

Central Nervous System Regeneration Inhibitors and their Intracellular Substrates

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Received: 14 July 2009 / Accepted: 27 August 2009 / Published online: 19 September 2009
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Abstract Injury to the central nervous system (CNS) initiates a cascade of responses that is inhibitory to the regeneration of neurons and full recovery. At the site of injury, glial cells conspire with an inhibitory biochemical milieu to construct both physical and chemical barriers that prevent the outgrowth of axons to or beyond the lesion site. These inhibitors include factors derived from myelin, repulsive guidance cues, and chondroitin sulfate proteoglycans. Each bind receptors on the axon surface to initiating intracellular signaling cascades that ultimately result in cytoskeletal reorganization and growth cone collapse. Here, we present an overview of the molecules, receptors, and signaling pathways that inhibit CNS regeneration, with a particular focus on the intracellular signaling machinery that may function as convergent targets for multiple inhibitory ligands.

Keywords Myelin-associated inhibitors · Nerve regeneration · Glial scar · Chondroitin sulfate proteoglycans

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Following injury to the adult mammalian central nervous system (CNS), axons do not spontaneously regenerate, and functional connections between neurons are not re-established. This failure to regenerate can be partly attributed to an unfavorable CNS environment [1]. The glial scar is a physical barrier that consists primarily of reactive astrocytes, which plays an important role in blocking axonal regeneration past the site of injury [2]. In addition, factors from the glial scar and from myelin debris create an inhibitory biochemical barrier that actively signals to the surface of regenerating axons to induce growth cone collapse and inhibit axon extension [3]. Several proteins that inhibit neurite outgrowth have been identified, including myelin-associated glycoprotein (MAG), nogo-A, oligodendrocyte-myelin glycoprotein (OMgp), netrin-1, semaphorin4D, ephrinB3, and chondroitin sulfate proteoglycans (CSPGs). Several of these proteins are upregulated by cells that form the glial scar [4], and the remainder are expressed by oligodendrocytes, which form the insulating myelin membrane. Although these inhibitory ligands engage multiple distinct receptors on the neuronal cell surface, they signal through overlapping pathways. This review will overview the inhibitory ligands and receptors through which they signal (Fig. 1), and then will focus on recent advances in our understanding of the intracellular signaling machinery activated in response to inhibitors of CNS regeneration.

Ligands Associated with CNS Injury

The Glial Scar and CSPGs

Damage to the CNS initiates a cascade of events that leads to the formation of a glial scar. Following injury, this scar beneficially stabilizes fragile CNS tissue, protects surrounding

