CCRII-Fc, CCRIV-Fc, or NgR^{OMNI}-Fc. All fusion proteins were conjugated to alkaline phosphatase (AP), and bound proteins were detected by developing the AP reaction with NBT/BCIP. Binding from 3 independent experiments was quantified using ImageJ software, and then averaged to yield the presented data points (+/- SEM). A sigmoidal line of best fit was used to assess binding affinities.

DISCUSSION

A detailed understanding of the mechanisms by which MAIs inhibit neuronal growth is of considerable interest, both biologically and clinically. While the CNS environment contains 2 distinct inhibitory factors, myelin and the glial scar, the delayed development of the glial scar implicates MAIs as the primary regenerative obstacle in acute spinal cord injury. As such, elucidation of the mechanism underlying MAI-mediated activation of RhoA is likely to yield invaluable insight into the therapeutic interventions that can block these effects. Interestingly, while there has been thorough characterization of identity of MAIs, as well as the downstream signaling events leading to activation of RhoA, there predominant facilitating receptor has yet to be revealed. As a result, current efforts at therapeutically targeting MAIs have been limited to disruption of the activation of RhoA, and neutralization of MAIs. Each of these approaches has distinct limitations, and blocking the mediating receptor would provide a preferable approach. As such, the identification of LRP1 as a novel MAG receptor may yield the critical piece of information for the development of agents capable of restoring the regenerative capacity of CNS neurons.

As discussed above, the identification of MAI receptors is of tremendous benefit for future generation of therapeutics. 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 re-dundancy by which MAIs signal growth inhibition[66,67,117]. Recently, there was the promising discovery of PirB as a promiscuous receptor for Nogo66, MAG, and

OMgp. Initial studies of the role of PirB in MAI-mediated regenerative inhibition indicated that 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[72]. However, subsequent studies were unable to confirm this effect and 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[74,75].

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[39,69,110,124]. Unfortunately, none of these mechanisms have demonstrated a reproducible ability to affect regenerative failure in a therapeutic manner. The work represented in this chapter identifies 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 with high affinity.

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. Interestingly, more recent studies showed that PirB also associates with p75NTR to signal growth inhibition[125]. NgR2 interacts with Troy[126]. Similar to NgR1 and PirB, we show that LRP1 associates with p75NTR in the presence of MAG. However, unlike previous studies with NgR, LRP1 does not seem to mediate regulated intermembrane

proteolysis of p75 (data not shown). Regardless, the interaction between LRP1 and p75 may be essential for activation of RhoA. 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[98,112,113,127]. These LRP1 ligands activate Src, transactivate Trk receptors, and activate ERK and AKT in a Trk-dependent manner[98]. NMDA receptors also may be involved[102,128]. 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 highly 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 is ligand-dependent and represents an important checkpoint in neuronal LRP1 signaling.

In the normal human brain, LRP1 is expressed by almost all neuronal populations[86,87,129]. In CNS injury, LRP1 expression significantly increases in reactive astrocytes[129]. Our previous studies suggest that LRP1-dependent phagocytosis of myelin debris occurs across diverse cell types[84]. 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 (which will be discussed in detail later) may be critical in determining the effects of MAIs on neuronal repair in the CNS, following injuries of diverse magnitudes. The discovery that LRP1 is a novel

receptor of MAG may represent a critical piece of information in understanding the chronic inhibitory effects of myelin after spinal cord injury.

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CHAPTER 3 - LRP1 IS SIALIC INDEPENDENT MAG RECEPTOR THAT IS RESPONSIBLE FOR MAG-MEDIATED INHIBITION OF NEURITE OUTGROWTH

ABSTRACT

The previous chapter demonstrated that LRP1 is a novel, high-affinity receptor of MAG. However, previously identified MAG receptors, which were also promiscuous binding partners for other MAIs, have not proven to be viable therapeutic targets as they do not appear to be facilitating the predominant inhibitory activities of MAIs on neuronal regeneration. The work in this chapter is aimed at deciphering the functional role of LRP1 in MAI-mediated inhibition of neurite outgrowth. In contrast with previously identified MAG receptors, binding of MAG to LRP1 occurs independently of terminal sialic acids. In primary neurons, PC12 cells, and N2a cells, functional inactivation of LRP1 with receptor-associated protein, depletion by RNAi knock-down, or *LRP1* gene deletion is sufficient to significantly reverse MAG-mediated inhibition of neurite outgrowth. Mechanistic studies in N2a cells showed that LRP1 and p75NTR associate in a MAG-dependent manner and that MAG mediated activation of RhoA may require both LRP1 and p75NTR. While MAG binding to LRP1 does not appear to result in RIP of p75 as is seen with binding to NgR1, it does result in efficient MAG-endocytosis and localization within Rab5 positive endosomes. When CCRII and CCRIV were expressed as Fc-fusion proteins, these products, like purified full-length LRP1 and shed LRP1, attenuated the inhibition of neurite

outgrowth caused by MAG in primary neurons. Collectively, our studies identify LRP1 as a novel MAG receptor that functions in neurite outgrowth inhibition.

INTRODUCTION

The search for MAI receptors has been a focus of spinal cord research for decades as they represent valuable therapeutic targets for neuronal regeneration. Previously identified receptors of MAG, which were also promiscuous for other MAIs, such as NgR1, PirB and gangliosides were extensively studied for their therapeutic potential. As the first discovered MAG and MAI receptor, NgR was the first to be investigated as a therapeutic target. NgR proved to be a complicated receptor, because while it does seem to play a viable mechanistic role in MAI-mediated growth cone collapse[66], abolishing NgR1 interaction with myelin proteins does not restore axonal growth[67]. Gangliosides were the next to be implicated as MAG receptors, and initial studies did indicate a functional role in regenerative failure[68,70]. Interestingly, gangliosides had been implicated in regenerative failure of CNS neurons for some time, and have been tried going back to the 1990's as a therapy in SCI[130]. Some evidence even suggested that NgR's and gangliosides shared overlapping functions and operated in a cell-specific manner, which was one explanation for conflicting evidence in the literature[69]. It was subsequently reported that in stark contrast to the evidence provided by Mehta et. al[69], glangliosides and NgR reportedly functioned in tandem with one another for MAG binding[71]. Regardless, the ganglioside evidence demonstrated significant promise, and even led to the development of a ganglioside-derived therapeutic that made it all the way to phase III

clinical trial[76]. Unfortunately, despite showing marked promise in phase II, phase III trials demonstrated no clinical efficacy[76].

The most recently identified potential MAI receptor is the paired immunoglobulin-like receptor B (PirB), which showed therapeutic potential in initial studies that indicated that PirB antagonism could restore neurite outgrowth of neurons[72]. PirB even was shown to associate with p75 in response to MAI binding, which provided the link to activation of RhoA[125]. However, genetic deletion studies and follow up experiments failed to validate PirB as a therapeutic target[75]. Unfortunately, this leaves no previously identified receptor of MAG or other MAIs as a viable therapeutic target in SCI. While this may indicate that the validity of the entire MAI pathway may be irrelevant therapeutically, a deeper look at the mechanisms of MAG mediated neurite outgrowth inhibition provides valuable insight into previous failures and where we should look going forward. As previously discussed, MAG is a lectin with sialic-acid binding functions independent of its neurite outgrowth inhibiting capabilities[110]. As such, any receptor capable of facilitating the inhibitory effects of MAG on neurite outgrowth must bind independent of sialic-acid. However, all previously identified MAI receptors failed to demonstrate MAG binding in the absence of receptor sialic-acid content[37,131–133]. As a result, previously identified receptors failed to demonstrate the characteristics of binding that would be anticipated from a receptor functionally involved in MAG-mediated neurite outgrowth inhibition.

The studies contained within this chapter are designed to elucidate the functional implications of LRP1 binding of MAG. As no previously described receptors of MAG have been proven to functionally facilitate MAG-mediated inhibition of neurite outgrowth, we hypothesize that LRP1 is the previously unappreciated functional mediator of regenerative failure caused by myelin proteins. As previously discussed, the sialic acid dependence is a critical factor in assessing the functional involvement of an MAI receptor in relation to regenerative failure. In addition, cell-signaling events leading to the activation of RhoA are critical for MAI-mediated regenerative failure. As such, assessing the implications of LRP1 binding to MAIs on RhoA signaling is also critical in elucidating LRP1's role. As p75 and other TNFR-family members have been reproducibly shown to be critical factors in RhoA activation downstream of MAI binding, assessing the relationship or association of p75 (or related proteins) with LRP1 could also yield valuable insight into the role of LRP1 in this process, as well as its mechanism.

Mechanistically, LRP1 is unique from other previously identified receptors for MAIs in that it is primarily recognized as an endocytic receptor for numerous ligands[89,99,134]. All LRP1 ligands undergo endocytosis. This is of particular interest in this system due to recent studies suggesting an endocytosis-dependent mechanism underlying chronic regenerative failure. In these studies, MAI treatment of neurons results in the formation of signaling endosomes which are capable of transport to the neuronal soma, where activated RhoA can be detected[14]. The importance of this mechanism lies in its ability to account for chronic signaling effects that would likely

be needed for the long-term suppression of regeneration characteristic of SCI. Previous models utilizing the tripartite receptor complex and p75 RIP are readily amenable to the dynamics of growth cone collapse characteristic of MAI binding to NgR[57,61]. However, the critical signaling factor in this process is the soluble intracellular cleavage fragment of p75 which interacts with the RhoGEF and leads to activation of RhoA[56]. This fragment is rapidly targeted for degradation and levels of p75 fragmentation within the cell are only detectable with robust inhibition of the proteasome. As such, this mechanism is unlikely to be capable of sustaining the type of chronic signal responsible for long-term regenerative failure. Additionally, retrograde transport of neurons is incapable of transporting such fragments rapidly enough for them to have a role in the types of transcriptional regulation that has been documented in neurite outgrowth inhibition. While the novel endocytic mechanism has gained traction over the traditional p75 RIP model within the scientific community and has been supported by other labs[135], the missing aspect of this process is the receptor involved. As such, the investigation of LRP1-mediated endocytosis of MAG is of particular relevance.

Finally, and perhaps most significantly, the assessment of the functional role of LRP1 in MAG and MAI-mediated inhibition of neurite outgrowth comes down to the ability of LRP1 manipulation to overcome or affect inhibition of neurite outgrowth and the associated cell-signaling. As such, the final step in this chapter is to assess the effect of LRP1 neutralization on the inhibition of neurite outgrowth by MAG.

MATERIALS AND METHODS

Recombinant and purified proteins

CCRII, which includes amino acids 804-1185 of mature LRP1; CCRIV, 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[72,107,117]. 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[84]. Shed LRP1 and full-length LRP1 were purified from human plasma and rat liver, respectively, by RAP-affinity chromatography and molecular exclusion chromatography[118]. OMgp-AP, AP-Nogo66, and AP-Fc were expressed in HEK293T cells as previously described[117].

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[72,107]. 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[136] 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[117]. N20.1 cells were a gift from Dr. Anthony Campagnoni (University of California Los Angeles) and were cultured as previously described[119].

Binding of CCRII-Fc and CCRIV-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°C 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 quantitated with ImageJ software and analyzed using GraphPad Prism software. In studies utilizing the enzyme *V. cholera* neuraminidase (VCN), which removes terminal sialic acids from proteins, VCN was added to recombinant proteins prior to addition to MAG-expressing cells.

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±SEM are presented.

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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[94]. 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).

Immunofluorescent 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°C and then with 25 nM MAG-Fc, RPTPσ-Fc, or Fc for 60 min at 4°C. The cells were then warmed to 37° C 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).

Immunoblot analysis of MAG endocytosis

For immunoblot studies assessing LRP1 mediated endocytosis of MAG, LRP1 positive and deficient cells were treated with Fc-receptor blocking antibody (1 μg/ml) for 30 min at 4°C and then with 25 nM MAG-Fc, RPTPσ-Fc, or Fc for 60 min at 4°C. The cells were then warmed to 37° C for 30 min. Surface-associated fusion protein was dissociated by treatment with acetic acid/sodium acetate pH 3.0 for 4 min on ice. Cells were then washed in cold PBS and 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 and actin as a loading control. Cellular protein

concentration was determined by a bicinchoninic acid assay (Pierce, Rockford, IL) and used to standardize sample concentrations.

Radiolabeled MAG Internalization

MAG-Fc was radioiodinated with 1 mCi of Na125-I using Iodobeads (Pierce, Rockford, IL, USA) and separated from free Na125-I by molecular exclusion chromatography. - cells were plated in 12-well plates and equilibrated in DMEM with 25 mM HEPES, pH 7.4, 0.1% BSA and Fc-Blocker (BD Biosciences, San Jose, CA, USA). 125I-MAG-Fc (25 nM) was incubated with cells for 2 h at 37°C. Unlabeled MAG (1.25 μM) was added to some wells. At the end of the incubation, cells were washed and treated with 0.25% Pronase (Roche, Pleasanton, CA, USA) 22 for 15 min to eliminate surface-associated 125I-MAG-Fc. Cell extracts were prepared in 0.1 M NaOH and 1% SDS. Cell-associated radioactivity was determined using a Wallac 1470 Wizard Gamma Counter (Perkin Elmer, Waltham, MA, USA). Cellular protein was determined by bicinchoninic acid assay (Pierce, Rockford, IL, USA). Specific MAG-Fc uptake was calculated as the fraction of total uptake that was inhibited by excess unlabeled MAG.

qPCR analysis

To ensure efficient silencing in experiments total RNA was isolated using the RNeasy kit (Qiagen). cDNA was synthesized using the iScript kit (Bio-Rad). qPCR was performed using an Applied Biosystems instrument and a one-step program: 95°C, 10 minutes; 95°C, 30 seconds; and 60°C, 1 minutes for 40 cycles. HPRT-1 gene expression was measured as a normalizer. Results were analyzed by the relative

quantity (ΔΔCt) method. All experiments were performed in triplicate with internal duplicate determinations.

Immunofluorescence microscopy

Cells were plated on glass coverslips, fixed in 4% paraformaldehyde, and as indicated, permeabilized in 0.2% Triton X-100, and incubated with antibodies specific for the specified antigens followed by secondary detection with antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594. Preparations were mounted on slides using ProLong Gold with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) and examined using a Leica DMIRE2 fluorescence microscope.

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[137]. 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^{o} C. 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.

RESULTS

The missing characteristic link in identification of the MAI receptor responsible for regenerative failure is the ability to bind MAG independently of sialic acid content. The previous chapter identifies LRP1 as a novel receptor of MAG, but to assess for functionality in the MAI-mediated inhibition pathway, we first wished to assess the role of sialic acid on LRP1 binding of MAG. To accomplish this we used the same cell-based binding assay described in the previous chapter where COS-7 cells were transfected to express membrane-bound MAG and incubated with recombinant CCRII, CCRIV and NgR^{OMN} Fc-fusion proteins. To first assess the role of sialic acid on the MAG/LRP1 interaction we pre-treated the recombinant proteins

with *V. cholera* neuraminidase (VCN), which is an enzyme that removes terminal sialic acids from glycosylated proteins. As a control we pre-incubated proteins with vehicle. As had been previously reported, VCN treatment ablated the binding of NgR^{OMNI}. However, CCRII and CCRIV binding to MAG was unaffected by removal of sialic acid (Fig. 3-1A). To further confirm the sialic independence of MAG binding to LRP1, 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 is within the sialic-acid binding domain and greatly reduces lectin activity[138]. Similar to the manner that VCN treatment affected MAG binding, MAG^{R118A} failed to bind NgR^{OMNI}-Fc, as previously demonstrated[107]; however, robust binding was still observed with CII-Fc and CIV-Fc (Fig. 3-1B). These results demonstrate that the interaction of MAG with LRP1 is not sialic acid-dependent, which leads to the conclusion that LRP1 is the first identified MAG receptor to bind in a manner characteristic of a receptor capable of transmitting the inhibitory signaling of MAIs. 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 (data not shown).

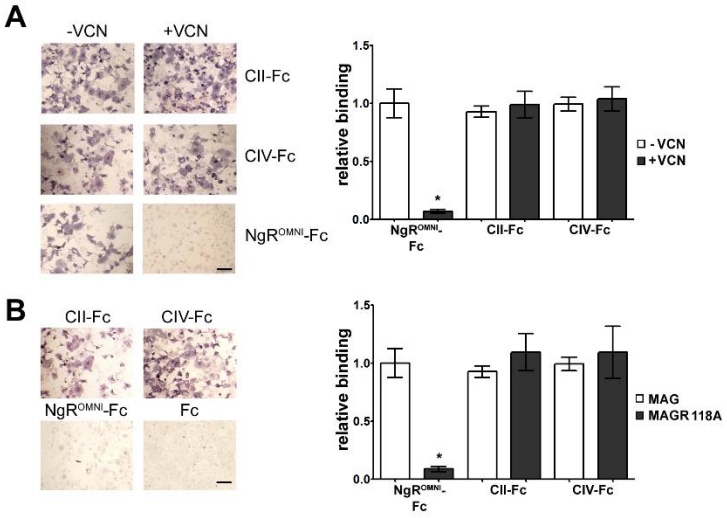


Figure 3-1 LRP1 is the first sialic-acid independent receptor of MAG.
A. MAG-expressing COS-7 cells were incubated with CII-Fc, CIV-Fc, or NgR^{OMNI}-Fc. In some cases, ligands were pretreated with VCN (1 mU/ml) for 1 h before incubation with COS-7 cells. The graph represents the degree of binding relative to NgR^{OMNI}-Fc to MAG-expressing COS-7 cells (+/- SEM, n=4 independent experiments). B. COS-7 cells were transfected with either membrane-bound MAG or MAG^{R118A}, a mutant with greatly reduced lectin activity, before addition of CII-Fc, CIV-Fc, or NgR^{OMNI}-Fc. The graph represents the degree of binding relative to NgR^{OMNI}-Fc to MAG-expressing COS-7 cells (+/- SEM, n=3 independent experiments). Scale bars, 20 μm.

In addition to sialic acid independent binding, recent studies have indicated that another potentially important binding characteristic of a receptor mediating regenerative failure caused by MAIs would be endocytic capacity[14]. In addition, LRP1 is known to facilitate endocytosis of all ligands, so confirmation of endocytosis of MAG serves the dual purpose of further establishing a functional ligand/receptor relationship between MAG and LRP1 as well as providing evidence that LRP1 may be facilitating the formation of the endosomes reported by Joset et. al[14]. To assess the endocytosis of MAG, and the LRP1-dependence, we first conducted immunofluorescence studies to assess MAG internalization in N2a cells. In these studies MAG-Fc was incubated with cells at 4 degrees, then warmed to 37 degrees for 30 minutes, then rinsed with cold PBS on ice to remove unbound protein. Surface bound, but not internalized proteins were then stripped off the cell surface using a mild acid wash. Cells were then fixed and permeabilized. As a control for MAG, RPTP-Fc and Fc alone were also used. Detection of internalized proteins was then accomplished with an anti-human FITC-conjugated antibody. Figure 3-2A demonstrates that in N2a cells MAG-Fc and RPTP-Fc, but not Fc alone, are readily endocytosed but when pre-treated with the LRP1 antagonist RAP, MAG-Fc internalization is blocked while RPTP-Fc is unaffected demonstrating the specificity of LRP1 for MAG. To confirm that MAG-endocytosis is LPR1 specific, we next utilized N2a cells in which LRP1 was stably silenced. Similar to experiments using RAP, LRP1 deficiency resulted in a deficiency in the endocytosis of MAG (fig. 3-2B). We next confirmed that LRP1 is responsible for MAG endocytosis using a similar experimental paradigm in which

instead of fixation and imaging of internalized Fc proteins, we instead prepared protein extracts from treated cells and assessed levels of internalized Fc by SDS-PAGE and immunoblot analysis. Figure 3-2C demonstrates that N2a cells endocytose both MAG and RPTP, but not Fc. In LRP1 deficient N2a cells MAG is once again not internalized while RPTP is unaffected. 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 protein/h (n=3). LRP1 gene-silencing completely abolished specific internalization of ^{125}I-MAG-Fc, further confirming the specific role of LRP1 in MAG endocytosis (Fig. 3-2D).

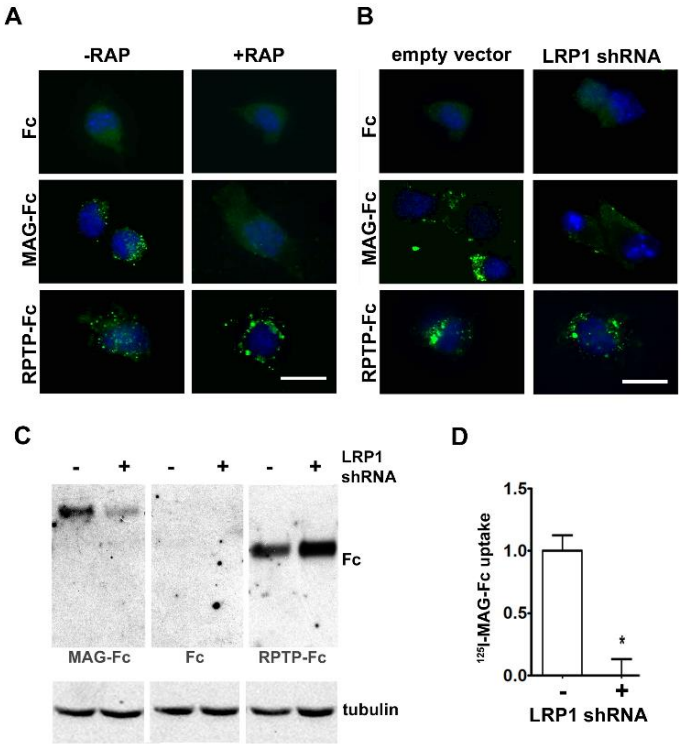


Figure 3-2 LRP1 is an endocytic receptor of MAG.
A. N2a cells were treated with RAP or GST (200nM) prior to addition of 25nM RPTP-Fc, MAG-Fc, or Fc control prior to fixation and mild acid washing to remove surface-associated proteins. Internalized proteins were then visualized by immunofluorescent detection of the Fc-tag. B. N2a cells stably transfected with LRP1-specific shRNA or empty vector were incubated with 25 nM RPTP-Fc, MAG-Fc, or Fc control prior to fixation and mild acid washing to remove surface-associated proteins. Internalized proteins were then visualized by immunofluorescent detection of the Fc-tag. C. N2a cells stably transfected with LRP1-specific shRNA or empty vector were incubated with 25 nM RPTP-Fc, MAG-Fc, or Fc control prior to mild acid washing to remove surface-associated proteins. Proteins extracts were prepared and immunoblotted for the Fc-tag. (D) To quantify the differences in MAG endocytosis, N2a cells stably transfected with LRP1-specific shRNA or empty vector were incubated with 25 nM ^{125}I-MAG-Fc, in the presence or absence of a 50-fold molar excess of unlabeled MAG-Fc. Specific MAG-Fc internalization was determined (***,** $p<0.01$).

LRP1 is known to facilitate endocytosis of ligands, so in addition to mediating MAG endocytosis we also wished to assess the type of endocytic vesicles formed. In neurons, the types of axonally transported endocytic vesicles capable of long range signaling transfer have been demonstrated to be, in many cases, Rab5 positive[139]. As such, we wished to assess the Rab5 positivity of MAG-containing endosomes. To accomplish this we modified the experimental paradigm used above to determine the endosome markers associated with the MAG-positive vesicles. Previously identified LRP1 ligands have been shown to localize to vesicles positive for early endosomal marker 1 (EEA1)[104,140–142]. We hypothesized that MAG would undergo similar trafficking, and to assess this theory we did co-immunofluorescence to detect both MAG and EEA1. Surprisingly, while LRP1 deficiency impaired cellular uptake of MAG as before, readily detectable MAG in LRP1 expressing cells did not co-localize with EEA1 (Fig. 3-3A). While unanticipated, this result opens the possibility of alternative endocytic pathways for LRP1. While LRP1 has been repeatedly demonstrated to mediate ligand endocytosis through both early and late endosomal compartments, this pathway is often reserved for ligand delivery to lysosomes[82,89,90,92,143]. As lysosomal delivery would negate the purpose of long-range retrograde transport of a signaling complex, alternative LRP1-mediated endocytic pathways would more easily account for such a mechanism. Because of this, our unanticipated findings led us to examine alternative endosomal markers for MAG-positive endosomes. Neuronal signaling is unique in that distal growth cones and synapses often need to be capable of transmitting signals across great distances, up to

1 meter, to the cell body to affect transcriptional change. As such, signaling endosomes in neurons must be capable of relatively rapid retrograde transport toward the cell nucleus. These signaling endosomes have been shown to be important for other ligand-induced neuronal signaling events, and p75 has been reported in multiple systems to be associated with these vesicles, which are often characteristically positive for the endosomal marker Rab5[59,139]. Because of the unique function of MAG as a newly discovered LRP1 ligand, we next hypothesized that MAG-positive vesicles in LRP1 expressing cells would also contain Rab5. To assess this we undertook the same experimental paradigm but instead of probing for EEA1, we performed co-immunofluorescence for MAG and Rab5. As anticipated, the LRP1-mediated endocytosis of MAG resulted in the formation of Rab5 positive vesicles (Fig. 3-3B).

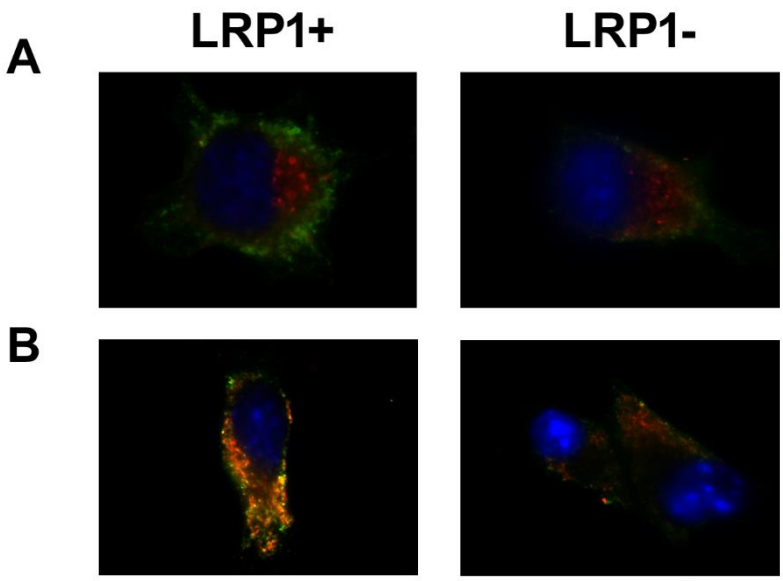


Figure 3-3 LRP1 endocytosis MAG, which localizes to Rab5, but not EEA1, positive endocytic vesicles.
A. N2a cells incubated with MAG prior to mild acid wash to remove surface bound proteins were fixed and permeabilized. MAG (488) and EEA1 (594) were then detected by IF. B. N2a cells incubated with MAG prior to mild acid wash to remove surface bound proteins were fixed and permeabilized. MAG (488) and Rab5 (594) were then detected by IF.

To this point we have characterized LRP1 as a novel MAG receptor that is the first to bind independently of sialic acid, and that LRP1-mediated endocytosis of MAG resulted in Rab5-positive, MAG-containing, endocytic vesicles. The next step in our assessment of LRP1's functional role in MAG mediated inhibition of neurite outgrowth was to examine the downstream signaling events known to results from MAI/neuronal interaction. As previously discussed, the necessary and sufficient cell signal for the disruption of the regenerative response of neurons is activation of RhoA[46,48,51,144]. To ascertain the ability of LRP1 to affect RhoA signaling downstream of MAG binding to neurons, we employed an N2a cell system in which LRP1 had been constitutively silenced to evaluate how MAG signaled in those cells. As a control for MAG-Fc, Fc protein alone was also used as a treatment. Fc alone had no effect on RhoA activation. However, in LRP1 positive cells, MAG treatment resulted in robust activation of RhoA. The absence of LRP1 attenuated the ability of MAG to activate RhoA (Fig. 3-4A). Figure 3-4B is a quantification of 7 independent studies in which the level of RhoA activation was assessed via densitometric analysis of the GTP-RhoA/total RhoA ratio. These studies indicate that LRP1 is essential for MAG-mediated activation of RhoA.

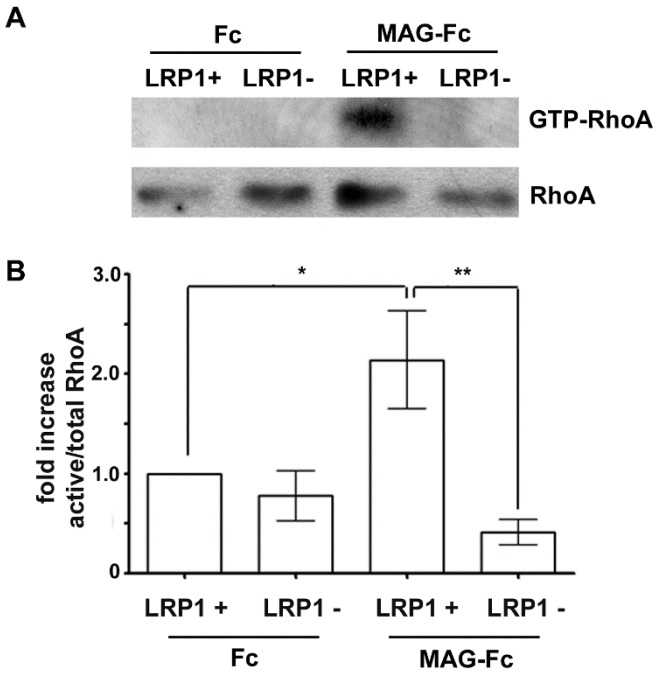


Figure 3-4 MAG-mediated activation of RhoA requires LRP1.
A. N2a cells in which LRP1 was stably silenced with shRNA, and cells transfected with empty vector (control) were treated with MAG-Fc or Fc (20 nM). GTP-RhoA was then affinity precipitated by GST-RBD pull-down and relative RhoA activity was determined as a ratio of GTP-RhoA to total RhoA, which was determined by analysis of whole cell extracts without affinity precipitation. B. The graph represents the relative RhoA activity normalized to the Fc treated NTC cells (+/- SEM, n=5 independent experiments).

The ability of MAIs to activate RhoA has been reported to be mediated through members of the TNFR family members, primarily the neurotrophin receptor p75, but also Troy/Taj[63,64,109,145]. Because we had shown that LRP1 was necessary for the activation of RhoA by MAG, we next wished to assess if p75 was also involved in this process. Our first step in accomplishing this was to examine the effects of inhibiting p75-mediated activation of RhoA in our N2a system as a means to confirm the role of p75 and rule out other TNFR family member function. Previous studies, which were pivotal for the identification of the role of p75 in this process, had identified and characterized a p75 interacting peptide that was capable of associating with the intracellular domain of p75 and blocking its interaction with Rho-GDI, which is a necessary step for the activation of RhoA in response to MAIs[109]. Because this peptide, referred to as pep5, had been thoroughly characterized and routinely used in studies p75, we chose to use this reagent to study the role of p75 in MAG-mediated activation of RhoA in N2a cells. N2a cells were treated pre-treated with pep5 or vehicle prior to incubation with MAG-Fc or Fc alone. While MAG readily activated RhoA, pep5 blocked this effect (Fig. 3-5A), indicating that both LRP1 and p75 are essential for MAG-mediated inhibition of neurite outgrowth in this system. It has been previously shown, and confirmed in our lab (data not shown), that p75 does not interact directly with MAG. As such, because LRP1 and p75 both appear to be necessary for activation of RhoA in our system, we hypothesized that LRP1 and p75 are functioning in conjunction with one another downstream of MAG binding. To assess this, we chose to evaluate the association of LRP1 and MAG in response to

LRP1 ligation with MAG. We found that under basal conditions p75 can be immunoprecipitated with MAG at very low levels (data not shown). However, when cells are treated with MAG, p75 readily and robustly immunoprecipitates with LRP1 from protein extracts (Fig. 3-5B).

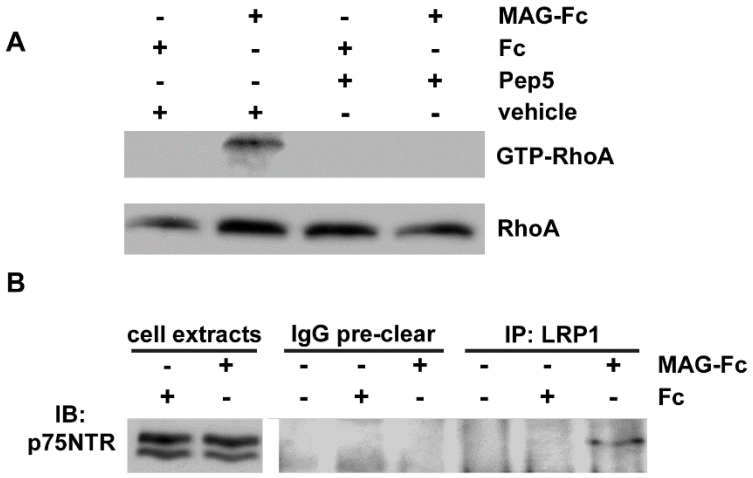


Figure 3-5 MAG-mediated activation of RhoA also requires p75, which forms a complex with LRP1 in response to MAG.

A. N2a cells were pre-treated with TAT-pep5, which blocks the activity of p75NTR, or vehicle. The cells were then treated with MAG-Fc or Fc (20 nM). GTP-loaded and total RhoA were determined. B. N2a cells were treated with 20 nM MAG-Fc or Fc, extracted, and subject to sequential immunoprecipitation with control IgG and then LRP1-specific antibody. Precipitated proteins were subjected to SDS-PAGE and immunoblot analysis for p75NTR. Whole cell extracts from N2a cells are shown in the left-hand lanes.

The data previously described in this chapter indicates a clear functional and mechanistic role for LRP1 in MAG mediated inhibition of neurite outgrowth. As such, LRP1 appears to be a viable therapeutic candidate and a receptor capable of affecting neurite outgrowth in the presence of myelin. As a final assessment of LRP1's role in this process, we wished to assess the morphological role of neuronal LRP1 in MAG-mediated neurite outgrowth inhibition. To accomplish this we employed a CHO-monolayer model that has been previously utilized in studies examining MAG's effects on neurite outgrowth in multiple neuronal systems[37,110,131,132,137,146]. In this model, CHO cells are transfected to either express or not express membrane-bound MAG and then grown into a monolayer for neuronal cells to be plated upon. In this model system, MAG significantly inhibited the neurite outgrowth of neuronal cells. However, when LRP1 function was lost either by antagonism with RAP or silencing of LRP1, neurite outgrowth was restored. Figure 3-6 demonstrates this effect and the quantification of the outgrowth in both PC12 and N2a cells with RAP treatment (3-6A) and silencing (3-6B).

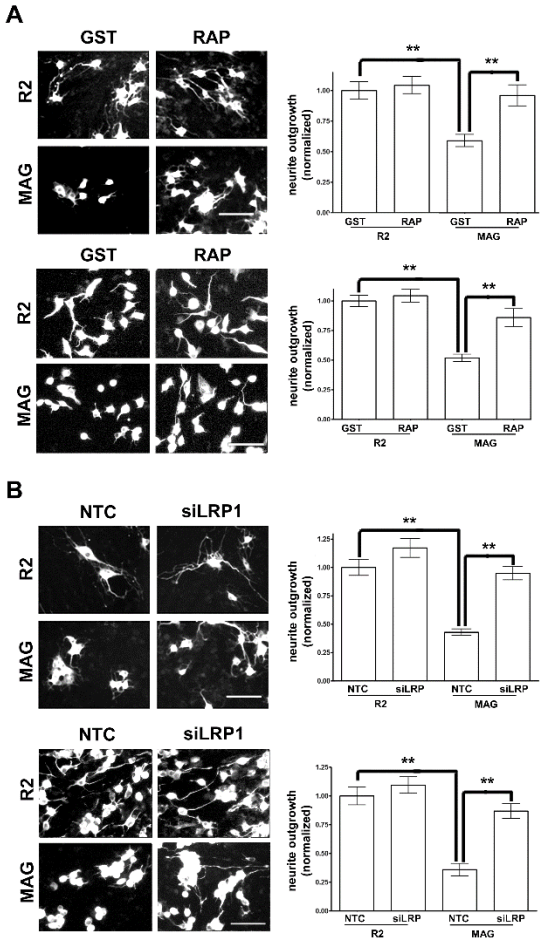


Figure 3-6 LRP1 inactivation via RAP antagonism or gene silencing restores neurite outgrowth of neuron-like cells on MAG-expressing CHO cells.

A. PC12 and N2a cells were plated on R2 control, or MAG-expressing CHO cells and allowed grown 48 h in the presence of GST-RAP or GST (200 nM). Neurite outgrowth was detected by immunofluorescent imaging of βIII-tubulin. Results were normalized against those obtained when cells were plated on R2 cells in the presence of GST. Graphs represent the mean neurite outgrowth (+/- SEM) per condition (n=3 independent experiments, **p<0.01). B. PC12 and N2a cells were transfected with non-targeting (NTC) or LRP1-specific (siLRP1) siRNA prior to plating on monolayers of R2 control, or MAG-expressing CHO cells and allowed grown 48 h. Neurite outgrowth was detected by immunofluorescent imaging of βIII-tubulin. Results were normalized against NTC transfected cells plated on R2 cells. Graphs represent the mean neurite outgrowth (+/- SEM) per condition (n=3 independent experiments, **p<0.01).