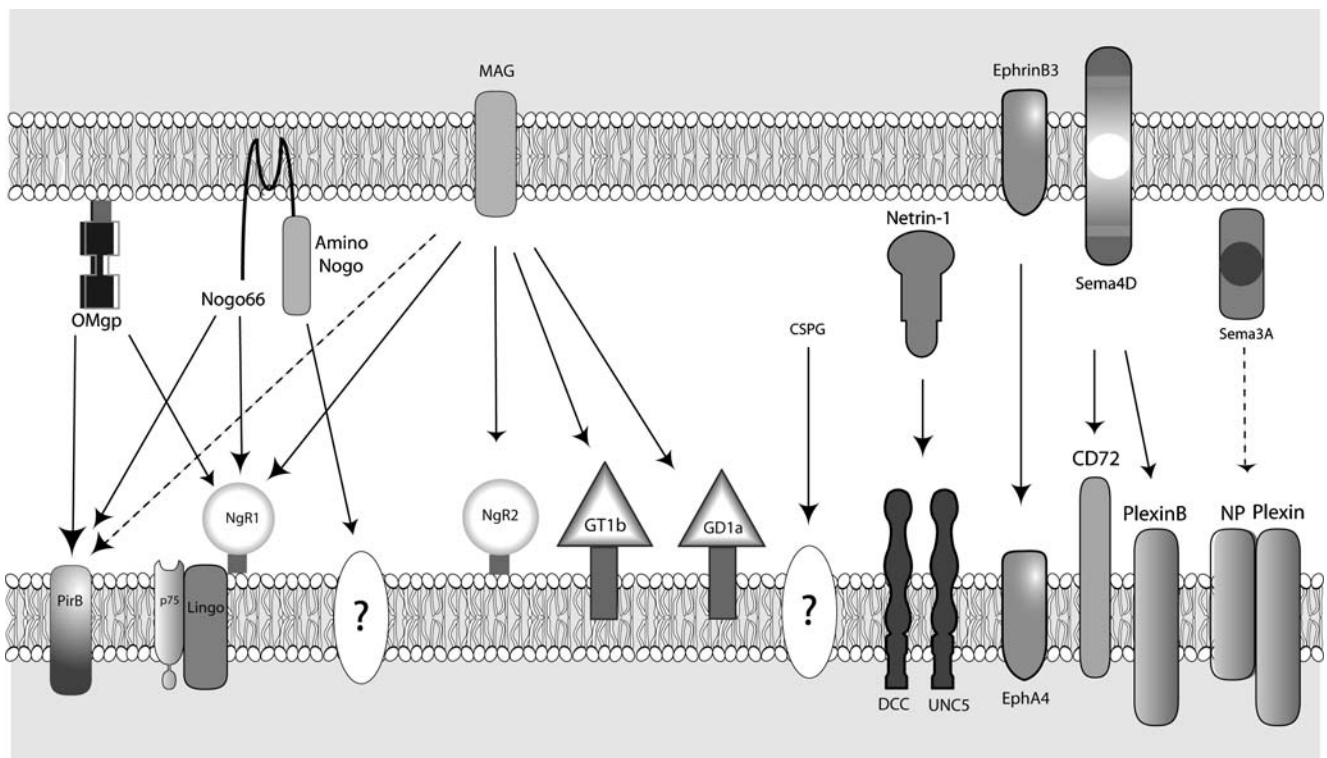


Fig. 1 The inhibitory ligands of neural regeneration and their receptors. Multiple inhibitory ligands prevent axonal repair in the central nervous system. Significant receptor redundancy exists as several ligands bind to multiple receptors; however, individual ligands can also bind to multiple receptors. This is highlighted by myelin-associated glycoprotein (MAG), which has been shown to interact with the gangliosides (GT1b and GD1a), the Nogo receptors (NgR1 and 2),

and PirB. Also shown is the binding of the various Noglilgands (Nogo66 and Amino Nogo, top left) with the tripartite Nogo receptor complex (NgR1, LINGO-1, and p75), PirB, and a third yet-to-be-identified receptor. Oligodendrocyte-myelin glycoprotein (OMgp) has similar binding partners and is shown in the top left. Netrin-1, EphrinB3, Sema4D, and Sema3A are shown to the right with their respective receptors

healthy tissue from inflammatory damage, and aids in the repair of the blood–brain barrier [3, 5]. However, the scar is also a critical barrier to regeneration; it forms a physical and biochemical barrier preventing the elongation of axons into damaged tissue [6]. The glial scar contains astrocytes, oligodendrocyte precursor cells, microglia, macrophages, and an extracellular matrix of secreted basal membrane components [2, 6].

Astrocytes undergo reactive gliosis and are the major contributor to the inhibitory nature of the scar [7]. Following injury, these astrocytes form tight junctions to create a physical barrier between injured and healthy CNS tissue. In addition, they secrete chondroitin sulfate proteoglycans (CSPGs), a class of extracellular matrix molecules comprising a protein core and numerous variable glycosaminoglycan (GAG) side chains [8]. The family of CSPGs includes more than a half dozen members, including aggrecan, brevican, neurocan, versican, NG2, and phosphacan, that have been demonstrated to be upregulated following certain types of CNS injury [9]. Collectively, they are potent inhibitors of neurite outgrowth in vitro from both adult and embryonic neurons, but the relative contribution of each member has not been fully character-

ized [8]. Interestingly, the neurite outgrowth inhibitory activity of CSPGs has been associated with both the core protein [10, 11] and the chondroitin sulfated GAG side chains [11–13]. Treatment with chondroitinase ABC to remove these side chains has demonstrated some promise in promoting plasticity and axon regeneration following CNS injury [9, 14].

Myelin-Associated Inhibitors

A number of inhibitors of neurite outgrowth are associated with myelin, including MAG, Nogo-A, and OMgp. MAG, also known as sialic acid binding Ig-like lectin (SIGLEC-4), is a glycoprotein member of the immunoglobulin (Ig)-like superfamily [15]. It is a 100-kDa type I transmembrane glycoprotein selectively localized to the periaxonal membrane of both peripheral nervous system Schwann cells and CNS oligodendrocytes. Its extracellular domain consists of five sequence-related glycosylated Ig-like domains, while the cytoplasmic domain is subject to developmentally regulated alternative splicing [16, 17]. MAG expression correlates with the onset of myelination and continues at high levels into adulthood; it may play multiple roles

during the development and maintenance of myelinated axons [18]. More than a decade ago, two groups independently identified MAG as a potent inhibitor of neurite outgrowth in vitro [19, 20]. MAG not only inhibits neurite outgrowth of various types of mature neurons but also promotes axon outgrowth at early developmental stages (discussed below) [20]. In MAG knockout mice, CNS compact myelin formation is relatively normal but it is significantly delayed and misguided in a small proportion of cells [21–23]. Furthermore, in aging MAG knockout mice, alterations in periaxonal oligodendrocyte processes are consistent with oligodendroglial pathology [24]. While myelin from MAG knockout mice is a less inhibitory substrate for neurite outgrowth of neonatal cerebellar or P5 DRG neurons in vitro [25], regeneration of retinal ganglion cells or corticospinal tract neurons is not enhanced in MAG knockout mice following optic nerve or spinal cord lesions, respectively [26]. Therefore, MAG does not account for a significant proportion of in vivo myelin inhibition or there is a non-additive redundancy between different myelin-associated inhibitors.