The ability of RAP and LRP1 silencing to restore neurite outgrowth of N2a and PC12 cells plated on MAG was substantial. We next chose to confirm these effects using cerebellar granule neurons to ensure that this phenomenon was reproducible in primary neuronal cultures. As previously seen in both PC12 and N2a cells, MAG significantly inhibited neurite outgrowth of CGNs. However, when CGNs were pre-treated with the LRP1 antagonist RAP neurite outgrowth was restored (Fig. 3-7A). We next sought to test the effect of LRP1 silencing in CGNs on the inhibitory capacity of MAG in this system. As before, LRP1 silencing resulted in a significant increase in neurite outgrowth on MAG. However, the extent to which neurite outgrowth was restored with LRP1 silencing was not as robust as observed in N2a and PC12 cells (Fig. 3-7B). This is likely due to the fact that primary neurons are more difficult to transfect than either N2a or PC12 cells, which is reflected in the decreased silencing efficiency[133]. Because the extent of neurite outgrowth restoration with transient LRP1 silencing was not as robust as observed in immortalized cell lines, we chose to confirm the silencing effect in a stably silenced system. To accomplish this, 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[147]. 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. 3-7C). 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 (Fig. 3-7C). Collectively, three different approaches show that functional ablation of LRP1 in primary neurons is sufficient to significantly attenuate MAG inhibition.

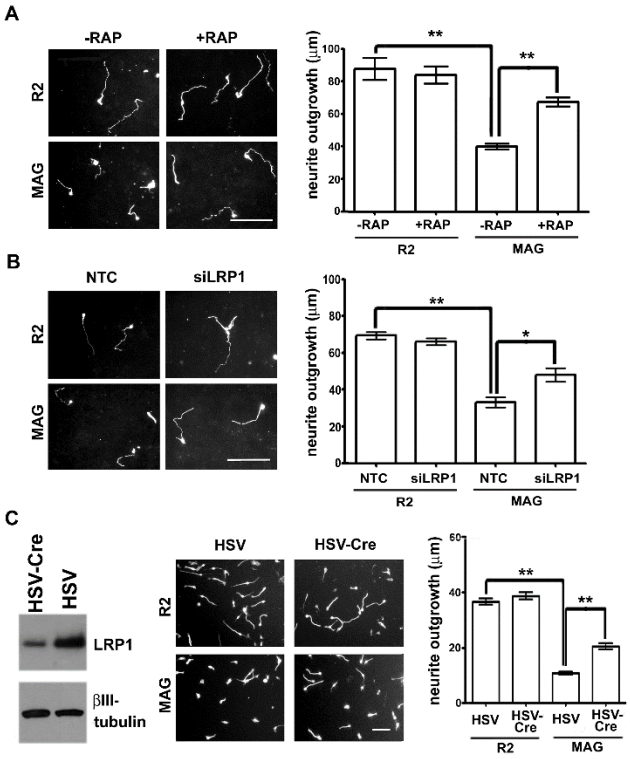


Figure 3-7 LRP1 inactivation restores neurite outgrowth of primary CGNs on MAG-expressing CHO cells.

A. CGNs pre-treated with GST-RAP or GST were plated on monolayers of MAG-expressing or control (R2) CHO cells and grown for 48 hours prior to fixation and immunofluorescent detection of βIII-tubulin. Graphs represent the mean neurite outgrowth (+/- SEM) per condition (n=3 independent experiments, **p<0.01). B. CGNs transfected with non-targeting (NTC) or LRP1 specific (siLRP1) siRNA were plated on monolayers of MAG-expressing or control (R2) CHO cells and grown for 48 hours prior to fixation and immunofluorescent detection of βIII-tubulin. Graphs represent the mean neurite outgrowth (+/- SEM) per condition (n=3 independent experiments, **p<0.01). C. Primary CGNs were isolated from LRP1$^{flox/flox}$ mice and were infected with HSV or HSV-Cre prior to plating on monolayers of MAG-expressing or control (R2) CHO cells and grown for 24 hours prior to fixation and immunofluorescent detection of βIII-tubulin. Extent of silencing is demonstrated as well as graphs representing the mean neurite outgrowth (+/- SEM) per condition (n=3 independent experiments, **p<0.01).

We hypothesized that purified LRP1, and LRP1 derivatives, that retain ligand-binding activity may compete for MAG-binding with membrane-anchored LRP1 and other MAG receptors and thereby reverse inhibitory activity. To test this hypothesis, we examined the effects of CCRII and CCRIV, the recombinant binding domains of LRP1, on neurite outgrowth in CGNs. Recombinant CCRII and CCRIV did not affect neurite outgrowth when it was added to CGNs that were cultured on control R2-CHO cells. However, when CGNs were cultured on MAG-CHO cells, CCRII and CCRIV significantly reversed the inhibition of neurite outgrowth observed (Fig. 3-8). These results further confirm the binding capacity, and functional relevance, of LRP1 in MAG-mediated inhibition of neurite outgrowth. Additionally, it appears that recombinant soluble receptor mimetics of LRP1 may have potential therapeutic value in themselves.

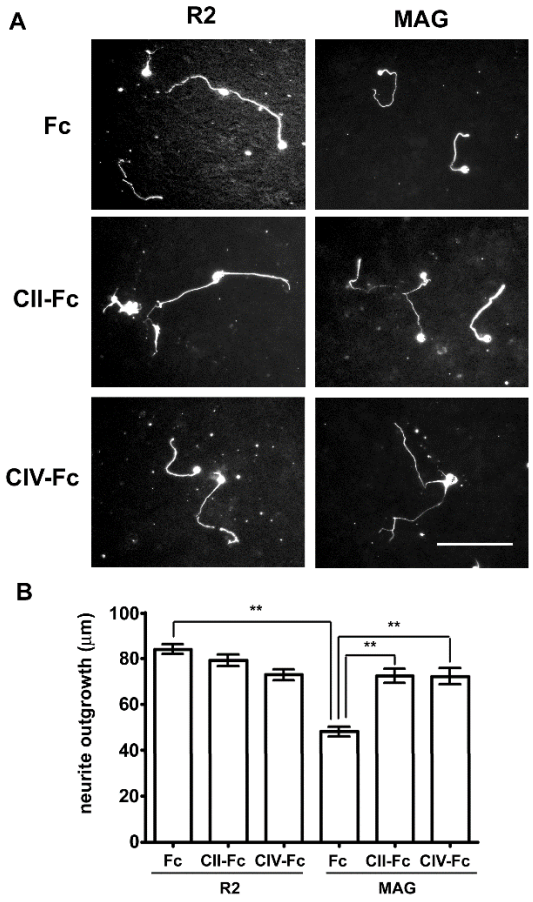


Figure 3-8 Soluble, purified LRP1 or recombinant binding domains of LRP1 block the effects of MAG on neurite outgrowth.

Pre-treatment of a monolayer of MAG-expressing CHO cells with purified CCRII and CCRIV leads to enhanced neurite outgrowth of CGNs. Monolayers of MAG-expressing and control (R2) CHO cells were incubated for 15 minutes with purified recombinant CCRs or Fc control prior to addition of CGNs, which were grown for 48 hours prior to fixation and immunofluorescent staining. B. Graphs represent the mean neurite outgrowth (+/- SEM) per condition (n=3 independent experiments, **$p<0.01$).

DISCUSSION

Chapter 1 laid out a detailed characterization of the binding capacity of LRP1 for MAG. The mechanism by which MAIs inhibit neuronal growth is of considerable interest, and the discovery of LRP1 as a novel receptor of MAG may yield significant insight into a critical piece of information that could have tremendous therapeutic implications. As previously discussed, clarification of the mechanism underlying MAI-mediated activation of RhoA is necessary for development of therapeutics targeted along this pathway. Currently, therapeutics are in development that address both the inhibitory ligands and downstream Rho signaling[20,77–79]. However, the piece of this pathway yet to be clarified is the receptor linking these 2 variables. While upstream and downstream effectors in MAI regenerative inhibition are valid therapeutic targets, the mediating receptor is a pharmacologically desirable alternative, if known. It is because of this that the detailed mechanistic characterization of LRP1 as a mediator of MAG-mediated inhibition of neurite outgrowth is of such potential importance.

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[66,67,117]. 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[72]. 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[39,69,110,124].

Herein, we report the identification of LRP1 as the first receptor for MAG to demonstrate sialic acid independent binding. Previously identified MAI receptors depend on sialic acid, despite the fact that that the inhibitory effects of membrane-associated MAG on neurite outgrowth are known to occur independently of sialic acid-binding[37,107,138]. 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. Further studies are needed to determine if there are additional associated partners in complex with LRP1 and p75 in response to MAG binding.

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. Additionally, ligand-dependent localization of p75 within signaling endosomes has also been reported in other neuronal signaling systems[139,148]. The previously

documented regulated intermembrane proteolysis of p75 downstream of MAG treatment does not produce a signal that is sustained and/or transported to the soma in a manner that would be capable of the transcriptional changes associated with chronic regenerative failure[57]. However, the formation of MAI induced signaling endosomes that have now been described are well suited for the type of signaling effects that are involved with chronic regenerative inhibition. However, previous studies of MAI endocytosis have not identified mediating receptors. 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 that indicates LRP1 is the previously unappreciated critical factor in this process. Interestingly, it has been reported that Nogo colocalizes with LRP1 in early endosomes in growth cone vesicles isolated from mouse brain, which further supports this theory[88].

Purified CCRII and CCRIV attenuated the inhibitory effects of MAG 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. We propose that the activity of MAG may be neutralized by any soluble LRP1 derivative that retains ligand-binding activity. An alternative approach involves direct targeting of the 2nd and 4th CCR domains of LRP1 with some form of competitive ligand to displace MAG.

In the normal human brain, LRP1 is expressed by almost all neuronal popu-lations. 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[66,67,117]. Recently, there was the promising discovery of PirB as a promiscuous receptor for Nogo66, MAG, and OMgp. Initial studies of the role of PirB in MAI-mediated regenerative inhibition indicated that 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[72]. However, subsequent studies were unable to confirm this effect and 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[74,75]. As a result, LRP1 arises as an exciting therapeutic target in SCI.

Acknowledgements

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CHAPTER 4 - LRP1 EFFECTS ACTIVATION OF RHOA AFTER *IN VIVO* SPINAL CORD INJURY AND MEDIATES SIGNALING AND INHIBITION OF NEURITE OUTGROWTH FROM PURIFIED MYELIN

ABSTRACT

The previous chapters chronicle a novel, functional role for LRP1 in MAG-mediated inhibition of neurite outgrowth. The work in this chapter aimed to decipher the relative contribution of the LRP1/MAG interaction to the overall regenerative inhibition from CNS myelin after SCI. We anticipated that LRP1 may contribute to the overall inhibitory effects of CNS myelin antagonism. However, LRP1 silencing, antagonism, and pre-treatment of inhibitory myelin substrate with LRP1 receptor mimetics all demonstrated a regeneration of neurite outgrowth comparable to those seen in experiments on MAG alone. As such, we hypothesized that LRP1 was playing a role beyond interaction with MAG. Herein we demonstrate LRP1 interacts directly with OMgp and both inhibitory domains of Nogo-A. Additionally, blocking LRP1 *in vivo* blocks LRP1 association with p75, and also attenuates activation of RhoA. In summary, the data in this chapter presents a case for LRP1 as a functional receptor for all major MAIs. As such, LRP1 arises as a valuable potential therapeutic target for overcoming the primary acute obstacle to neuronal regeneration after injury to the CNS such as seen in SCI.

INTRODUCTION

Previous chapters clearly demonstrate a functional, pivotal role for LRP1 in MAG-mediated inhibition of neurite outgrowth. The characterization of LRP1 as the first sialic acid-independent MAG receptor capable of mediating activation of RhoA, formation of Rab5 positive endosomes, and conferring neurite outgrowth inhibition may potentially represent the critical missing piece in understanding the mechanism underlying MAG-mediated inhibitory effects. However, what is not addressed in chapters 1 and 2 is the overall contribution of LRP1 in the regenerative inhibition caused by disorganized complete CNS myelin. In this chapter we sought to assess to what extent, if any, the LRP1/MAG interaction contributed to the overall inhibitory effects of CNS myelin.

It has been known for decades that CNS myelin is potently inhibitory to axonal extension/neuronal regeneration[5,17]. The isolation and characterization of the specific components of myelin that are responsible for regenerative inhibition have resulted in the identification of MAG, OMgp, and Nogo proteins as the primary components responsible for the inhibitory effects of myelin. Interestingly, previously identified receptors have shown promiscuous binding of these proteins despite the existence of any prominent common binding motif. Unfortunately, these receptors have not been shown to be responsible for the majority of the inhibitory activities of MAIs. As LRP1 has now been demonstrated as a novel receptor for MAG that facilitates the inhibitory signaling of this particular MAI, its unique binding characteristics and ability to mediate MAI endocytosis into Rab5 endosomes indicate that LRP1 may be the yet-

unappreciated missing link in the mechanism by which myelin proteins inhibit regeneration of neurons.

The fact that LRP1 seems to be solely responsible, at least in our system, for MAG endocytosis is of particular interest in the context of the recent mechanistic insight by Joset et. al indicating that endocytosis is necessary for Nogo-A chronic inhibition of neurite outgrowth. Additionally, previously identified MAI receptors have demonstrated promiscuity for other MAIs, demonstrating that these diverse ligands are capable of sharing common receptors. Regardless, the robust effect of LRP1 on MAG-mediated signaling and neurite outgrowth inhibition warrants investigation into the ability of LRP1 to affect the inhibitory influence of isolated CNS myelin on neurons.

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[72,107,117]. 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[84]. Shed LRP1 and full-length LRP1 were purified from human plasma and rat liver, respectively, by RAP-affinity chromatography and

molecular exclusion chromatography[118]. OMgp-AP, AP-Nogo66, and AP-Fc were expressed in HEK293T cells as previously described[117].

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[72,107]. 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[136] and cultured in DMEM with 50 mM glucose, 10% FBS, 25 mM KCl, and P/S.

CNS myelin purification

Myelin vesicles were purified from rat brain, as described by Norton and Poduslo[120]. The purity of the preparation was determined by Coomassie Blue staining and by immunoblot analysis for myelin basic protein, as previously described[84].

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[149] 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, 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 was 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^{o}C. Bound proteins were detected using appropriate antibodies.

Neurite outgrowth experiments

MAG-CHO and R2-CHO cells were cultured on glass slides until confluent[137]. CGNs, PC12 cells, or N2a cells were then added and cultured for 48 h. 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[94]. 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).

Basic animal care

Experimental animals were female Sprague-Dawley rats from Charles River Labs that were 9-10 weeks old at the start of the experiments in the same manner as previously reported by Fu et al[150]. It is noteworthy that we normally obtain Sprague-Dawley rats from Harlan Labs, and we found that rats from Charles River Labs were more sensitive to anesthetic than the Sprague-Dawley rats. Anesthetic doses were adjusted to account for abnormally high mortality. All experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Irvine.

Spinal cord injury

Rats were anesthetized with ketamine 75 mg/kg and xylazine 10 mg/kg and placed in a stereotaxic device with the head stabilized by ear and bite bar. A clamp (Kopf, Tujunga, California) was attached to the lower one-third of the tail to apply traction to straighten the thoracic curvature. Respiratory pattern was monitored throughout the time rats were in traction. A laminectomy was performed at thoracic level 6 and 7 (T6 and T7) and the dura mater was opened with a 23-gauge needle. Dorsal over-hemisection injuries were produced first by using micro scissors (Roboz, Gaithersburg, MD; RS-5600) at a depth of 1.5 mm and then a no. 11 blade attached to a stereotaxic arm was passed 4X through the lesion at a depth of 1.9mm. The goal was to completely transect the dorsal half of the spinal cord extending slightly past the

central canal, but sparing the ventral column and ventral gray matter. This lesion completely transects the two main components of the corticospinal tract (CST) in the ventral part of the dorsal column (dCST) and dorsal part of the lateral column (dlCST), sparing the small component of CST axons in the ventral column (ventral CST). Spinal muscles were closed with 4-0 chromic gut (Henry Schein, Melville, NY) and the skin was closed with 9 mm staples (Fisher Scientific, Pittsburg, PA).

Minipump implantation

Osmotic minipumps (Alzet, Cupertino, CA, model 2ML4) were filled either with GST or GST-RAP in a 10 µM stock in PBS. Minipumps were filled 16hrs prior to surgery, and pumps were then primed by incubating the pumps in saline at 37ºC until the time of implantation. Minipumps were implanted subcutaneously immediately after producing the dorsal over-hemisections. All staff members involved in the experiment were blind to the treatment groups for the entire experiment.

General post-surgical care

Following surgery, the rats were immediately placed on a water circulating jacketed heating pad until they recovered from the anesthetic. Post-surgical care included delivery of lactated ringers (5mg/100g, subcutaneously) for hydration for 3-5 days and Baytril (Enroflaxacin 2.5mg/kg, subcutaneously, Western Medical Supplies, Arcadia, CA) for 3-5 days for prophylaxis against urinary tract infections (UTI's). The analgesic Buprnex (Buprenorphine, 0.01 mg/kg, Western Medical Supplies, Arcadia, CA) was given for 3-5 days for pain management. Rats were housed 3-4 per cage and were monitored twice daily for general health, coat quality (indicative of normal

grooming activity) and mobility within the cage. Rats with dorsal over-hemisection injuries typically resume these activities the day following injury. In addition, signs of paralysis were monitored, including lack of hind limb movement, tail flaccidity, and instability/ uncoordinated movement. Animals were also monitored for skin lesions on the paralyzed limbs or autophagia of the toes.

Because autophagia was observed, we developed a rating scale to characterize the mutilation of each hind paw separately. Each hindlimb was scored, and scores were averaged (Table 2.). Rats exhibiting autophagia received extensive care to prevent further self-mutilation. Each affected paw was treated with a mixture of 1ml New-Skin liquid bandage (Prestige Brands, Inc., Irvington, NY) and 500 mg Metronidazole (Teva Pharmaceuticals, Sellersville, PA). A thin layer was placed on the paw to prevent further mutilation and then an Elizabethan collar (EC404VS-5, Kent Scientific, Torrington, CT) was placed on the rat.

RhoA assessment

To assess RhoA-inhibiting effects of RAP, we performed an assay for Rho-GTP as described by Fu et al. 2007 in rats that received dorsal hemisection injuries at T6/7. Ten rats received dorsal over-hemisections as described above. Three died during the surgery due to anesthetic complications. Surviving rats received RAP (n=3), or GST (n=3). 2 un-operated rats were also prepared.

Five days following the over-hemisections and osmotic pump placement, rats were euthanized with Euthasol and transcardially perfused with cold 1X PBS for ten minutes. Immediately following perfusion, animals were transferred to a 4˚ room and

an approximately 10mm block of spinal cord containing the lesion site was collected. Tissue was immediately homogenized via dounce in 1.2 ml of lysis buffer (50 mM HEPES pH 7.4, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 10mM $MgCl_2$, 10% glycerol, protease inhibitor cocktail, 2 mM EDTA, 1 mM sodium orthovanadate). Extracts were clarified by centrifugation at 15,000 rpm for 1 minute. GTP-loaded RhoA was then affinity precipitated using the Rho binding domain (RBD) of Rhotekin conjugated to agarose beads (Millipore, Temecula, CA). For affinity precipitation of RhoA, 900µl of tissue extract was incubated with 40µl of RBD-agarose beads for 1 hour at 4^oC. Beads were then washed 3X in lysis buffer and precipitated protein was dissolved in 40µl of 2x-concentrated Lamelli sample buffer. The remainder of the tissue extract was collected and an equal concentration of 2X-concentrated Lamelli sample buffer was added and saved for immundetection of total RhoA. Affinity-precipitated active RhoA and total RhoA were determined by immunoblot analysis using a mouse monoclonal anti-RhoA antibody (SC-418, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Equal volumes of sample were loaded for affinity-precipitated and total extracts. Quantification of RhoA activation was by densitometric analysis of GTP-RhoA/total RhoA, using ImageJ software.

RESULTS

The previous 2 chapters demonstrated LRP1 to be a novel, functional receptor of MAG which mediates inhibition of neurite outgrowth. In this chapter, we aimed to follow up on this finding to assess the relative contribution of this interaction to the inhibitory effects of CNS myelin in its entirety. To accomplish this, we used a model

in which purified CNS myelin was immobilized on the culture surface and used as a substrate for neuronal cells and compared neurite outgrowth compared to cells on permissive substrate. In our initial studies using PC12 cells, we first assessed the ability of LRP1 antagonism via RAP to affect neurite outgrowth on myelin. As expected, PC12 cells plated on myelin exhibited significantly less neurite outgrowth than those plated on collagen. The addition of RAP attenuated this effect in a manner similar to what was previously observed in our MAG system (Fig. 4-1A). As we had done in previous studies, we sought to confirm the LRP1 specificity of the RAP effect using transient gene silencing. Again, the restoration of neurite outgrowth with LRP1 silencing was similar to that observed in our MAG system (Fig. 4-1B).

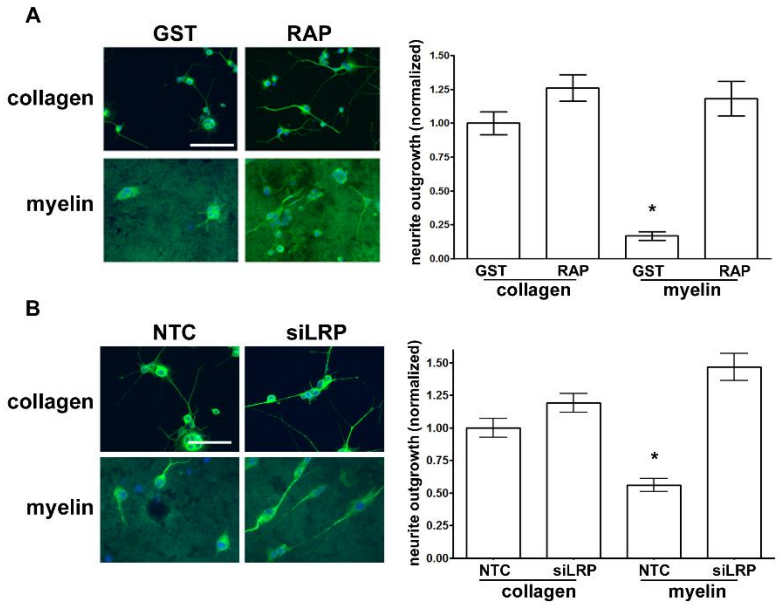


Figure 4-1 Antagonism and deletion of LRP1 restores neurite outgrowth of PC12 cells on purified myelin.

A. PC12 cells were pre-treated with GST or GST-RAP (200nM) prior to plating on substrate with and without myelin and allowed to grow for 48 hours prior to fixation and immunofluorescent imaging. Graphs represent the mean neurite outgrowth (+/- SEM) per condition (n=3 independent experiments, **p<0.01). B. PC12 cells were transfected with non-targeting (NTC) or LRP1-specific (siLRP1) siRNA, plated on substrate with and without myelin, and allowed to grow for 48 hours. Graphs represent the mean neurite outgrowth (+/- SEM) per condition (n=3 independent experiments, **p<0.01). Scale bar 100 µm.

While we anticipated that neutralization of LRP1 with RAP or siRNA would result in enhanced neurite outgrowth on myelin, the degree to which neurite outgrowth was enhanced was unanticipated. However, before making any conclusions about the extent of LRP1's effect on myelin-mediated neurite outgrowth inhibition, we felt it was important to confirm the PC12 result in primary neurons. As such, we repeated the myelin study using primary CGNs. However, myelin is incredibly inhibitory not only to neurite outgrowth of CGNs, but also to adherence. Because of this, serial dilutions of myelin solution were used to coat culture plates and analyzed conditions were chosen wherein CGNs adhered readily, but were still inhibited in neurite length. Under these conditions, we once again observed that while myelin was inhibitory to neurite outgrowth, pre-treatment of neurons with RAP resulted in significant restoration of outgrowth on purified myelin (Fig. 4-2A). Additionally, siRNA against LRP1 also restored neurite outgrowth of CGNs on myelin, however, due to a much lower transfection efficiency of CGNs, the extent to which silencing LRP1 restored neurite outgrowth on myelin was not as robust as seen in PC12 cells (Fig. 4-2B). It is of note that the extent of neurite outgrowth restoration was quite significant relative to the degree of silencing, which is reminiscent of the results observed in similar studies of CGNs on MAG-expressing CHO cells.

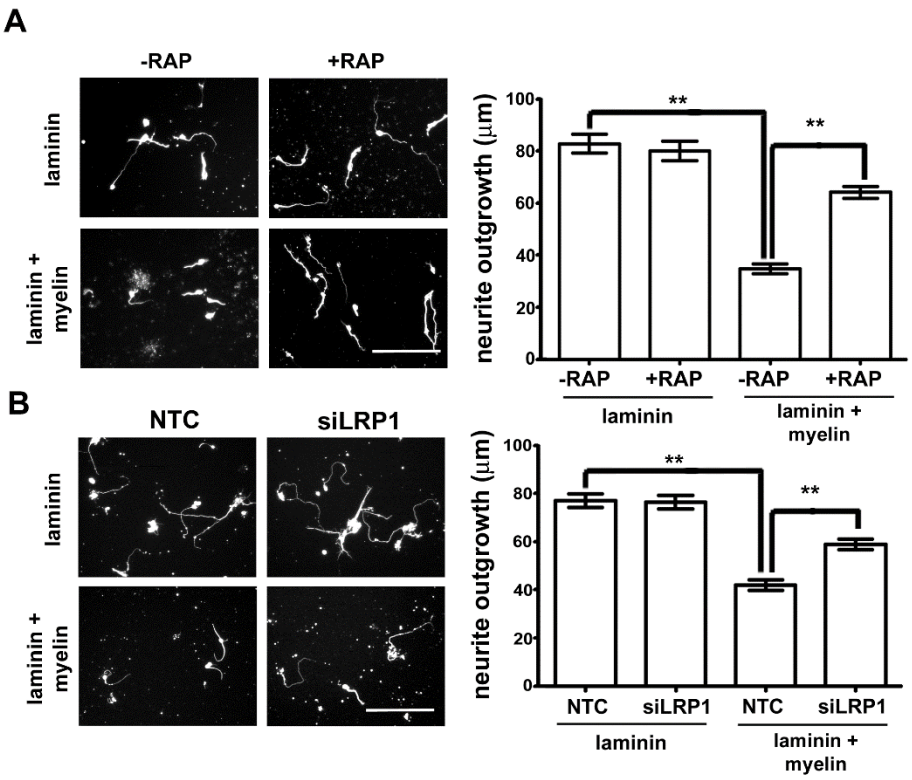


Figure 4-2 Antagonism and deletion of LRP1 restores neurite outgrowth of CGNs cells on purified myelin.

A. CGNs pre-treated with GST-RAP or GST were plated on laminin or laminin + myelin and grown for 48 hours prior to fixation and immunofluorescent detection of βIII-tubulin. Graphs represent the mean neurite outgrowth (+/- SEM) per condition (n=3 independent experiments, **p<0.01). B. CGNs transfected with non-targeting (NTC) or LRP1 specific (siLRP1) siRNA were plated on laminin or laminin + myelin and grown for 48 hours prior to fixation and immunofluorescent detection of βIII-tubulin. Graphs represent the mean neurite outgrowth (+/- SEM) per condition (n=3 independent experiments, **p<0.01).

As a third approach to elucidating the LRP1 role in mediating the inhibitory effects of complete myelin, we went back to the model used in the previous chapter where soluble CCRII and CCRIV were pre-incubated with the immobilized inhibitory substrate prior to addition of CGNs. As these recombinant proteins likely compete with neuronal LRP1 for binding of ligands, saturation of inhibitory epitopes with these binding domains would restrict neuronal interaction and thereby restore growth, which we observed previously (Fig. 3-8). If LRP1's role does, in fact, extend beyond MAG alone, similar results should be obtainable on purified myelin. As hypothesized, figure 4-3 demonstrates that CCRII and CCRIV pre-treatment of immobilized myelin does attenuate the inhibitory effects on neurite outgrowth.

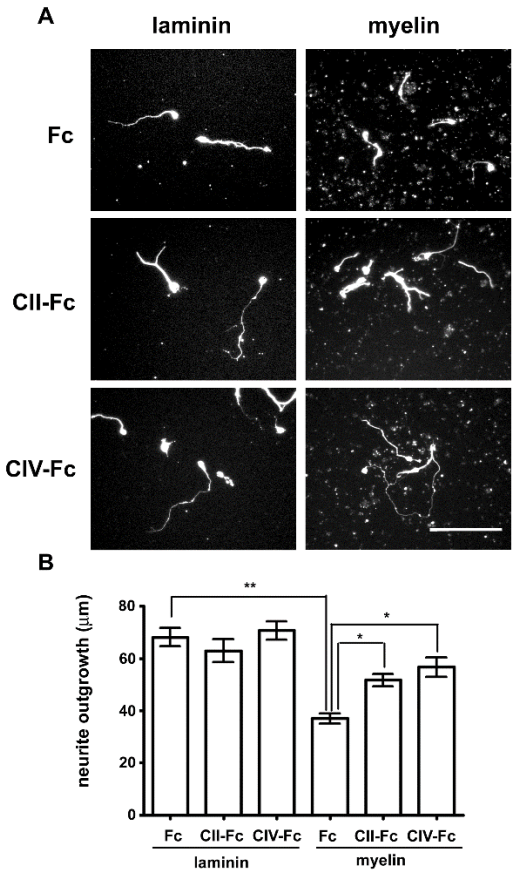


Figure 4-3 Soluble, purified LRP1 or recombinant binding domains of LRP1 block the effects of myelin on neurite outgrowth.
Pre-treatment of immobilized CNS myelin with purified LRP1 leads to enhanced neurite outgrowth of CGNs. Monolayers of MAG-expressing and control (R2) CHO cells were incubated for 15 minutes with purified LRP1 or vehicle control prior to addition of CGNs, which were grown for 48 hours prior to fixation and immunofluorescent staining. B. Graphs represent the mean neurite outgrowth (+/- SEM) per condition (n=3 independent experiments, **$p<0.01$).

LRP1 is released from cells as a shed product by α-secretase and accumulates in blood and cerebrospinal fluid[151]. Shed LRP1 retains the entire α-chain and ligand-binding activity[152]. To confirm that the effects we observed with CCRII and CCRIV could be reconstituted by intact LRP1, we purified shed LRP1 from human plasma for use in neurite outgrowth studies[118]. Similar to what was observed with recombinant LRP1 binding domains, 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. 4-4). These results demonstrate that the role of LRP1 in myelin-mediated regenerative failure likely extends beyond interaction with MAG alone.

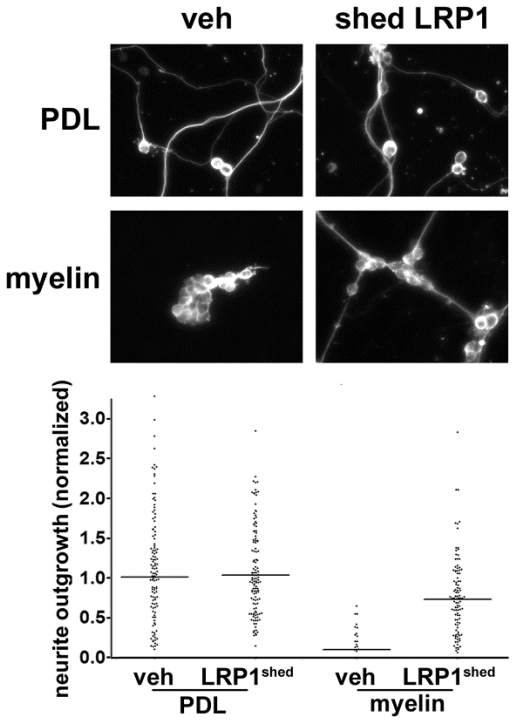


Figure 4-4 Purified soluble LRP1 incubated on purified myelin results in restoration of neurite outgrowth of CGNs.

Pre-treatment of a monolayer of purified myelin with purified LRP1 leads to enhanced neurite outgrowth of CGNs. Surfaces coated with laminin or laminin + myelin incubated for 15 minutes with purified LRP1 or vehicle control prior to addition of CGNs, which were grown for 48 hours prior to fixation and immunofluorescent staining. Graphs represent the mean neurite outgrowth (+/- SEM) per condition (n=3 independent experiments, **$p<0.01$).

While the LRP1/MAG interaction is critical for the inhibitory effects of MAG, complete myelin is composed of other inhibitory components which, in combination, account for a much greater portion of myelin's inhibitory capacity than MAG alone. Because of this, the ability of LRP1 to have such robust effects on the inhibition of neurite outgrowth by intact myelin indicates that LRP1 is likely mediating inhibition by other MAIs as well. Such promiscuous binding of MAIs would not be unprecedented as both NgRs and PirB have been shown to bind all 3 major MAIs[52,73]. This led us to hypothesize that LRP1 also binds OMgp and Nogo-A. To test this, we first assessed Nogo-A/ LRP1 endogenous interaction from brain tissue. Figure 4-5A shows that when LRP1 or Nogo-A are immunoprecipitated from rat brain tissue, both proteins are detectable in either sample, which shows that LRP1 and Nogo-A have significant endogenous interaction in the brain. Additionally, CCRII and CCRIV efficiently and specifically pulled-down Nogo-A from purified myelin extracts (Fig. 4-5B). CCRII and CCRIV also efficiently pull down OMgp from purified myelin extracts (Fig. 4-5C). Finally, to confirm that these interactions are direct, we showed that recombinant, purified OMgp and one inhibitory domain of Nogo-A, Nogo66, selectively associate with CCRII and CCRIV (Fig. 4-5D).

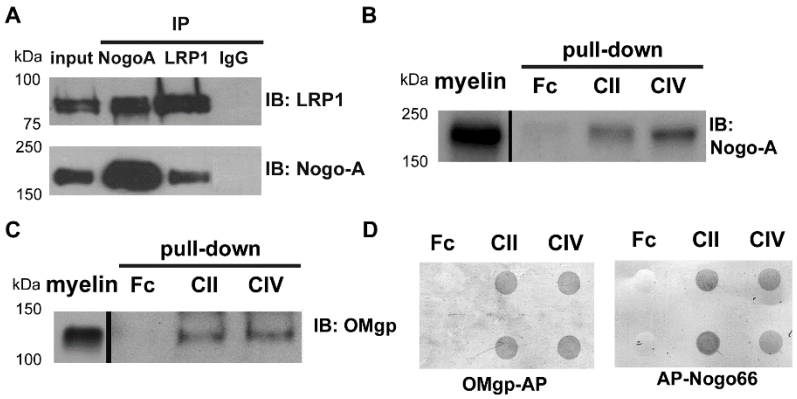


Figure 4-5 LRP1 binds Nogo-A and OMgp.
A. Endogenous Nogo-A and LRP1 were precipitated from membrane preparations from adult rat brain using LRP1 and Nogo-A-specific antibodies, as well as non-specific IgG as a control. Precipitated proteins were the immunoblotted for Nogo-A and LRP1. B. CII-Fc, CIV-Fc, and Fc were incubated with purified myelin extracts followed by precipitation with Protein A-Sepharose. Immunoblot analysis was performed to detect Nogo-A. C. CII-Fc, CIV-Fc, and Fc were incubated with purified myelin extracts followed by precipitation with Protein A-Sepharose. Immunoblot analysis was performed to detect OMgp. D. OMgp-AP and AP-Nogo66 were expressed in HEK293T cells and conditioned medium was recovered from these cells as a source of the fusion proteins. CII-Fc, CIV-Fc, and Fc (40 pmol) were immobilized in duplicate on nitrocellulose membranes and incubated with equivalent amounts of conditioned medium. Binding was detected by NBT/BCIP AP detection. In control experiments, no alkaline phosphatase activity was detected when control medium was incubated with the immobilized Fc fusion proteins.

As we had now demonstrated LRP1 is capable of mediating neurite outgrowth inhibition by complete myelin, and that LRP1 was capable of binding to other MAIs, we next sought to determine if LRP1 was also capable of mediating the inhibitory signaling caused by myelin debris. To accomplish this, we chose to use an *in vivo* system to assess levels of RhoA activation after SCI. RhoA is well understood to be a critical regulator of neuroregenerative failure after SCI, and it has been repeatedly shown that RhoA is robustly activated after spinal cord injury[47,48,144,150,153]. Inhibition of RhoA after SCI has been demonstrated to have regenerative effects on neurons as well[50,77,78]. Because in the acute phase of injury after SCI the primary inhibitory factor is myelin debris[11,17], we sought to assess the effect of LRP1 antagonism on RhoA activation 5 days post-SCI. What we found was that after 5 days of RAP infusion into the site of injury, the antagonism of LRP1 had a remarkable effect on the attenuation of RhoA (Fig. 4-6). Of additional interest, we also found that relative association of p75 with LRP1 was dramatically upregulated by injury, and RAP attenuated this effect as well (Fig. 4-7). Interestingly, SCI also seemed to result in decreased total levels of LRP1 and p75, and RAP treatment seemed to stabilize this effect, although the significance of this is unknown.