The most widely studied myelin-associated inhibitor is Nogo-A. Nogo-A was initially described as biochemical fractions of myelin approximately 250 and 35 kDa (NI-35/250) in size that were non-permissive substrates for neurite outgrowth [27]. Subsequently, Schwab and colleagues generated a function-blocking antibody (IN-1) against NI-35/250 that could alleviate the inhibition of neurite outgrowth by CNS white matter both in vitro [28] and in vivo [29–31]. More than a decade following the discovery of NI-35/250, NI-250 was identified as Nogo-A, also known as reticulon 4A [32–34]. Nogo-A is encoded by the *nogo/RTN-4* gene. This locus also generates two other isoforms, Nogo-B and Nogo-C, by alternative splicing [35]. The Nogo-A transcript is expressed in adult CNS tissue, including oligodendrocytes and neurons in the brain and spinal cord [33, 36, 37]. Nogo-A protein is predominantly expressed by oligodendrocytes and localizes to the outer and inner myelin loops [35, 37]. Nogo-B and Nogo-C are also expressed by neurons although Nogo-C appears to be the predominate species on northern blots [33, 37]. Given the neuronal pattern of Nogo-A, the protein has been proposed to play a role in neuronal development and maturity beyond its role as a myelin-associated inhibitor.

The three Nogo splice variants contain a common C-terminal domain with an inhibitory 66 amino acid luminal/extracellular domain found between two hydrophobic regions. This domain is termed Nogo-66 [33]. In addition, the amino terminus of Nogo-A is also inhibitory [32, 38]. Of the three Nogo splice variants, Nogo-A is considered to be the major player in myelin inhibition because it is expressed in oligodendrocytes, the IN-1 antibody recog-

nizes Nogo-A, and the inhibitory activity residing in the amino terminal region is unique to Nogo-A [38]. However, Nogo-A is a reticulon, and the majority of the protein is found associated with the endoplasmic reticulum intracellularly. The potential functions of Nogo-A and other isoforms beyond their role at myelin membranes are not clear. Rapoport and colleagues have proposed that Nogo-A is critical for the formation of ER tubules. However, the restricted expression pattern and mild phenotype of several different strains of Nogo-A mutant mice are inconsistent with the protein such a central role in ER morphology [39].

Three groups independently generated mutant mice in which the *rtn4* locus was perturbed. [40–42]. The inhibitory activity of myelin prepared from all three strains of mutant mice is significantly diminished as demonstrated with in vitro neurite outgrowth assays. Two of the three strains [41, 42] demonstrated some limited corticospinal tract (CST) regeneration following a dorsal hemisection injury to the spinal cord but no improvement was detectable in the third line [40]. Follow-up studies have revealed that strain background, age, and the nature of the Nogo mutant allele all influence the regenerative capacity of the corticospinal tract [43, 44]. Instructively, when the two strains of Nogo-A mutant with the greatest and least anatomical plasticity and functional recovery following spinal cord injury were examined following a more precise lesion to the corticospinal tract, intact uninjured CST exhibited greater local sprouting in both lines [45]. Thus, each of these mutant Nogo strains possess some degree of enhanced outgrowth or sprouting following CNS injury. Collectively, these studies have largely dispelled concerns regarding artifactual tract tracing in the Nogo regeneration studies but the specific differences between the strains that account for the different initial reports of recovery, or lack thereof, after spinal cord injury have not been fully elucidated [46]. More recently, a deletion mutant line that is null for all Nogo isoforms was generated [47]. CST regeneration is not significantly enhanced in this strain following experimental spinal cord injury; however, the targeting vectors used to generate the strain are identical to those used for the previously generated Nogo-C and Nogo-A, B targeted mutants that showed no regeneration [40].

Oligodendrocyte-myelin glycoprotein (OMgp) is another myelin-associated inhibitor [48, 49]. OMgp is a membrane glycoprotein anchored to the plasma membrane through a glycosylphosphatidylinositol (GPI) lipid moiety [48, 50]. This 433 amino acid protein contains a series of leucine-rich repeats (LRRs). LRRs are present in a spectrum of proteins, but these domains are generally considered to provide an interface for interacting with other proteins [51]. Expression of OMgp correlates with the onset of myelination but can also be detected on pre-myelinated axons as well as on the surface of oligodendrocytes [50, 52]. Mikol

and colleagues [50] demonstrated that OMgp does not execute a direct role in myelination itself but mediates the adhesion of membranes at the nodes of Ranvier between either two oligodendrocytes or between an oligodendrocyte and axon. Further, OMgp plays a role in inhibition of axonal sprouting at the nodes of Ranvier [53]. Similar to Nogo-A, OMgp appears to constrain neurite outgrowth and can induce growth cone collapse [48, 50]. Myelin from OMgp knockout mice is less inhibitory for neurite outgrowth and there are improvements in the numbers of ascending sensory axons and 5-HT-positive descending axons following spinal cord injury in OMgp knockout mice; however, this effect is dependent on the strain background [54].

Other Myelin-Associated Inhibitory Ligands: Netrins, Semaphorins, and Ephrins

Netrin-1 is a secreted protein that acts as a long-range chemotropic axon guidance cue during neuronal development [55]. Netrins function both as a chemoattractant and chemorepellent, depending on the types of receptors expressed by the responding axon [56]. Netrin was first described as a chemoattractant expressed by the floor plate of the developing neural tube [57]. Subsequent studies have demonstrated that netrin-1 is also expressed by neurons and oligodendrocytes in the adult brain and spinal cord [58]. Netrin-1 is associated with the extracellular matrix in the periaxonal myelin allowing for close contact between axon and oligodendrocyte [58]. Consistent with this expression pattern, Low and colleagues reported an inhibitory role of netrin-1 on axonal growth [59] and demonstrated that neutralization of netrin-1 in myelin with soluble Unc5 receptor increases neurite outgrowth from spinal neurons. Furthermore, following an *in vivo* dorsal column lesion, axonal penetration into a graft of netrin-1-secreting fibroblasts is significantly diminished compared to a control graft. Thus, netrin-1 is another category of inhibitor to CNS regeneration associated with myelin.

A second class of axon guidance cues implicated in inhibiting axon regeneration is the semaphorins. Semaphorins are potent mediators of growth cone collapse and axon repulsion [60]. Subsequent studies have demonstrated that semaphorins are upregulated following CNS injury and play roles in limiting regeneration [61, 62]. Expression of both secreted and transmembrane semaphorins have been reported within the glial scar and in preparations of myelin membranes. Sema4D, also called CD100, is a transmembrane semaphorin expressed by myelinating oligodendrocytes and is upregulated following CNS injury [63, 64]. In a stripe assay, Sema4D repels post-natal sensory and cerebellar granule cells. Therefore, it may contribute to failed regeneration *in vivo*. Sema3A also contributes to

axonal inhibition. Increased expression of Sema3A appears mainly within scar tissue following damage to the spinal cord [65, 66]. A selective Sema3A inhibitor created from fungal extract, SM-216289, enhances repair and functional recovery in a spinal cord transection model [67].

Members of the ephrin family of guidance molecules have also been implicated as inhibitors of CNS regeneration [68]. Ephrin-B3 is expressed by post-natal myelinating oligodendrocytes and is dramatically enriched in spinal cord white matter. Purified recombinant Ephrin-B3 inhibits growth of EphA4-positive cortical neurons and myelin from ephrin-B3 knockout mice is significantly less inhibitory than myelin from wild-type mice [69].

Receptors: NgRs, PirB, and Gangliosides

The relative contribution of individual receptor components to inhibitory signaling in response to CSPGs and repulsive guidance cues is not well defined. Receptor components for CSPGs have yet to be identified. Netrin-1, semaphorin4D, and ephrinB3 are likely to signal through their respective receptors, DCC/UNC5 [59], CD72 [64], and EphA4 [69], but the specific roles of these receptors in regeneration *in vivo* have yet to be fully addressed. Receptor partners for the classic MAIs have been more widely studied and these include members of the Nogo receptor (NgR) family, Paired immunoglobulin-like receptor B (PirB) as well as gangliosides and integrins.

NgR

Despite their differing structural properties, MAG, Nogo-66, and OMgp all bind to a common receptor, Nogo-66 receptor 1 (NgR1) [70–72]. Two similar proteins are also present in the mammalian genome, NgR2 and NgR3 [73]. NgR3 fails to bind to myelin-associated outgrowth inhibitors but NgR2 is a high affinity receptor for MAG [74]. NgR family members are attached to the plasma membrane by GPI anchor and lack an intracellular signaling domain. NgR1 has been proposed to function together with p75 neurotrophin receptor (p75^{NTR}) and LRR and Ig domain-containing *Nogo* receptor-interacting protein-1 (LINGO-1) to form a tripartite Nogo receptor complex [70, 75, 76]. The expression of p75^{NTR} decreases postnatally, and TROY (tumor necrosis factor receptor superfamily, member 19) has been suggested as a molecular substitute for p75^{NTR} in some adult axons [77, 78] (Fig. 1). Blockade of NgR1 function with the soluble extracellular domain of the protein (NgREcto) or with a small peptide antagonist (NEP1-40) enhances neurite outgrowth on inhibitory substrates *in vitro* and axon regeneration *in vivo* [79–84]. Neurons from NgR1 knockout mice are resistant to myelin-

dependent growth cone collapse. Following spinal cord injury, regeneration of the raphespinal and rubrospinal axon regeneration is enhanced in NgR1 knockout mice; however, corticospinal tract regeneration remains poor [85, 86]. Subsequent studies have suggested that the physiologic role of NgR1 may be in sensitizing neurons to more acute myelin responses [87]. NgR1 knockout mice have an extended critical period for ocular dominance plasticity [88], demonstrate mild differences in dendritic spine morphology and activity-dependent synaptic strength under specific conditions [89], and have enhanced plasticity in a variety of injury models [45].

PirB

More recently, a second receptor for Nogo-66, MAG, and OMgp was identified as Paired immunoglobulin-like receptor B (PirB) [90]. PirB is a major histocompatibility complex class I (MHC1) receptor that is expressed in subsets of neurons within the brain. Ocular dominance plasticity is enhanced in mice carrying a loss of function PirB allele suggesting that it may function to stabilize neural circuits [91]. Its identification as a receptor mediating myelin-associated inhibition is particularly intriguing in light of the recent findings implicating NgR1 in synaptic plasticity [45, 89]. Cerebellar granule neurons from the PirB mutant mice or wild-type neurons treated with a PirB function-blocking antibody are less inhibited by Nogo-66 or total myelin in an *in vitro* neurite outgrowth assay. When the PirB function-blocking antibody was used to treat NgR1-null neurons, myelin inhibition was completely relieved. The *in vivo* contribution of PirB to regeneration has yet to be reported.