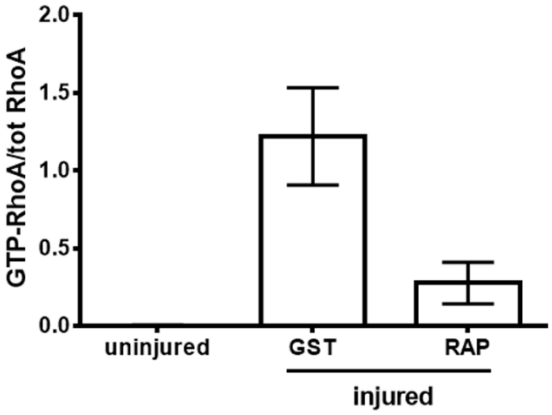


Figure 4-6 RAP blocks the activation of RhoA in the injured spinal cord.
RAP or GST (vehicle) were infused into the injury site post-SCI for 5 days prior to extraction of tissue and assessment of RhoA activation. Uninjured tissue was also assessed as a control. As anticipated, SCI resulted in marked activation of RhoA comparted to uninjured controls. RAP infusion blocked this effect to statistically insignifcant levels compared to control ($p<0.05$).

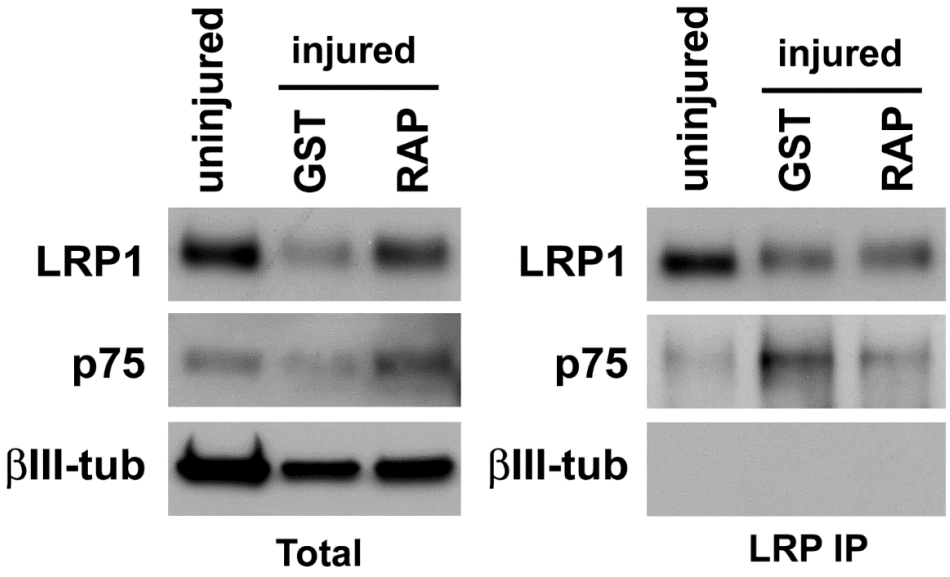


Figure 4-7 RAP disrupts co-immunoprecipitation of LRP1 with p75 after SCI.
Rats were given dorsal hemisection injuries and infused intrathecally for 5 days with GST or RAP. After 5 days, the damaged section of spinal cord was removed and LRP1 was immunoprecipitated from protein extracts. While the total levels of LRP1 seemed to be decreased by injury, the relative association of LRP1/p75 was robustly increased after SCI compared to uninjured controls. Interestingly, RAP infusion blocked this effect and appears to stabilize total LRP1 levels.

DISCUSSION

This chapter demonstrates that LRP1 is a potent facilitator of not just MAG-mediated regenerative inhibition, but rather plays an important role in the inhibitory effects of myelin in general. LRP1 associates directly with both OMgp and the Nogo66 domain of Nogo-A. LRP1 also associates endogenously in rat brain with both MAG and Nogo-A. Antagonism and genetic silencing of LRP1 restored neurite outgrowth of neuronal cells on purified myelin. Similarly, pre-treatment of inhibitory myelin substrate with soluble receptor mimetics of LRP1 also had the ability to promote neurite outgrowth on myelin. In summary, these results indicate that LRP1 is a potent facilitator of myelin-mediated regenerative failure and, as such, stands as an exciting potential therapeutic target. This will be discussed in further detail in the following section.

Acknowledgements

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CHAPTER 5 - GENERAL CONCLUSIONS

Conclusions

The goal of this thesis work was to identify the interacting components of myelin with LRP1 and determine if these interactions may have pathological implications. Herein I present extensive evidence that LRP1 is a receptor for MAG. Strong evidence exists for LRP1 interaction with other MAIs as well, but further work is being conducted to assess the functionality of these interactions. Regardless, LRP1 is presented in this work as a critical facilitator of the inhibitory effects of MAG and myelin on neurite outgrowth. Furthermore, antagonism of LRP1 blocks the signaling events known to be responsible for regenerative failure *in vivo.* These results support the hypothesis that LRP1 is a potentially exciting and potent therapeutic target for the purpose of regenerating neurons of the CNS after injury.

This work began as a follow-up on the observation that LRP1 is a novel receptor for myelin debris. We wished to identify the specific components of CNS myelin that were capable of LRP1 ligation. My particular interest lied in assessing the potential pathological role these interactions might play. Myelin components have been demonstrated in CNS pathology for some time. For example, disorganized myelin in the event of demyelination in multiple sclerosis suppresses oligodendrocytes differentiation and subsequent remyelination[154]. Nogo proteins have been shown to be involved in the progression of motor neuron disease such as amyotrophic lateral sclerosis[28,155]. And finally, myelin proteins have been known for decades to be a major

regenerative obstacle to CNS neurons after injury[10,18,146]. Identifying receptors mediating these pathological effects would be of profound therapeutic interest.

Our initial studies involved a mass spectrometric screen of LRP1 interacting partners from CNS myelin. Several candidates were identified, but those that stood out for my interests were MAG, identified in 3 of 3 studies, and OMgp, which was identified in 2 of the 3. Because MAG was identified in all 3 of our studies, we chose to focus on this protein for our initial work. MAG binding to LRP1 was confirmed, and additionally shown to be direct, reversible, and high-affinity. Several receptors for MAG have been previously identified such as gangliosides[70,71,156], Nogo-receptors[51,157], and PirB[72,73]. MAG is both a lectin and MAI. However, these activities are independent of one another and deletion of the sialic acid-binding capacity of MAG has no effect on its inhibitory capacity. Interestingly, previously identified MAG receptors depend on sialic acid for MAG binding. This work identifies LRP1 as the first confirmed receptor capable of binding MAG independent of sialic acid, which led me to believe that LRP1 may be the first identified MAG receptor capable of robust facilitation of regenerative inhibition.

In addition to being the first sialic acid-independent receptor of MAG, we also demonstrated LRP1 binding to MAG to mediate MAG endocytosis. This appeared to be completely dependent on LRP1. This was important as neuronal signals often must travel long distances, and the formation of signaling endosomes which can be transported in the axon to the soma are necessary for influencing genetic changes[59,88,135,139]. The formation of signaling endosomes has also been shown

recently to function in the chronic regenerative inhibitory effects of MAIs[14]. As such, the dominant endocytic function of LRP1 in relation to MAG further indicates an important role in its ability to inhibit neurite growth.

The unique and novel characteristics of the LRP1 MAG interaction led me to hypothesize that LRP1 robustly facilitates the inhibitory effects of MAG. We confirmed this hypothesis in multiple cell systems using both antagonism and genetic silencing of LRP1. While these results revealed LRP1 as a novel and critical mediator of MAG mediated inhibition of neurite outgrowth, I next wished to assess the contribution of this interaction to the inhibitory effects of whole CNS myelin.

I hypothesized that because LRP1 played a robust role in MAG-mediated neurite outgrowth inhibition we would see a proportionate effect in models using complete myelin. We were surprised when the ability of LRP1 antagonism or silencing to attenuate the inhibitory effects of myelin seemed comparably robust to the results observed in our MAG-only systems. Because initial mass spec studies also indicated and LRP1 interaction with OMgp, I hypothesized that LRP1 may be a promiscuous MAI receptor as had been demonstrated with NgRs and PirB. While additional studies are being conducted, we have demonstrated that LRP1 is capable of direct binding to both OMgp and Nogo-A.

The robust *in vitro* effects of LRP1 antagonism and silencing led us to assess if we could observe a role for LRP1 *in vivo.* RhoA activation is a critical signaling event required for regenerative inhibition shared by myelin and the glial scar[46–48,50,51,144]. Additionally, RhoA has demonstrated to be an effective therapeutic target clinically

and agents blocking RhoA activation have shown efficacy in clinical trial[77]. We had previously shown that LRP1 deletion attenuated RhoA activation in response to MAG. I hypothesized that RAP infusion to the site of spinal cord injury would have the same effect. We were able to confirm this, and in addition we demonstrated that p75 association with LRP1 occurs *in vivo* and is also blocked by RAP. In sum, LRP1 arises as an important therapeutic target in SCI.

Proposed Mechanism

From the data presented in this disseration, I propose that LRP1 functions as a direct receptor for MAG and other MAIs. Binding to LRP1 results in a formation of a receptor complex with p75. The participation of other receptors in this complex is likely and need be investigated. This complex undergoes endocytosis and a signaling endosome is formed with the intracellular domain of p75 exposed on the outer domain of the endosome. Both p75 and LRP1 are needed for MAG-mediated activation of RhoA in our system. I propose that the necessity of LRP1 is due to both its binding capacity and its endocytic function. The importance of endocytosis in chronic inhibition of neurite outgrowth has recently been demonstrated[14]. The essential nature of p75 in this model is a result of the ability of the intracellular domain to interact with Rho-GDI and displace RhoA and facilitate its activation[109]. Because the intracellular domain is exposed on the outer surface of the MAI-containing endosome, it facilitates activation of RhoA to be sustained as the signaling endosome is transported toward the neuronal soma. This mechanism accounts for chronic signaling events and transmission of inhibitory signaling at the distal growth cone to the nucleus.

FUTURE DIRECTIONS

Based on the findings of this thesis work, I have identified a few potential lines of future work which could contribute greatly to both the LRP1 field, and to the development of novel therapeutics for SCI.

Characterization of differential LRP1 co-receptors resulting from distinctive ligand interactions.

A prevailing issue in the LRP1 field is the diverse array of LRP1 effects in cells. Significant evidence exists that much of this may be explained by the ability of LRP1 to form receptor complexes in response to different ligands. In this work, we identify p75 as an important co-receptor with LRP1 participating in regenerative failure of neurons. Further work should be done to correlate LRP1 associated membrane proteins to the diverse array of cell-signaling and morphological effects observed in response to the array of LRP1 ligand binding events.

Investigate relationship between unique ligand-specific binding domains and specific co-receptor formations

The idea that different LRP1 ligands can result in stark differences in cell signaling may be explained by the formation of ligand-specific co-receptor complexes. However, if this model were to hold true, LRP1 ligands would almost certainly need to exhibit differences in how they bind LRP1. These differences are likely to be uncovered in the specific binding domains within LRP1 with different ligands. As such, characterization of these specific domains using molecular dissection and site-directed mutation studies will likely yield critical information into understanding these

phenomena. Additionally, as is particularly the case with MAIs, uncovering the identities of these domains to the minimal essential level would greatly aid in development of competitive inhibitors of binding that could be translated into novel drug therapies.

Investigate RAP, or derivatives thereof, as a potential therapeutic for SCI

The pronounced effects of RAP on the activation of RhoA after SCI demonstrate a unique therapeutic opportunity for potentially regenerating neurons after injury. Further studies must be conducted to confirm that reduced RhoA activation with RAP infusion results in restoration of function and regeneration of damaged axons after SCI. RAP has been demonstrated to readily cross the blood brain barrier and has been experimentally investigated as a chemotherapeutic conjugate for delivery to the CNS for brain cancers[158]. As a result, if RAP is confirmed to be capable of restoring nerve growth after SCI, intravenous delivery would likely be a viable delivery mechanism. To investigate this, experiments should be conducted on the utility of IV RAP delivery in the suppression of RhoA activation after SCI.

Develop competitive agonists capable of MAI displacement from LRP1 as putative therapeutics in SCI

While RAP has demonstrated significant promise as a potential therapeutic agent in the treatment of spinal cord injury, the promiscuity of this protein for other LDLR family members make it less than ideal in terms of specificity. Even in the consideration of its effects on LRP1 alone, RAP shuts down all binding functions, which may have detrimental ramifications over the course of long term treatment.

These issues may potentially be responsible for undesirable toxicity and off-target effects. To potentially overcome these issues, development of agents capable of LRP1 specific competitive displacement of MAIs. To accomplish this, detailed characterization of the LRP1 binding sites within LRP1 responsible for MAI interaction must first be identified. Molecular dissection and site-directed mutagenesis studies would likely provide the necessary information. Once identified, large-scale screens of compound libraries to identify other agents capable of binding to these domains could identify lead candidates for development as additional drug candidates.

As an alternative approach, *in silico* computational prediction of potential drugs may also be a useful approach. To accomplish this, high-quality crystal structures of LRP1/MAI complexes would be of great utility. Such studies are impossible with full-length LRP1, and the relatively large nature of MAIs are somewhat prohibitive as well. As such, assuming the Nogo-66 interaction with LRP1 is further confirmed to have similar effects as MAG, this short peptide could be a useful tool for creating a crystal structure of the ligand binding pocket within LRP1. However, as previously mentioned this could not be done with full-length LRP1 and would need to be assessed using recombinant versions of the CCRII or CCRIV domain, or truncated versions thereof corresponding to the minimal essential interacting sequence. Once this structure is obtained, computational modeling of protein interactions would then be able to predict agents capable of high-affinity binding to the same site of interest.

Investigate soluble receptor mimetics as a therapeutic approach to regeneration of CNS neurons after injury.

It appears that the ability to bind MAIs is reconstituted in both the CCRII and CCRIV domains of LRP1. MAG, OMgp, and Nogo-A all associated selectively with both of these domains. We hypothesized that saturating inhibitory epitopes of MAIs with soluble forms of LRP1 may compete with neuronal LRP1 for MAI binding, thereby attenuating the inhibitory effects on neurite outgrowth. We confirmed this using 3 different models of purified LRP1; shed LRP1, purified full-length LRP1 and recombinant CCRII/IV. In each case, pretreatment of inhibitory substrata with these soluble receptors/receptor mimetics released neuronal cells from inhibition. These results suggest that soluble receptor mimetics may also be a useful therapeutic approach in SCI. Conceptually, this approach is therapeutically very similar to a monoclonal antibody therapeutics in that, in practice, you are creating a therapeutic benefit by restricting undesirable epitopes of proteins from interacting with pathologically relevant cellular receptors. This approach is already being pursued in the form of anti-Nogo antibodies, which are being examined clinically[20,79]. However, while MAIs share common receptors, they lack sequence homology that would provide a ready explanation for the common binding and antibodies generated against individual MAIs are limited to only affecting that particular molecule. As such, soluble receptor mimetics of LRP1 capable of MAI binding may yield greater therapeutic utility due to the ability to affect multiple MAIs. Unfortunately, it is unlikely that such molecules would have RAP's ability to cross the blood brain barrier

and would likely need to be delivered intrathecally. Regardless, the therapeutic potential of such molecules should be evaluated.

Summary

The presented work demonstrates an important novel role for LRP1 in regenerative failure of CNS neurons after injury. Perhaps more importantly, the data presented unveils a novel therapeutic approach potentially capable of restoring the regenerative capacity of neurons in the CNS. Further *in vivo* validation of the therapeutic validity of LRP1 as a drug target needs to be conducted, and variations upon direct antagonism, competitive displacement and receptor mimicry are amendable to further refinement. Additionally, if RAP demonstrates the ability to restore nerve growth and function in animal models, the RhoA studies presented in this work represent an exciting tool for use in *in vivo* screening of potentially therapeutic compounds that can be accomplished on the order of days to weeks as opposed to months, which may result in much more rapid translation and validation of experimental compounds. In conclusion, the work presented herein represents an exciting advancement in the field of neuroregeneration that could yield valuable insights into therapeutic approaches and pre-clinical testing for future studies.

APPENDIX

The following is a selection of optimized, specific protocols from the author. Not all protocols were applied to the work presented herein, but are included for the sake of preservation.

Dot Blot binding assay

In dot blotting studies, 1.0 µg of CII-Fc, CIV-Fc or Fc was immobilized on nitrocellulose that was secured in Bio-Rad bio-dot apparatus. The membrane was blocked with 5% bovine serum albumin (BSA) in PBS. Incubations with MAG-Fc (5 µg/ml) were conducted for 1 h at 22^{o} C. The membranes were then washed and immunoblotted for MAG.

1) Using nitrocellulose membrane, secure in Bio-Rad dot blot apparatus. Make sure its really tight.
2) Aspirate 1ug of protein per well in 100-200ul of buffer. Buffer can vary. Sometimes PBS alone is fine, but using a more stringent incubation buffer might yield better results. This may take some trial and error so perhaps try 1 or 2 different types. I suggest; a) PBS/TBS alone, b) PBS/TBStween alone.
3) Block for 1 hour in "incubation" buffer. For incubation buffer you can use PBS/TBS with or without tween plus a protein. For the protein you can use the classic BSA, milk, or serum. Personally, I recommend using 5% of the serum from the host secondary (ie: if it's a goat anti-rabbit HRP secondary, block in goat serum). However, this is something you might have to play with to get as clean as possible.
4) Incubate ligand and control (tag or vehicle) at desired concentration for 20 minutes to 1 hour at room temperature. Overnight results in too much background. Incubate in the same buffer as used for blocking.

5) Perform immunodetection as you would for a western. However, you can lower the antibody concentration and only go 1 hour at RT each for primary and secondary.

Competitive Binding Assay

Binding Solution (EHB):

Earle's balanced salts solution (EBSS)

10mg/ml BSA

10mM HEPES

pH 7.4

Procedure

1) Wash cells 2x's with EHB
2) Add radiolabeled a2M-MA to the cells at equal concentrations (500pM-1nM) with increasing molar concentrations of competing ligand from 0-200 times molar excess (100-200nM).
3) Incubate at 4° C for 5 hours.
4) Wash 3x's with EHB.
5) Lyse cells using 100mM NaOH in 1% SDS using equal volume of buffer for each sample.
6) Quantify the radioactivity of each solution using gamma counter.
7) Adjust the radioactivity to the amount of protein as determined via BCA
8) BASELINE MEASURE OF NON-SPECIFIC BINDING
9) Use cold a2M-MA as the competitive ligand.
10) Determine non-specific binding as the radioactivity recovered in 200x molar excess of cold ligand.
11) Specific binding is the difference between total binding and this value.

*experiments should be performed in triplicate.

CII and CIV pull-down from purified rat myelin

Myelin protein solubilization

1) Add 2mM EDTA and protease inhibitor cocktail to RIPA buffer.
2) Incubate with purified myelin at a 1:5 ratio by volume for 30 minutes at RT with agitation.
3) Clarify myelin extracts by centrifugation at 15000 rpm for 15 min at 4 degrees.
4) Determine protein concentration by BCA analysis.

Bead preparation

1) Wash protein-A sepharose beads
2) Incubate 30 ug of Fc, CII, and CIV on 30 ul beads for 1 hour at RT
3) Wash thoroughly, final wash in extraction buffer.

Myelin incubation

1) Incubate 1-3mg of protein extracts from myelin with beads for 1 hour at RT
2) Wash beads thoroughly

Neuronal Co-Immunoprecipitation protocol for LRP1, p75, NgR

IP lysis buffer:

50mM HEPES pH 7.4

1% Triton X-100

150mM NaCl

10% glycerol

protease inhibitor cocktail

2mM EDTA

1mM sodium orthovanadate

Procedure

1) In T175 flasks/15cm dish, lyse cells in 1.5ml lysis buffer and thoroughly scrape and collect on ice.

   *BCA and balance concentration.

2) Save lysate for blotting of total protein.

   *Between 50-100ug

3) If necessary split the remaining lysate into separate incubations.

   *ie: IP LRP1 IB p75; IP p75 IB NgR, etc

4) Incubate IgG or antibody with beads in the quantities described below for 30 minutes prior to adding to lysates. In the case of double IPs and 2 conditions, 30ul of beads and 7.5ug of antibody will be pre-incubated for 30 minutes in 500ul of IP lysis buffer, then spun down and split into the separate reactions.

5) Perform sequential IP

Pre-clear

a) 2.5ug Rabbit IgG and 10ul Protein A beads (washed)
b) Incubate with agitation for 2.5 hours
c) Spin down and remove supernatant

   *This will be used for the next step

d) Wash beads 2 times
e) Add 2x sample buffer and DTT and store

Immunoprecipitation

a) 2.5ug antibody (ie: LRP1 or p75) and 10ul Protein A beads (washed)
b) Incubate with agitation overnight
c) Spin down and remove supernatant
d) Wash beads 2 times
e) Add 2x sample buffer and DTT
f) Boil sample along with pre-clear beads
g) Spin at max speed for at least 3 min and load

Endocytosis Immunofluorescence assay for MAG-Fc and RPTP-Fc

*specific antibodies and dilutions will vary

1) Plate approximated 10,000 cells/well on sterile glass slides in 12-well plate and recover overnight.
2) Serum starve, or otherwise differentiate, for desired time frame (overnight preferable). This step is not for starvation purposes as much as differentiation.
3) Suspend FcR blocker in ice-cold serum free media at concentration of 1ug/ml (each well will require 500 ul so plan accordingly). * for Fc-conjugated protiens
4) In sterile hood, add cold SFM/FcR blocker mix to cells at 500ul per well.
5) Transfer in sterile secondary container immediately to cold room, incubate for 30 minutes.

   *sterilize in 10% bleach 30 min, spray with 70% EtOH and wipe down.
6) Bring back to sterile hood and quickly add 20ug/ml MAG-Fc to cells, and quickly take back to cold room for 60 more minutes.

   *For surface binding experiments, after 60 minutes immediately wash in cold PBS, fix and continue with IF (skip to step 8).
7) Aspirate media and add 500ul warm N2a media and place in incubator for 30 minutes.
8) Wash 1x with cold PBS on ice.

   *For surface stripping, incubate on ice on rocker for 4 min in 0.2M acetic acid in PBS and wash 2 times.
9) fix with 4% PFA in PBS for 10 min.

10) wash cold PBS.

11) Permeabilize/block with 0.1% Triton X-100 5% goat serum in PBS or TBS for 1 hour.

12) Put glass slides on parafilm (or in wells) and incubate with primary antibodies 150ul per slide overnight.

*Goat anti-MAG, 1:500

*Rabbit or mouse anti-EEA1, 1:250

*Mouse anti-Rab5, 1:100

*Rabbit anti-p75, 1:250

14) Wash 2x's PBS in original plate, 1x blocking buffer.

15) Put slides back on parafilm and incubate with secondary antibody 150ul/slide for 2.5 hours in dark (optional as needed)

-488, 1:500

-594, 1:250

16) Wash 2x's PBS, 1x blocking buffer in original plate, rinse briefly in deionized water and mount.

rFc-fusion protein purification (pFUSE vector system)

Cells are selected in 300ug/ml of Zeocin

Cells are very durable, so you can begin antibiotic selection immediately upon thawing, but it is not critical to do so.

CHO media

DMEM low glucose

5ml non-essential amino acid (100x NEAA; Gibco 15140)

5ml L-glutamine (200mM stock, final conc 2mM; Cellgro 25-005)

10% FBS (Hyclone SH30088.03)

5ml pen/strep (Gibco 15140)

Expression media

Lonza Power CHO2 CD (cat #12-771Q)

10ml L-glutamine (same as above, double the amount because the media comes in 1L size)

P/S if desired, zeocin if desired (not really needed in my opinion).

Procedure

1) Plate cells, once confluent pass into desired number of flasks (T175 flasks are what we use, and I usually plate 15-20). Keep zeocin in the media.
2) At approximately 70% confluency (err on the side of less not more) change media to expression media and let express for 5-7 days.
3) Collect conditioned media (a little shaking can help increase yield a little bit) and spin down to get rid of cell debris (5000xg for 10 min).

4) Add protease inhibitor cocktail (Roche #11 873 580 001) 1 tab per 150ml, and 2mM EDTA.
5) Filter through vacuum filter into sterile container.
6) Concentrate to approximately 50ml (If you think you have a LOT of protein, for example if it appears that you are getting precipitation, concentrate to 100ml and do a double incubation with beads).
7) Add protein A beads (500ul pre-washed beads, or 1ml of slurry before washing…can use a little more).
8) Bind O/N at 4 degrees.
9) Wash 3x's with PBS and pack elution column (can wash the tube with a little PBS and add to column to get all the beads out.)
10) Elute with 1 column volume of 0.1M citric acid (ph 3.0, made fresh). For 25ml, 0.48g citric acid pH to 3.0. Incubate citric acid with beads 5 minutes before elution for each fraction.
11) Collect 4-8 fractions *important: add final concentration of 100mM Tris Ph8 to each fraction tube before elution to minimize time protein is in acid (you can either do coomassie on each fraction or just pool them).
12) Desalt with either desalting column or dialysis (2 x 2L, first volume for 1 hour at RT, then second O/N at 4 degrees).
13) Concentrate and calculate concentration. If desired, you can filter the unconcentrated the solution through a syringe driven filter to make sure of sterility.

MAG-Fc spot assay protocol

Purpose

MAG has been shown to be highly inhibitory to neurite outgrowth. Typical neurite outgrowth assays using MAG either immobilize the protein on a surface, or utilize a confluent monolayer of MAG-expressing CHO cells on which neurons are plated. However, these model systems lack the physiologic significance of demonstrating the ability of neurites to penetrate the inhibitory milieu seen *in vivo.* This model system creates a natural gradient with a distinct border that allows for visualization of neuritic extension from the permissive surface into the inhibitory MAG border, indicating loss of inhibitory activity.

Protocol

1) Coat glass slides with collagen (see collagen coating protocol).
2) Dilute MAG-Fc to 134µg/ml in PBS
3) 25µl will be used per 18mm circle glass slides, so make MAG solutions at appropriate volume.
4) Dilute Fc control to 44µg/ml in PBS
5) Place 25µl of MAG or Fc solution on middle of glass slide.
6) Allow to dry, wash 1x with PBS.
7) For 12 well plate, seed PC12 cells at a density of 50,000 cells/well.
8) Continue as with any neurite outgrowth assay.

Modified CGN purification/MAG experimental protocol

Travis Stiles 2-10-2010

This is an amended protocol derived from the combination of 2 distinct protocols contained in "protocols" binder. These protocols should be used for reference as they contain specifics important to this modified protocol

CHO-MAG/CHO-R2 medium (500ml)		
Ingredients	Final Concentration	Amount per 500ml
DMEM high glucose		
Dialyzed FBS (Gibco #26400-004)	10%	50ml
Glutamine (probably already added) and P/S	2mM	5ml
*Proline (Biochemika #81709) Make 50mg/ml stock	40mg/L	400ul
*Thymidine (Sigma #T-1895) make 10mg/ml stock	0.73mg/L	36.5uL
*Methotrexate or MTX make 5mM stock	1uM	100uL
*Glycine make 10mg/ml stock	7.5 mg/L	375uL

*Can be made as a 1ml pre-aliquot stock

**add G418 for CHO-MAG only

CGN medium make fresh

DMEM high glucose

2.25 g glucose (added to high glucose, final concentration 50mM)

10% heat-inactivated FBS

25 mM KCl (for 500ml 0.932g)

P/S

Standard 10x BSSH (pH 7.3-7.5)

Pt 1		
Ingredients	For 250 ml	For 1 L
NaCl	20g	80g
KCl	650mg	2.6g
KH_2PO_4	135mg	540mg
Na_2HPO_4	445mg	1.78g
HEPES-NaOH (1M stock)	12.5 ml	100 ml
Pt 2		
$CaCl_2$-$2H_2O$	808 mg	3.23 g
$MgSO_4$-$7H_2O$	600 mg	4.9 g
Glucose	4.5 g	18 g

1% stock DNase (Roche #104-159) specific activity 2000U/mg:

Mix 1.25 ml BSSH parts 1 and 2 in 10ml ddH_2O (total volume = 12.5ml)

Take 10 ml and add to 100mg DNase

Use at 100ul/2ml = 0.05% final concentration

TDT (TBS-T with 5% donkey serum)

50ml:

-2.5 ml donkey serum

-50 µl Triton

-47.45 ml TBS

Materials

10cm dishes coated with 1.5% agarose (.75 g in 50ml water)

Fill with cold HBSS

60mm dishes with ice-cold SF L15 antibiotic media (SFM)

Surgical tools

Dissection scope and light source

Body bags

Paper towels

PDL coated T75 flasks

Dissect neonatal rat cerebellum (laminar flow hood)

1) Wearing surgical gloves, spray the pup with ethanol and rapidly decapitate with large dissection scissors. Discard the body.
2) Using small dissection scissors, cut the skin in a straight line from the base of the skull to the nose and spread the skin apart.

3) Using the same small scissors, start at the base of the skull (foramen magnum) and carefully cut up the midline of the skull to the eyes. IT IS CRITICAL THAT YOU REMAIN AS SUPERFICIAL AS POSSIBLE IN THIS PROCESS TO AVOID DAMAGING OR LOSING CEREBELLAR TISSUE!
4) Cut perpendicular to the skull incision across the eyes to facilitate easier exposure of the brain.
5) Carefully pull skull-flaps apart being careful to not damage the cerebellum.
6) Using curved #7 forceps, clip the optical nerves and gently pull the brain out. It helps to use the forceps to pull the base-flaps away from the cerebellum as this makes the full flaps come apart without damaging the tissue.
7) Place into the cold HBSS in 10cm agarose coated dish.
8) Place brain ventrally and begin dissecting away the meninges and blood vessels using #5 forceps.
9) Flip and continue cleaning away meninges until the cerebellum is exposed and clear of meninges.
10) Pull cerebellum away from the brain gently and place into 60mm dishes of cold SFM.
11) Pool up to 4 cerebella
12) Isolation of CGNs (take cerebella to tissue culture hood on ice)
13) Aspirate off excess media and mince the tissue using a razor blade
14) Add 2ml of SFM medium and put everything in 15ml tube without triturating
15) Add 2ml of trypsin/EDTA(0.25%) + 200µl of DNase solution.

16) Incubate at 37° for 30 min, shaking every 5 min.
17) Wash 2x with 4ml CGN medium by letting the big chunks fall to the bottom, aspirating extra media, and adding more CGN medium
18) Remove SFM trypsin media first
19) Wait until chunks fall to the bottom of the tube and aspirate with Pasteur pipette
20) All media RT from here forward
21) After last wash add CGN medium to cells DO NOT TRITURATE
22) Triturate 5x's with 20G needle until completely homogenized and there are no chunks

    *NO BUBBLES! THEY WILL KILL CGNS!
23) Using a 10ml serologic pipette, strain the cells through a 100µm nylon mesh into a 50ml conical tube.
24) Allow astrocytes to attach
25) Transfer homogenate into PDL coated T75 flask. Incubate 15min at 37° C.
26) No more than 4 cerebella per flask
27) Shake flask back and forth for 1min to detach neurons, and collect medium using 10ml serologic pipette.
28) Shaking should be rapid but not so much as to turn the media foamy.
29) Separate and Plate Neurons
30) Strain the medium through a 70µm mesh strainer into a new 50ml tube.
31) Repeat using a 40µm strainer.
32) Count cells and plate as desired

*For morphology, approximately 1 x 10^5 to 1 x 10^6 cells per 12 well.

*For signaling, approximately 15 x 10^6 cells per well in 6-well plate.

*Add Arac to kill proliferating cells (1μM).

1) Clear the fix and wash bottle lines of any air bubbles. Sufficient perfusion pressure is obtained with the bottles 100-120 cm above the animal (but no more!).
2) Run wash solution through cannula to remove any fix from lines. Set at a slow drip.
3) Place the heavily anesthetized animal on its back on a rack or paraffin block over a sink. Note: A barbiturate type of anesthesia is highly preferred to gaseous types such as Fluothane, with which vessels tend to constrict resulting in poor perfusion.
4) Spread the forelimbs and secure each paw to the rack with a surgical towel clamp or a pin if on a paraffin block. Start running faucet water.
5) Make a cut along the sternum ~8 cm long, low enough to expose the sternum's end.
6) Grasp end of sternum with a 5" hemostat. Use sharp scissors to cut diaphragm laterally on both sides and cut toward the head across ribs and parallel to lungs.
7) If brain tissue is all that is desired, clamp the descending aorta (not necessary for mice). With a mosquito hemostat, reflect one lung and locate the descending aorta as it runs along the spinal column. Clamp it and allow the lung to return.
8) Release the hose clamp on the perfusion tube so that the fluid is just barely passing out of the cannula tip.
9) With left hand, use a small pair of rat tooth forceps to grasp ventral tip of the heart.

10) With right hand, use a #11 scalpel with blade edge out (away from heart) and pierce the left ventricle, allowing the blade to penetrate far enough to make the slit large enough for the cannula tip.
11) Quickly remove blade and lower heart to prevent spurts from the long incision.
12) With right hand, insert the cannula and direct it up through the left ventricle into the ascending aorta. Stop when the tip of the cannula is visible within the aorta.
13) Maintain placement of the cannula in the left hand, holding the cannula and forceps together, side by side. With the right hand free, use the rubber-tipped hemostats to clamp around the aorta, holding the tip of the cannula in place. (Clamping is difficult or not feasible in mice so the cannula/ needle must be held while perfusing.)
14) With left hand, release the cannula and forceps from heart and close the wash line clamp. Or you may choose to allow more wash before fix.
15) Fully open the fix line clamp and perfusion tube clamp.
16) With left hand, use the teeth of the rat tooth forceps or scissors to puncture the right auricle, allowing the escape of return circulation.
17) For rats, allow 200 to 250 cc of fix to flow. For mice, allow 50-100 cc of fix to flow. Consider the perfusion a dialysis process, exchanging formaldehyde and water, rather than a ‘flushing’ process.
18) Close both clamps and remove perfusion instruments. Remove skin from head and decapitate at a level even with forelimbs. Place head in a container filled with perfusion fix solution. Removal of the skin is preferred.

Myelin associated inhibitor activation of RhoA assay

1) Pre-aggregate MAG-Fc with anti Rabbit-Fc antibody at 2:1 molar ratio for 30 min at room temp.

   *cells to be treated with final concentration of 20nM MAG-Fc, in 10ml SFM = 26ug

   *pre-aggregate 26ug of MAG-Fc or 75ug Fc with 2.5ug Goat anti-Rabbit IgG, Fc specific antibody

   *Fc antibody, Jackson ImmunoResearch #111-005-008, 2.3mg/ml = 1ul

2) Add preclustered protein to cells in SFM for 10min (15?).

3) Rinse 3 times in ice cold TBS

4) Add lysis buffer (50mM HEPES pH 7.4, 1% Triton X-100, 150mM NaCl, 10% glycerol, 10 mM MgCl2, protease inhibitor cocktail, 2mM EDTA, 1mM sodium orthovanadate).

5) Spin lysate at 13,000 g at 4° C for 2 min

6) Incubate cleared lysate with GST-TRBD (40ul) beads on ice for 60 minutes.

7) Wash beads 4 times with lysis buffer.

8) Spin down and add 30ul SDS sample buffer + DTT.

9) Boil for 10 min.

10) Load and run normal western against RhoA.

Myelin coating

Prepare poly-lysine coated 50-100 ug/ml plastic dishes

1) Coat with poly-lysine overnight. The next day rinse the plate 3x with PBS and once with water, dry the plate completely.
2) Incubate the plate with different concentrations of CNS myelin (depending on how clean you myelin prep is, you will have to use different amounts of myelin).

   *Determining the exact protein concentration of myelin isn't easy, therefore making standardizing between preps and experiments difficult. One good way to do this the first time you use a new myelin prep is to do a serial dilution of myelin, starting at 100ug/ml and then dilute down to 1 ug/ml. Whatever concentration of myelin leads to a ~ 70-80% inhibition of neurite length (compare to BSA) is a good dose for your functional studies (remember, nothing will ever growth is you use a very high dose of myelin, it's just too inhibitory).
3) Adsorb (but NEVER dry) the myelin for 2-3 h at RT to your plate (overnight at 4degrees works too) and rinse the plate 2x with PBS.
4) Coat for 2h with 2ug/ml of laminin in PBS at RT, rinse plates twice immediately before plating the neurons. Let cultures go for ~ 24-48 hours.

Myelin Vesicle Preparation from Rat Brain

Materials:

Rat brains

0.32 M sucrose* (109.54 g/L)

0.85 M sucrose* (291 g/L)

*sterile filters for stock

PBS

Protease inhibitor

-200mM PMSF

50 ml conical tubes

High speed centrifuge tubes

-Polyallomer 1x31/2" Beckman

Polytron (or preferred homogenizing tool)

-medium probe, small can't reach bottom of 50 ml tube

Dounce (A and B probe)

High speed centrifuge

-Beckman Coulter Avanti J 30 I

-rotor JS-24.38

30 ml syringe

16G 5" needle

Isolation of crude myelin

1) Place each rat brain in 50ml conical

2) Add 20 ml 0.32 M sucrose and 1/100 ratio PMSF (stock 200mM to 2mM final concentration in 20ml sucrose).
3) Using polytron medium probe completely homogenize brains.
4) Use dounce to further homogenize at a max volume of 35ml at a time.
5) Complete 10 full, slow strokes with probe 'B'
6) 10 more full slow strokes with 'A'
7) After polytron blending and dounce, add 20 ml of homogenate into as many high speed plastic centrifuge tubes as needed/this should be 1 tube per brain. (*tubes compatible with JS-24.38 rotor hold about 40ml and should fit loosely in Styrofoam 50ml falcon holder).
8) Using 30ml syringe and 16Gx5" needle, draw up 0.85M sucrose and add 15ml to bottom of centrifuge tubes, under homogenate.