Gangliosides and NgR2

MAG has several additional binding partners that have been implicated in its neurite outgrowth inhibitory activity. It binds the gangliosides GT1b and GD1a [92] and the NgR1 homolog NgR2. NgR2 acts selectively to mediate MAG inhibitory responses [74]. Gangliosides play an essential role in mediating MAG inhibition [93, 94]; however, the extent of this role appears to depend on neuronal cell type [95, 96]. The signaling mechanism utilized by these receptors remains unclear. It is known that GT1b interacts with NgR1 [97] and p75^{NTR} [98], and that both NgR1 and NgR2 bind MAG in a sialic acid-dependent manner [74]. Gangliosides may be components of the Nogo receptor complex. However, studies using biochemical [95] or genetic [96] methods to ablate NgR1 or p75^{NTR} receptors indicate that gangliosides are able to mediate MAG inhibition even in the absence of the Nogo receptor complex in some types of neurons. Thus, ganglio-

sides may bind yet another receptor to mediate MAG inhibition.

Integrin receptors also appear to play a role in signaling by myelin-associated inhibitors. Amino-Nogo inhibits cell adhesion and axon outgrowth as a function of extracellular matrix composition. It reduces focal adhesion kinase (FAK) activity in response to fibronectin and its inhibition can be partially overcome by β 1-integrin activation [99]. MAG also appears to signal through beta1-integrin and FAK to induce growth cone turning [100].

Intracellular Signaling Events Inhibitory to CNS Regeneration

Intracellularly, inhibitors of axonal regeneration signal through Ca^{2+} -dependent activation of protein kinase C (PKC) and epidermal growth factor receptor (EGFR), and activation of RhoA GTPase and its downstream effectors to affect cytoskeletal rearrangements. Further, loss of PTEN (phosphatase and tensin homolog) promotes robust retinal ganglion cell survival and regeneration in an optic nerve injury model. This likely occurs by relieving inhibition of mTOR (mammalian target of rapamycin)-dependent protein synthesis. Responses to myelin-associated inhibitors are further subject to modulation by cAMP-dependent signaling. These signals act in a concerted fashion to induce growth cone collapse and/or inhibition of axon extension during regeneration.

Ca^{2+} -Dependent Activation of PKC and EGFR

Calcium-dependent signaling in response to MAIs was first noted by Kater and colleagues [101]. Follow-up studies suggest that MAG induces a rapid rise in intracellular Ca^{2+} which is dependent on the co-expression and interaction of p75^{NTR} and NgR1 [75] and that a reduction of extracellular Ca^{2+} abolishes growth cone turning in response to a MAG gradient [102]. MAG-induced calcium elevation in neurons is blocked by inhibitors of phospholipase C (PLC), the heterotrimeric GTP-binding protein G_i , and the IP_3 receptor [103]. The conventional PKCs α , β_1 , and β_2 are activated in a PLC-dependent manner by MAG. Neurite outgrowth inhibition and growth cone collapse in response to MAG, Nogo-A, and myelin substrates are mitigated by PKC inhibitors [103]. Similar results were obtained by Sivasankaran et al., who showed that both myelin and CSPGs activate the conventional PKCs α and β , as well as that overexpression of dominant-negative forms of these PKCs overcomes myelin- and CSPG-dependent neurite outgrowth inhibition [104]. Furthermore, intrathecal infusion of Gö6976 (a selective inhibitor of PKC α and PKC β_1) to adult rats with spinal cord dorsal hemisection stimulates