   *Be slow and gentle!
9) Balance tubes to within 50mg of one another.
10) Centrifuge at 75,000g for 30 min

    *rotor has buckets that tubes go into. ALWAYS MAKE SURE THAT THE APPROPRIATE BUCKET IS IN THE APPROPRIATE SWING AND THAT THE LIDS ARE TIGHTENED SO THAT THE BUCKET NUMBER ON THE LID AND BODY ARE ALIGNED!

    *Do not leave in centrifuge! Take out soon after done spinning!

11) Remove from centrifuge and CAREFULLY aspirate top layer, do not aspirate any of the median layer myelin! A little sucrose will remain, it is impossible to get it all without losing myelin.
12) Using P1000 pipette, carefully collect the myelin layer by placing tip right in the middle of the myelin layer. Transfer myelin into clean high speed centrifuge tube.
13) Rinse and soak tubes with remaining sucrose overnight and wash for re-use.
14) Sucrose wash-out
15) Add nanopure water to myelin mixtures to approximately 35-40ml careful to not overfill.
16) Balance the tubes to within 50mg of each other with nanopure water.
17) Centrifuge as before at 75,000g's for 15 minutes.
18) Collect the myelin pellet.

    *pellet is fairly compact, but pouring out supernatant will lose small amount of lipid material so it may be better to aspirate.
19) First osmotic shock and removal of small membrane fragments
20) Each tube should have 1 brain worth of myelin pellet, re-suspend each pellet in nanopure water approximately 30ml and make sure tubes are balanced to within 50mg of each other.
21) Centrifuge at 12,000g's for 10min.
22) Aspirate cloudy supernatant being careful to avoid loosely packed pellet.
23) Second osmotic shock
24) Repeat re-suspension and centrifugation as in step 3.

25) Discontinuous gradient centrifugation to obtain purified myelin
26) Re-suspend pellets in 20ml 0.85M sucrose.
27) Using 30ml syringe and 16Gx5" needle, draw up 0.85M sucrose and add 15ml to bottom of centrifuge tubes, under homogenate.

    *Again be slow and gentle.
28) Centrifuge as in step 1.
29) Collect purified myelin from the interface as before and transfer into clean centrifuge tube.
30) Sucrose wash out
31) Repeat as in step 2
32) Collection and storage
33) Re-suspend pellet in sterile PBS (approximately 1ml/brain but depends on pellet size).
34) Aliquot and store at -80º C.
35) Vesicles are good at 4º for around 2 days.

Western blot endocytosis protocol

1) Plate N2a cells in 6-well plate at 200,000/well.
2) Serum starve to induce differentiation for 4 hours.
3) Suspend FcR blocker in ice-cold serum free media at concentration of 1ug/ml (each well will require 2ml so plan accordingly).
4) In sterile hood, add cold SFM/FcR blocker mix to cells at 1ml per well.
5) Transfer in sterile secondary container immediately to cold room, incubate for 30 minutes.
6) sterilize in 10% bleach 30 min, spray with 70% EtOH and wipe down.
7) Bring back to sterile hood and quickly add 20ug/ml fusion proteins to cells, and quickly take back to cold room for 60 more minutes.
8) For surface binding experiments, after 60 minutes immediately wash in cold PBS, surface strip, rinse with PBS and keep at 4 degrees.
9) Aspirate media and add warm N2a media and place in incubator for 30 minutes.
10) Wash 1x with cold PBS on ice.
11) surfacce stripping, incubate on ice on rocker for 4 min in 0.2M acetic acid 0.5M NaCl
12) wash 2 times cold PBS
13) Extract protein in RIPA buffer (protease inhibitor coctain, 2uM EDTA).
14) Perform WB analysis for detection of Fc-tag (species specific, HRP conjugated).

BIBLIOGRAPHY

1. Dent, E. W. & Gertler, F. B. Cytoskeletal dynamics and transport in growth cone motility and axon guidance. *Neuron* 40, 209–27 (2003).

2. Oyinbo, C. A. Secondary injury mechanisms in traumatic spinal cord injury: a nugget of this multiply cascade. *Acta neurobiologiae experimentalis* 71, 281–99 (2011).

3. Christopher and Dana Reeve Foundation *One Degree of Separation: Paralysis and Spinal Cord Injury in the United States*. (2009).at <http://www.christopherreeve.org/atf/cf/{3d83418f-b967-4c18-8ada-adc2e5355071}/8112REPTFINAL.PDF>

4. Bradke, F., Fawcett, J. W. & Spira, M. E. Assembly of a new growth cone after axotomy: the precursor to axon regeneration. *Nature reviews. Neuroscience* 13, 183–93 (2012).

5. Berry, M. Post-injury myelin-breakdown products inhibit axonal growth: an hypothesis to explain the failure of axonal regeneration in the mammalian central nervous system. *Bibl Anat* 1–11 (1982).at <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7138484>

6. Benfey, M. & Aguayo, A. J. Extensive elongation of axons from rat brain into peripheral nerve grafts. *Nature* 296, 150–152 (1982).

7. Yiu, G. & He, Z. Glial inhibition of CNS axon regeneration. *Nat Rev Neurosci* 7, 617–627 (2006).

8. Busch, S. A. & Silver, J. The role of extracellular matrix in CNS regeneration. *Current opinion in neurobiology* 17, 120–127 (2007).

9. Kuhn, T. B., Brown, M. D., Wilcox, C. L., Raper, J. A. & Bamburg, J. R. Myelin and collapsin-1 induce motor neuron growth cone collapse through different pathways: inhibition of collapse by opposing mutants of rac1. *J Neurosci* 19, 1965–1975 (1999).

10. Ng, W. P., Cartel, N., Roder, J., Roach, A. & Lozano, A. Human central nervous system myelin inhibits neurite outgrowth. *Brain Res* 720, 17–24 (1996).

11. Filbin, M. T. Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. *Nat Rev Neurosci* 4, 703–713 (2003).

12. Leskovar, a, Moriarty, L. J., Turek, J. J., Schoenlein, I. a & Borgens, R. B. The macrophage in acute neural injury: changes in cell numbers over time and levels of cytokine production in mammalian central and peripheral nervous systems. *The Journal of experimental biology* 203, 1783–95 (2000).

13. Campana, W. M. *et al.* The low-density lipoprotein receptor-related protein is a pro-survival receptor in Schwann cells: possible implications in peripheral nerve injury. *J Neurosci* 26, 11197–11207 (2006).

14. Joset, A., Dodd, D. A., Halegoua, S. & Schwab, M. E. Pincher-generated Nogo-A endosomes mediate growth cone collapse and retrograde signaling. *J Cell Biol* 188, 271–285 (2010).

15. Xie, F. & Zheng, B. White matter inhibitors in CNS axon regeneration failure. *Exp Neurol* 209, 302–312 (2008).

16. Chaudhry, N. & Filbin, M. T. Myelin-associated inhibitory signaling and strategies to overcome inhibition. *J Cereb Blood Flow Metab* 27, 1096–1107 (2007).

17. Caroni, P. & Schwab, M. E. Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. *J Cell Biol* 106, 1281–1288 (1988).

18. Spillmann, A. A., Bandtlow, C. E., Lottspeich, F., Keller, F. & Schwab, M. E. Identification and characterization of a bovine neurite growth inhibitor (bNI-220). *J Biol Chem* 273, 19283–19293 (1998).

19. Chen, M. S. *et al.* Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. *Nature* 403, 434–439 (2000).

20. Liebscher, T. *et al.* Nogo-A antibody improves regeneration and locomotion of spinal cord-injured rats. *Ann Neurol* 58, 706–719 (2005).

21. Prinjha, R. *et al.* Inhibitor of neurite outgrowth in humans. *Nature* 403, 383–384 (2000).

22. Karnezis, T. *et al.* The neurite outgrowth inhibitor Nogo A is involved in autoimmune-mediated demyelination. *Nat Neurosci* 7, 736–744 (2004).

23. Zheng, B. *et al.* Lack of enhanced spinal regeneration in Nogo-deficient mice. *Neuron* 38, 213–224 (2003).

24. Oertle, T. *et al.* Nogo-A inhibits neurite outgrowth and cell spreading with three discrete regions. *J Neurosci* 23, 5393–5406 (2003).

25. Magnusson, C., Libelius, R. & Tågerud, S. Nogo (Reticulon 4) expression in innervated and denervated mouse skeletal muscle. *Molecular and Cellular Neuroscience* 22, 298–307 (2003).

26. GrandPré, T., Nakamura, F., Vartanian, T. & Strittmatter, S. M. Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. *Nature* 403, 439–44 (2000).

27. Huber, A. B., Weinmann, O., Brosamle, C., Oertle, T. & Schwab, M. E. Patterns of Nogo mRNA and Protein Expression in the Developing and Adult Rat and After CNS Lesions. *J. Neurosci.* 22, 3553–3567 (2002).

28. Schwab, M. E. Functions of Nogo proteins and their receptors in the nervous system. *Nature reviews. Neuroscience* 11, 799–811 (2010).

29. Dodd, D. A. *et al.* Nogo-A, -B, and -C are found on the cell surface and interact together in many different cell types. *The Journal of biological chemistry* 280, 12494–502 (2005).

30. Trapp, B. D. Myelin-Associated Glycoprotein Location and Potential Functions. *Annals of the New York Academy of Sciences* 605, 29–43 (1990).

31. Minuk, J. & Braun, P. E. Differential intracellular sorting of the myelin-associated glycoprotein isoforms. *Journal of neuroscience research* 44, 411–20 (1996).

32. Trapp, B. D., Andrews, S. B., Cootauco, C. & Quarles, R. The myelin-associated glycoprotein is enriched in multivesicular bodies and periaxonal membranes of actively myelinating oligodendrocytes. *The Journal of cell biology* 109, 2417–26 (1989).

33. Trapp, B. D., Bernier, L., Andrews, S. B. & Colman, D. R. Cellular and subcellular distribution of 2’,3'-cyclic nucleotide 3'-phosphodiesterase and its mRNA in the rat central nervous system. *Journal of neurochemistry* 51, 859–68 (1988).

34. Li, C. *et al.* Myelination in the absence of myelin-associated glycoprotein. *Nature* 369, 747–50 (1994).

35. Montag, D. *et al.* Mice deficient for the glycoprotein show subtle abnormalities in myelin. *Neuron* 13, 229–246 (1994).

36. Fruttiger, M., Montag, D., Schachner, M. & Martini, R. Crucial Role for the Myelin-associated Glycoprotein in the Maintenance of Axon-Myelin Integrity. *European Journal of Neuroscience* 7, 511–515 (1995).

37. Cao, Z. *et al.* The inhibition site on myelin-associated glycoprotein is within Ig-domain 5 and is distinct from the sialic acid binding site. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 27, 9146–9154 (2007).

38. Tang, S. *et al.* Myelin-associated glycoprotein interacts with neurons via a sialic acid binding site at ARG118 and a distinct neurite inhibition site. *The Journal of cell biology* 138, 1355–1366 (1997).

39. Worter, V. *et al.* Inhibitory activity of myelin-associated glycoprotein on sensory neurons is largely independent of NgR1 and NgR2 and resides within Ig-Like domains 4 and 5. *PloS one* 4, e5218 (2009).

40. Kelm, S. *et al.* Sialoadhesin, myelin-associated glycoprotein and CD22 define a new family of sialic acid-dependent adhesion molecules of the immunoglobulin superfamily. *Current Biology* 4, 965–972 (1994).

41. Tang, S., Qiu, J., Nikulina, E. & Filbin, M. T. Soluble myelin-associated glycoprotein released from damaged white matter inhibits axonal regeneration. *Mol Cell Neurosci* 18, 259–269 (2001).

42. Mikol, D. D. & Stefansson, K. A phosphatidylinositol-linked peanut agglutinin-binding glycoprotein in central nervous system myelin and on oligodendrocytes. *The Journal of cell biology* 106, 1273–9 (1988).

43. Habib, A. A. *et al.* Expression of the oligodendrocyte-myelin glycoprotein by neurons in the mouse central nervous system. *Journal of neurochemistry* 70, 1704–11 (1998).

44. Mikol, D. D. *et al.* Structure and chromosomal localization of the gene for the oligodendrocyte-myelin glycoprotein. *The Journal of cell biology* 111, 2673–9 (1990).

45. Hughes, J. T. The Edwin Smith Surgical Papyrus: an analysis of the first case reports of spinal cord injuries. *Paraplegia* 26, 71–82 (1988).

46. Kozma, R., Sarner, S., Ahmed, S. & Lim, L. Rho family GTPases and neuronal growth cone remodelling: relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. *Mol Cell Biol* 17, 1201–1211 (1997).

47. Tan, E. Y., Law, J. W., Wang, C. H. & Lee, A. Y. Development of a cell transducible RhoA inhibitor TAT-C3 transferase and its encapsulation in biocompatible microspheres to promote survival and enhance regeneration of severed neurons. *Pharm Res* 24, 2297–2308 (2007).

48. Jeon, C. Y. *et al.* Control of neurite outgrowth by RhoA inactivation. *J Neurochem* (2011).doi:10.1111/j.1471-4159.2011.07564.x

49. Reid, T. *et al.* Rhotekin, a new putative target for Rho bearing homology to a serine/threonine kinase, PKN, and rhophilin in the rho-binding domain. *J Biol Chem* 271, 13556–13560 (1996).

50. Boato, F. *et al.* C3 peptide enhances recovery from spinal cord injury by improved regenerative growth of descending fiber tracts. *J Cell Sci* 123, 1652–1662 (2010).

51. Niederost, B., Oertle, T., Fritsche, J., McKinney, R. A. & Bandtlow, C. E. Nogo-A and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA and Rac1. *J Neurosci* 22, 10368–10376 (2002).

52. Fournier, A. E., GrandPre, T. & Strittmatter, S. M. Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. *Nature* 409, 341–346 (2001).

53. Mi, S., Sandrock, A. & Miller, R. H. LINGO-1 and its role in CNS repair. *Int J Biochem Cell Biol* 40, 1971–1978 (2008).

54. Ji, B. *et al.* LINGO-1 antagonist promotes functional recovery and axonal sprouting after spinal cord injury. *Mol Cell Neurosci* 33, 311–320 (2006).

55. Zampieri, N., Xu, C. F., Neubert, T. A. & Chao, M. V Cleavage of p75 neurotrophin receptor by alpha-secretase and gamma-secretase requires specific receptor domains. *J Biol Chem* 280, 14563–14571 (2005).

56. Kanning, K. C. *et al.* Proteolytic processing of the p75 neurotrophin receptor and two homologs generates C-terminal fragments with signaling capability. *J Neurosci* 23, 5425–5436 (2003).

57. Domeniconi, M. *et al.* MAG induces regulated intramembrane proteolysis of the p75 neurotrophin receptor to inhibit neurite outgrowth. *Neuron* 46, 849–855 (2005).

58. Gil, C., Cubi, R. & Aguilera, J. Shedding of the p75NTR neurotrophin receptor is modulated by lipid rafts. *FEBS Lett* 581, 1851–1858 (2007).

59. Bronfman, F. C., Tcherpakov, M., Jovin, T. M. & Fainzilber, M. Ligand-induced internalization of the p75 neurotrophin receptor: a slow route to the signaling endosome. *J Neurosci* 23, 3209–3220 (2003).

60. Jung, K. M. *et al.* Regulated intramembrane proteolysis of the p75 neurotrophin receptor modulates its association with the TrkA receptor. *J Biol Chem* 278, 42161–42169 (2003).

61. Ceni, C. & Barker, P. A. Getting RIP'd Stunts Your Growth. *Neuron* 46, 839–844 (2005).

62. Ahmed, Z. *et al.* TACE-induced cleavage of NgR and p75NTR in dorsal root ganglion cultures disinhibits outgrowth and promotes branching of neurites in the presence of inhibitory CNS myelin. *Faseb J* 20, 1939–1941 (2006).

63. Mi, S. Troy/Taj and its role in CNS axon regeneration. *Cytokine Growth Factor Rev* 19, 245–251 (2008).

64. Shao, Z. *et al.* TAJ/TROY, an orphan TNF receptor family member, binds Nogo-66 receptor 1 and regulates axonal regeneration. *Neuron* 45, 353–359 (2005).

65. Park, J. B. *et al.* A TNF receptor family member, TROY, is a coreceptor with Nogo receptor in mediating the inhibitory activity of myelin inhibitors. *Neuron* 45, 345–351 (2005).

66. Chivatakarn, O., Kaneko, S., He, Z., Tessier-Lavigne, M. & Giger, R. J. The Nogo-66 receptor NgR1 is required only for the acute growth cone-collapsing but not the chronic growth-inhibitory actions of myelin inhibitors. *J Neurosci* 27, 7117–7124 (2007).

67. Zheng, B. *et al.* Genetic deletion of the Nogo receptor does not reduce neurite inhibition in vitro or promote corticospinal tract regeneration in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 102, 1205–1210 (2005).

68. Vinson, M. *et al.* Myelin-associated glycoprotein interacts with ganglioside GT1b. A mechanism for neurite outgrowth inhibition. *J Biol Chem* 276, 20280–20285 (2001).

69. Mehta, N. R., Lopez, P. H., Vyas, A. A. & Schnaar, R. L. Gangliosides and Nogo receptors independently mediate myelin-associated glycoprotein inhibition of neurite outgrowth in different nerve cells. *J Biol Chem* 282, 27875–27886 (2007).

70. Vyas, A. A. *et al.* Gangliosides are functional nerve cell ligands for myelin-associated glycoprotein (MAG), an inhibitor of nerve regeneration. *Proceedings of the National Academy of Sciences of the United States of America* 99, 8412–8417 (2002).

71. Williams, G. *et al.* Ganglioside inhibition of neurite outgrowth requires Nogo receptor function: identification of interaction sites and development of novel antagonists. *J Biol Chem* 283, 16641–16652 (2008).

72. Atwal, J. K. *et al.* PirB is a functional receptor for myelin inhibitors of axonal regeneration. *Science* 322, 967–970 (2008).

73. Filbin, M. T. PirB, a second receptor for the myelin inhibitors of axonal regeneration Nogo66, MAG, and OMgp: implications for regeneration in vivo. *Neuron* 60, 740–742 (2008).