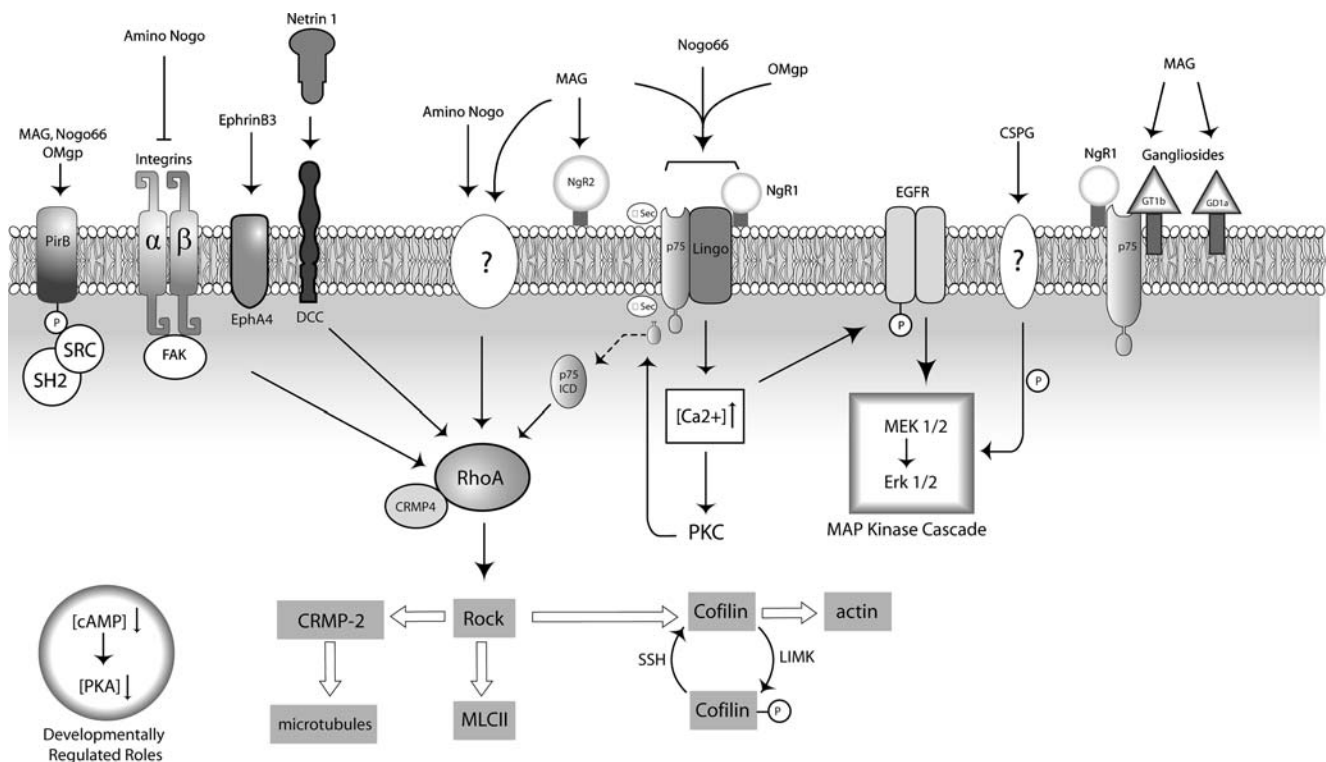


Fig. 2 Signaling pathways mediated by myelin-associated inhibitors and CSPGs. Inhibitors of axonal regeneration signal through Ca^{2+} -dependent activation of PKC, the activation of the epidermal growth factor receptor (EGFR), and the activation of RhoA. RhoA inhibits outgrowth through the remodeling the cytoskeleton. ROCK, CRMP-2, and Cofilin are all downstream of RhoA and have significant impacts on microtubule and actin polymerization. Calcium-dependent

activation of PKC also impacts the RhoA pathway. The sequential cleavage of p75 by α - then γ -secretases generates an intracellular domain of 25 kDa that activates RhoA-mediated inhibition of neurite outgrowth. Finally, the developmental regulation of cAMP and PKA plays a role in the inhibition of neuronal outgrowth. A decrease in cAMP levels is coincident with a developmental switch from outgrowth promotion to inhibition in response to MAG

regeneration of the dorsal column but not of the corticospinal tract. Taken together, the results of Hasegawa et al. [103] and Sivasankaran et al. [104] strongly implicate PKC in the signaling pathways mediated by myelin-associated inhibitors and CSPGs (see Fig. 2).

From a small molecule screen using a neurite outgrowth assay on myelin substrates, Koprivica et al. discovered that inhibition of the epidermal growth factor receptor (EGFR) counters outgrowth inhibition. Nogo-66 and OMgp trigger rapid EGFR phosphorylation in neurons, requiring expression of full-length NgR1. However, direct binding or a physical association between EGFR and NgR1 or p75^{NTR} could not be detected suggesting that EGFR is transactivated in response to myelin inhibitors. Further, calcium chelators can prevent Nogo-66-dependent EGFR phosphorylation, indicating that transactivation likely occurs via a calcium-dependent signal. Interestingly, CSPGs elevate cytoplasmic calcium levels in DRG neurons [105] and signal in a calcium-dependent manner to rapidly phosphorylate EGFR [106]. Furthermore, downstream of EGFR, CSPGs activate the MAPK signaling pathway through phosphorylation of MEK-1/2 and ERK [67]. These findings

imply that myelin-associated inhibitors and CSPGs share components of the intracellular signaling pathways blocking axonal regeneration in the CNS.

Activation of RhoA GTPase and its Downstream Effectors

Inhibitors of axonal regeneration must signal to the growth cone cytoskeleton if they are to cause growth cone collapse and inhibition of axonal extension. The Rho family of small GTPases are well characterized as transducers of extracellular cues to the growth cone actin cytoskeleton. These GTPases, which include RhoA, Rac1, and Cdc42, cycle between an active GTP-bound state and an inactive GDP-bound state. Nucleotide state is controlled by several inactivating GTPase-activating proteins (GAPs), activating guanine nucleotide exchange factors (GEFs), and guanine nucleotide dissociation inhibitors (GDI) that maintains GTPases in the inactive GDP-bound state [107]. While Cdc42 and Rac1 activation are associated with growth cone attraction via filopodial and lamellipodial formation, RhoA activation is associated with growth cone collapse and neurite retraction [108].

RhoA has been implicated as an essential component of the myelin inhibitory signaling pathway during regeneration of CNS axons [109–111]. Retinal ganglion neurons grown on an inhibitory substrate of MAG or myelin and in the presence of C3 transferase—an enzyme which selectively ADP-ribosylates RhoA and thereby inactivates it—exhibit greater neurite outgrowth. In vivo regeneration of optic nerve axons past a crush lesion site as well as corticospinal tract regeneration and BBB locomotor score are improved by C3 transferase treatment [110, 112]. Biochemical work by Niederost et al. [109] indicates that a rapid and transient increase in RhoA-GTP, as well as a concomitant but persistent decrease in Rac1-GTP levels, occurs following treatment with either MAG, Amino-Nogo, or Nogo-66 in cerebellar granule neurons. The increase in active RhoA can be blocked by C3 transferase treatment.

Signaling events upstream of RhoA activation involve the regulated PKC-dependent cleavage of the p75^{NTR} intracellular domain (ICD). Upon binding MAG, the p75^{NTR} component of the Nogo receptor complex undergoes sequential proteolytic cleavage by α - and then γ -secretases generating a final ICD fragment of about 25 kDa [113]. It is γ -secretase-mediated cleavage that is dependent on PKC activity. Pharmacological inhibition of either α -secretase, γ -secretase, or PKC prevents formation of the ICD fragment, the activation of RhoA, and reverses MAG inhibition of neurite outgrowth. Overexpression of a cleavage-resistant form of p75^{NTR} can prevent MAG-induced inhibition, while the overexpression of the ICD fragment alone can mimic inhibition. Yamashita and Tohyama suggest that MAG and Nogo stimulation strengthens an interaction between Rho-GDI and p75^{NTR} allowing for RhoA activation [114]. Thus, there are two possible mechanisms for signaling through the p75^{NTR} component of the Nogo receptor complex to activate RhoA.

Results from Sivasankaran et al. also support a central role for PKC in MAI-dependent activation of RhoA. Application of the PKC inhibitor Gö6976 prevents RhoA activation caused by either MAG, Nogo-66, and CSPGs, indicating a convergence of signaling pathways activated by inhibitory ligands binding to different receptors [104]. However, some of these findings are not supported by Hasegawa et al., who reports that pharmacological inhibition of PKC does not impair the RhoA-GTP increase caused by MAG or Nogo-66 treatment [103].

Intriguingly, some inhibitors of axonal regeneration that do not signal through NgR1 do signal through RhoA. Treatment with either C3 transferase or the pharmacological rho-associated kinase (ROCK) inhibitors Y-27632 or HA1077 enhances neurite outgrowth on CSPG [115–117]. Therefore, together with evidence implicating activation of the PKC and EGFR pathways in response to CSPGs [104, 106], it appears that CSPGs activate a set of signaling

pathways in common with MAIs. Netrin-1 and ephrinB3 also may signal to inhibit regeneration via RhoA given that their receptors, DCC and ephA4, respectively, can signal through RhoA [118, 119].

Downstream of RhoA activation, ROCK is an important relay point for signal transduction to cytoskeletal regulators. The pharmacological ROCK inhibitors Y-27632 and HA1077, as well a dominant-negative form of ROCK, protect neurons from myelin-induced growth cone collapse and neurite outgrowth inhibition [111, 112, 117, 120]. Nogo-66 treatment activates ROCKII activity, which in turn increases phosphorylation of the actomyosin regulator myosin light chain II (MLCII) [120]. In agreement with this finding, Kubo et al. showed that neurite outgrowth inhibition, growth cone collapse, and the reduction in F-actin density caused by repulsive guidance molecule (RGMa) require MLCII phosphorylation. Furthermore, phosphorylation of MLCII increases after spinal cord injury [121]. Thus, MLCII likely acts downstream of RhoA and ROCK as an effector for cytoskeletal remodeling in response to regeneration inhibitors (Fig. 2).

In addition to MLCII, cofilin is a major regulatory protein of the actin depolymerizing factor/cofilin family, which has also been shown to mediate inhibitory responses to the cytoskeleton [122]. Cofilin regulates actin dynamics by increasing F-actin depolymerization and by severing F-actin. It cycles between active (dephosphorylated) and inactive (phosphorylated) states, as regulated by two kinases, LIM Kinase (LIMK) and TES Kinase (TESK), and two phosphatases, Slingshot (SSH) and Chronophin (CIN) [123]. Hsieh et al. demonstrated that cofilin phosphorylation is regulated by Nogo-66, in a manner that is dependent on LIMK1 and SSH1. In turn, Nogo-66-mediated LIMK1 and SSH1 regulation was shown to be dependent on ROCK. Myelin-dependent growth cone collapse and neurite outgrowth inhibition were blocked by introduction of dominant-negative LIMK1, indicating that an active ROCK–LIMK1–Cofilin–SSH signaling pathway impedes neurite outgrowth through modulation of the actin cytoskeleton [122].