74. GrandPre, T. *et al.* Paired immunoglobulin-like receptor B knockout does not enhance axonal regeneration or locomotor recovery after spinal cord injury. *J Biol Chem* 286, 1876–1883 (2011).

75. Omoto, S., Ueno, M., Mochio, S., Takai, T. & Yamashita, T. Genetic deletion of paired immunoglobulin-like receptor B does not promote axonal plasticity or functional recovery after traumatic brain injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 30, 13045–13052 (2010).

76. Fehlings, M. G. & Bracken, M. B. Summary statement: the Sygen(GM-1 ganglioside) clinical trial in acute spinal cord injury. *Spine* 26, S99–100 (2001).

77. Fehlings, M. G. *et al.* A phase I/IIa clinical trial of a recombinant Rho protein antagonist in acute spinal cord injury. *Journal of neurotrauma* 28, 787–96 (2011).

78. McKerracher, L. & Guertin, P. Rho as a target to promote repair: translation to clinical studies with Cethrin. *Current pharmaceutical design* (2013).at <http://www.ncbi.nlm.nih.gov/pubmed/23360272>

79. Gonzenbach, R. R. *et al.* Delayed anti-nogo-a antibody application after spinal cord injury shows progressive loss of responsiveness. *Journal of neurotrauma* 29, 567–78 (2012).

80. Qu, Z., Sun, D. & Young, W. Lithium promotes neural precursor cell proliferation: evidence for the involvement of the non-canonical GSK-3β-NF-AT signaling. *Cell & bioscience* 1, 18 (2011).

81. Lu, P. *et al.* Long-distance growth and connectivity of neural stem cells after severe spinal cord injury. *Cell* 150, 1264–73 (2012).

82. Strickland, D. K., Gonias, S. L. & Argraves, W. S. Diverse roles for the LDL receptor family. *Trends Endocrinol Metab* 13, 66–74 (2002).

83. Lillis, A. P. *et al.* Murine low-density lipoprotein receptor-related protein 1 (LRP) is required for phagocytosis of targets bearing LRP ligands but is not required for C1q-triggered enhancement of phagocytosis. *J Immunol* 181, 364–373 (2008).

84. Gaultier, A. *et al.* Low-density lipoprotein receptor-related protein 1 is an essential receptor for myelin phagocytosis. *J Cell Sci* 122, 1155–1162 (2009).

85. Fernandez-Castaneda, A. *et al.* Identification of the LDL Receptor-Related Protein-1 Interactome in Central Nervous System myelin suggests a role in the clearance of necrotic cell debris. *J Biol Chem* (2012).doi:M112.384693 [pii] 10.1074/jbc.M112.384693

86. Wolf, B. B., Lopes, M. B., VandenBerg, S. R. & Gonias, S. L. Characterization and immunohistochemical localization of alpha 2-macroglobulin receptor (low-density lipoprotein receptor-related protein) in human brain. *Am J Pathol* 141, 37–42 (1992).

87. Bu, G., Maksymovitch, E. A., Nerbonne, J. M. & Schwartz, A. L. Expression and function of the low density lipoprotein receptor-related protein (LRP) in mammalian central neurons. *J Biol Chem* 269, 18521–18528 (1994).

88. Steuble, M. *et al.* Molecular characterization of a trafficking organelle: dissecting the axonal paths of calsyntenin-1 transport vesicles. *Proteomics* 10, 3775–3788 (2010).

89. Brown, M. D., Banker, G. A., Hussaini, I. M., Gonias, S. L. & VandenBerg, S. R. Low density lipoprotein receptor-related protein is expressed early and becomes restricted to a somatodendritic domain during neuronal differentiation in culture. *Brain Res* 747, 313–317 (1997).

90. Gaultier, A., Salicioni, A. M., Arandjelovic, S. & Gonias, S. L. Regulation of the composition of the extracellular matrix by low density lipoprotein receptor-related protein-1: activities based on regulation of mRNA expression. *J Biol Chem* 281, 7332–7340 (2006).

91. Hu, K., Wu, C., Mars, W. M. & Liu, Y. Tissue-type plasminogen activator promotes murine myofibroblast activation through LDL receptor-related protein 1-mediated integrin signaling. *J Clin Invest* 117, 3821–3832 (2007).

92. Salicioni, A. M., Gaultier, A., Brownlee, C., Cheezum, M. K. & Gonias, S. L. Low density lipoprotein receptor-related protein-1 promotes beta1 integrin maturation and transport to the cell surface. *J Biol Chem* 279, 10005–10012 (2004).

93. Gaultier, A., Hollister, M., Reynolds, I., Hsieh, E. H. & Gonias, S. L. LRP1 regulates remodeling of the extracellular matrix by fibroblasts. *Matrix biology : journal of the International Society for Matrix Biology* 29, 22–30 (2010).

94. Gaultier, A. *et al.* Regulation of tumor necrosis factor receptor-1 and the IKK-NF-kappaB pathway by LDL receptor-related protein explains the antiinflammatory activity of this receptor. *Blood* 111, 5316–5325 (2008).

95. Webb, D. J., Thomas, K. S. & Gonias, S. L. Plasminogen activator inhibitor 1 functions as a urokinase response modifier at the level of cell signaling and thereby promotes MCF-7 cell growth. *The Journal of cell biology* 152, 741–752 (2001).

96. Boucher, P., Gotthardt, M., Li, W. P., Anderson, R. G. & Herz, J. LRP: role in vascular wall integrity and protection from atherosclerosis. *Science* 300, 329–332 (2003).

97. Zilberberg, A., Yaniv, A. & Gazit, A. The low density lipoprotein receptor-1, LRP1, interacts with the human frizzled-1 (HFz1) and down-regulates the canonical Wnt signaling pathway. *The Journal of biological chemistry* 279, 17535–17542 (2004).

98. Shi, Y., Mantuano, E., Inoue, G., Campana, W. M. & Gonias, S. L. Ligand binding to LRP1 transactivates Trk receptors by a Src family kinase-dependent pathway. *Sci Signal* 2, ra18 (2009).

99. Parkyn, C. J. *et al.* LRP1 controls biosynthetic and endocytic trafficking of neuronal prion protein. *J Cell Sci* 121, 773–783 (2008).

100. Ulery, P. G. *et al.* Modulation of beta-amyloid precursor protein processing by the low density lipoprotein receptor-related protein (LRP). Evidence that LRP contributes to the pathogenesis of Alzheimer's disease. *J Biol Chem* 275, 7410–7415 (2000).

101. Nathan, B. P. *et al.* Apolipoprotein E4 inhibits, and apolipoprotein E3 promotes neurite outgrowth in cultured adult mouse cortical neurons through the low-density lipoprotein receptor-related protein. *Brain Res* 928, 96–105 (2002).

102. May, P. *et al.* Neuronal LRP1 functionally associates with postsynaptic proteins and is required for normal motor function in mice. *Mol Cell Biol* 24, 8872–8883 (2004).

103. Donahue, J. E. *et al.* RAGE, LRP-1, and amyloid-beta protein in Alzheimer's disease. *Acta Neuropathol* 112, 405–415 (2006).

104. Rushworth, J. V, Griffiths, H. H., Watt, N. T. & Hooper, N. M. Prion protein-mediated toxicity of amyloid-β oligomers requires lipid rafts and the transmembrane LRP1. *The Journal of biological chemistry* (2013).doi:10.1074/jbc.M112.400358

105. Schwab, M. E., Kapfhammer, J. P. & Bandtlow, C. E. Inhibitors of neurite growth. *Annual review of neuroscience* 16, 565–595 (1993).

106. Fawcett, J. Molecular control of brain plasticity and repair. *Progress in brain research* 175, 501–509 (2009).

107. Robak, L. A. *et al.* Molecular basis of the interactions of the Nogo-66 receptor and its homolog NgR2 with myelin-associated glycoprotein: development of NgROMNI-Fc, a novel antagonist of CNS myelin inhibition. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29, 5768–5783 (2009).

108. Kim, J. E., Liu, B. P., Park, J. H. & Strittmatter, S. M. Nogo-66 receptor prevents raphespinal and rubrospinal axon regeneration and limits functional recovery from spinal cord injury. *Neuron* 44, 439–451 (2004).

109. Yamashita, T. & Tohyama, M. The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI. *Nat Neurosci* 6, 461–467 (2003).

110. Venkatesh, K., Chivatakarn, O., Sheu, S. S. & Giger, R. J. Molecular dissection of the myelin-associated glycoprotein receptor complex reveals cell type-specific mechanisms for neurite outgrowth inhibition. *The Journal of cell biology* 177, 393–399 (2007).

111. Cai, D., Shen, Y., De Bellard, M., Tang, S. & Filbin, M. T. Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism. *Neuron* 22, 89–101 (1999).

112. Qiu, Z., Strickland, D. K., Hyman, B. T. & Rebeck, G. W. alpha 2-Macroglobulin exposure reduces calcium responses to N-methyl-D-aspartate via low density lipoprotein receptor-related protein in cultured hippocampal neurons. *The Journal of biological chemistry* 277, 14458–14466 (2002).

113. Hayashi, H., Campenot, R. B., Vance, D. E. & Vance, J. E. Apolipoprotein E-containing lipoproteins protect neurons from apoptosis via a signaling pathway involving low-density lipoprotein receptor-related protein-1. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 27, 1933–1941 (2007).

114. Qiu, Z., Hyman, B. T. & Rebeck, G. W. Apolipoprotein E receptors mediate neurite outgrowth through activation of p44/42 mitogen-activated protein kinase in primary neurons. *The Journal of biological chemistry* 279, 34948–34956 (2004).

115. Mantuano, E. *et al.* The hemopexin domain of matrix metalloproteinase-9 activates cell signaling and promotes migration of schwann cells by binding to low-density lipoprotein receptor-related protein. *J Neurosci* 28, 11571–11582 (2008).

116. Fuentealba, R. A., Liu, Q., Kanekiyo, T., Zhang, J. & Bu, G. Low density lipoprotein receptor-related protein 1 promotes anti-apoptotic signaling in neurons by activating Akt survival pathway. *The Journal of biological chemistry* 284, 34045–34053 (2009).

117. Dickendesher, T. L. *et al.* NgR1 and NgR3 are receptors for chondroitin sulfate proteoglycans. *Nat Neurosci* advance on, (2012).

118. Gorovoy, M., Gaultier, A., Campana, W. M., Firestein, G. S. & Gonias, S. L. Inflammatory mediators promote production of shed LRP1/CD91, which regulates cell signaling and cytokine expression by macrophages. *J Leukoc Biol* 88, 769–778 (2010).

119. Wight, P. A. & Dobretsova, A. The first intron of the myelin proteolipid protein gene confers cell type-specific expression by a transcriptional repression mechanism in non-expressing cell types. *Gene* 201, 111–117 (1997).

120. Norton, W. T. & Poduslo, S. E. Myelination in rat brain: method of myelin isolation. *Journal of neurochemistry* 21, 749–757 (1973).

121. Gaultier, A. *et al.* LDL Receptor-related Protein 1 Regulates the Abundance of Diverse Cell-signaling Proteins in the Plasma Membrane Proteome. *J Proteome Res* (2010).doi:10.1021/pr1008288

122. Neels, J. G. *et al.* The second and fourth cluster of class A cysteine-rich repeats of the low density lipoprotein receptor-related protein share ligand-binding properties. *The Journal of biological chemistry* 274, 31305–31311 (1999).

123. Willnow, T. E., Orth, K. & Herz, J. Molecular dissection of ligand binding sites on the low density lipoprotein receptor-related protein. *The Journal of biological chemistry* 269, 15827–15832 (1994).

124. Goh, E. L. *et al.* beta1-integrin mediates myelin-associated glycoprotein signaling in neuronal growth cones. *Molecular brain* 1, 10 (2008).

125. Fujita, Y., Takashima, R., Endo, S., Takai, T. & Yamashita, T. The p75 receptor mediates axon growth inhibition through an association with PIR-B. *Cell Death Dis* 2, e198 (2011).

126. Wills, Z. P. *et al.* The nogo receptor family restricts synapse number in the developing hippocampus. *Neuron* 73, 466–81 (2012).

127. Mantuano, E., Mukandala, G., Li, X., Campana, W. M. & Gonias, S. L. Molecular dissection of the human alpha2-macroglobulin subunit reveals domains with antagonistic activities in cell signaling. *J Biol Chem* 283, 19904–19911 (2008).

128. Rebeck, G. W. Nontraditional signaling mechanisms of lipoprotein receptors. *Science signaling* 2, pe28 (2009).

129. Lopes, M. B., Bogaev, C. A., Gonias, S. L. & VandenBerg, S. R. Expression of alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein is increased in reactive and neoplastic glial cells. *FEBS letters* 338, 301–305 (1994).

130. Geisler, F. H., Dorsey, F. C. & Coleman, W. P. The New England Journal of Medicine Downloaded from nejm.org at UC SHARED JOURNAL COLLECTION on May 10, 2013. For personal use only. No other uses without permission. Copyright © 1991 Massachusetts Medical Society. All rights reserved. *New England Journal of Medicine* 324, 1829–39 (1991).

131. Collins, B. E. *et al.* Sialic acid specificity of myelin-associated glycoprotein binding. *J Biol Chem* 272, 1248–1255 (1997).

132. Venkatesh, K. *et al.* The Nogo-66 receptor homolog NgR2 is a sialic acid-dependent receptor selective for myelin-associated glycoprotein. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 25, 808–822 (2005).

133. Stiles, T. L. *et al.* 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. *Journal of cell science* 126, 209–20 (2013).

134. Takayama, Y., May, P., Anderson, R. G. & Herz, J. Low density lipoprotein receptor-related protein 1 (LRP1) controls endocytosis and c-CBL-mediated ubiquitination of the platelet-derived growth factor receptor beta (PDGFR beta). *The Journal of biological chemistry* 280, 18504–18510 (2005).

135. Winckler, B. & Yap, C. Endocytosis and Endosomes at the Crossroads of Regulating Trafficking of Axon Outgrowth-Modifying Receptors. *Traffic* 12, 1099–1108 (2011).

136. Oberdoerster, J. Isolation of Cerebellar Granule Cells from Neonatal Rats. *Current Protocols in Toxicology* (2001).

137. Domeniconi, M. *et al.* Myelin-associated glycoprotein interacts with the Nogo66 receptor to inhibit neurite outgrowth. *Neuron* 35, 283–290 (2002).

138. Tang, S. *et al.* Soluble myelin-associated glycoprotein (MAG) found in vivo inhibits axonal regeneration. *Mol Cell Neurosci* 9, 333–346 (1997).

139. Deinhardt, K., Reversi, A., Berninghausen, O., Hopkins, C. R. & Schiavo, G. Neurotrophins Redirect p75NTR from a clathrin-independent to a clathrin-dependent endocytic pathway coupled to axonal transport. *Traffic* 8, 1736–1749 (2007).

140. Perrot, G. *et al.* LRP-1--CD44, a new cell surface complex regulating tumor cell adhesion. *Molecular and cellular biology* 32, 3293–307 (2012).

141. Muratoglu, S. C., Mikhailenko, I., Newton, C., Migliorini, M. & Strickland, D. K. Low density lipoprotein receptor-related protein 1 (LRP1) forms a signaling complex with platelet-derived growth factor receptor-beta in endosomes and regulates activation of the MAPK pathway. *The Journal of biological chemistry* 285, 14308–14317 (2010).

142. Galliano, M.-F. *et al.* Binding of alpha2ML1 to the low density lipoprotein receptor-related protein 1 (LRP1) reveals a new role for LRP1 in the human epidermis. *PloS one* 3, e2729 (2008).

143. Liu, Q. *et al.* Neuronal LRP1 knockout in adult mice leads to impaired brain lipid metabolism and progressive, age-dependent synapse loss and neurodegeneration. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 30, 17068–17078 (2010).

144. Dubreuil, C. I., Winton, M. J. & McKerracher, L. Rho activation patterns after spinal cord injury and the role of activated Rho in apoptosis in the central nervous system. *J Cell Biol* 162, 233–243 (2003).

145. Yamashita, T., Higuchi, H. & Tohyama, M. The p75 receptor transduces the signal from myelin-associated glycoprotein to Rho. *J Cell Biol* 157, 565–570 (2002).

146. Mukhopadhyay, G., Doherty, P., Walsh, F. S., Crocker, P. R. & Filbin, M. T. A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron* 13, 757–767 (1994).

147. Rohlmann, A., Gotthardt, M., Hammer, R. E. & Herz, J. Inducible inactivation of hepatic LRP gene by cre-mediated recombination confirms role of LRP in clearance of chylomicron remnants. *The Journal of clinical investigation* 101, 689–95 (1998).

148. Khursigara, G., Orlinick, J. R. & Chao, M. V Association of the p75 neurotrophin receptor with TRAF6. *J Biol Chem* 274, 2597–2600 (1999).

149. Winters, J. J. *et al.* Congenital CNS hypomyelination in the Fig4 null mouse is rescued by neuronal expression of the PI(3,5)P(2) phosphatase Fig4. *J Neurosci* 31, 17736–17751 (2011).

150. Fu, Q., Hue, J. & Li, S. Nonsteroidal anti-inflammatory drugs promote axon regeneration via RhoA inhibition. *J Neurosci* 27, 4154–4164 (2007).

151. Liu, Q. *et al.* LRP1 shedding in human brain: roles of ADAM10 and ADAM17. *Molecular neurodegeneration* 4, 17 (2009).

152. Quinn, K. A., Pye, V. J., Dai, Y. P., Chesterman, C. N. & Owensby, D. A. Characterization of the soluble form of the low density lipoprotein receptor-related protein (LRP). *Experimental cell research* 251, 433–441 (1999).

153. Madura, T. *et al.* Activation of Rho in the injured axons following spinal cord injury. *EMBO Rep* 5, 412–417 (2004).

154. Kotter, M. R., Li, W.-W., Zhao, C. & Franklin, R. J. M. Myelin impairs CNS remyelination by inhibiting oligodendrocyte precursor cell differentiation. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 26, 328–32 (2006).

155. Dupuis, L. *et al.* Nogo receptor antagonizes p75NTR-dependent motor neuron death. *Proc Natl Acad Sci U S A* 105, 740–745 (2008).

156. Fujitani, M. *et al.* Binding of soluble myelin-associated glycoprotein to specific gangliosides induces the association of p75NTR to lipid rafts and signal transduction. *J Neurochem* 94, 15–21 (2005).

157. Wang, K. C., Kim, J. A., Sivasankaran, R., Segal, R. & He, Z. P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. *Nature* 420, 74–78 (2002).

158. Pan, W. *et al.* Efficient transfer of receptor-associated protein (RAP) across the blood-brain barrier. *Journal of cell science* 117, 5071–8 (2004).