The collapsin-response mediator protein (CRMP) family of cytosolic phosphoproteins has also been implicated in the signaling pathways mediated by myelin-associated inhibitors [124, 125]. CRMP-2 promotes microtubule assembly during axon extension [126] and is phosphorylated in response to Nogo-66 and MAG in a ROCK-dependent manner [124]. Furthermore, mutation of threonine 555 of CRMP-2, a ROCK phosphorylation site, counteracts MAG inhibition of neurite outgrowth in cerebellar neurons [124]. These findings raise the possibility that MAIs act not only via the actin cytoskeleton to reduce outgrowth but may also directly regulate microtubule assembly.

Alabed et al. identified CRMP4b as a protein which physically and functionally interacts with RhoA to mediate

neurite outgrowth inhibition in a screen for proteins which interact with constitutively active RhoA in response to Nogo-66 treatment. The interaction between RhoA and CRMP4b is dependent on Nogo-66 stimulation, but independent of the nucleotide binding state of RhoA, indicating that upstream signaling events may differ from those leading to RhoA activation. In fact, the interaction depends on the phosphorylation status of CRMP4, with phosphorylation of CRMP4b preventing the interaction with RhoA. Disruption of the RhoA-CRMP4b interaction attenuates neurite outgrowth inhibition by both myelin and aggrecan. Overexpression of CRMP4b promotes an actin-based filopodial phenotype in growth cones and neurites, suggesting that CRMP4b may participate in outgrowth inhibition by modulating actin dynamics [125]. Furthermore, all members of the CRMP family bind tubulin [126], and CRMP4b is known to interact with intersectin, an endocytic–exocytic adaptor protein [127], raising the possibility that CRMP4b may act through microtubule- or endocytosis-dependent mechanisms. Alternatively, the interaction between RhoA and CRMP4b may be necessary for RhoA-dependent activation of ROCK and its downstream effectors.

Developmental Regulation of cAMP and its Modulation of Myelin Inhibition

A clearer picture of the signaling pathways acutely activated following CNS injury is emerging. However, equally important is the differential interpretation of inhibitory signals by neurons at different developmental stages and the underlying molecular signals responsible for these changes. For example, the response to MAG undergoes a developmental switch from neurite outgrowth promotion to inhibition [20]. In rodents, this occurs around post-natal day 3 or 4 for DRG neurons. The basis for this switch is a developmentally regulated decrease in endogenous levels of cAMP [128].

Several lines of evidence indicate that modulation of cAMP or downstream signaling events can alter the response of neurons to myelin-associated inhibitors. First, in older neurons, which have comparatively lower cAMP levels, elevation of cAMP using an analog such as db-cAMP blocks myelin or MAG inhibition of neurite outgrowth [128]. Further, when applied prior to myelin exposure, the neurotrophins BDNF and GDNF, but not NGF, can elevate cAMP by preventing its degradation, thereby blocking myelin inhibition [129, 130]. Finally, the ability of DRG neurons to regenerate their axon innervating the CNS following a pre-conditioning lesion of the peripheral branch is cAMP and PKA dependent, and can be mimicked by a cAMP analog [131, 132].

cAMP elevation activates the transcription factor cAMP response element binding protein (CREB) that is required to overcome myelin inhibition [133]. This likely initiates long-lasting changes through transcription of a set of genes responsible for cAMP-dependent blockade of myelin inhibition. For example, transcription of Arginase I, a key enzyme in polyamine synthesis, is upregulated, and this results in increased putrescine levels [134]. Both exogenous putrescine and overexpression of Arginase I overcome myelin inhibition in older neurons. It is not clear how polyamines may contribute to block myelin inhibition, but possible mechanisms include initiation of a second round of transcription, modulation of NMDA and AMPA receptor channels, or the promotion of microtubule assembly [134].

The cytokine interleukin-6 (IL-6) is also upregulated in neurons in response to high cAMP levels or a conditioning lesion [135]. Both in vitro and in vivo, IL-6 overcomes the inhibitory effects of myelin on DRG neurons. However, while IL-6 is sufficient to mimic the effects of cAMP elevation or the pre-conditioning lesion, it is not necessary for either response [135, 136].

The Intrinsic Ability of Adult CNS Neurons to Regenerate Following Injury

While inhibitory signaling pathways continue to be elucidated, numerous studies are also assessing ways to enhance the intrinsic ability of adult CNS neurons to regenerate following injury. The reduced intrinsic ability of neurons to re-extend axons following injury and an insufficient ability of non-injured axons to branch and form new synapses constitute a significant limitation to functional recovery. In this regard, strategies altering the activity of several signaling components critical for neuronal development have proven effective in enhancing regeneration.

The optic nerve injury system has been used extensively to study intrinsic limitations to adult CNS axon regeneration (for an extensive review see [137]). In a recent study, Park et al. used the optic nerve injury system to study the effects of ablating an number of evolutionarily conserved genes responsible for controlling cell growth and size, including *Rb*, *p53*, *Smad4*, *Dicer*, *LKB1*, and *PTEN* [138]. *PTEN* deletion results in both increased retinal ganglion cell (RGC) survival and sustained axon regeneration following an optic nerve crush. Deletion of *TSC1*, which, like *PTEN*, negatively regulates the mTOR pathway, was also beneficial to RGC survival and regeneration. While axotomy appeared to reduce phosphorylation levels of ribosomal s6—a protein downstream of mTOR kinase—deletion of *PTEN* maintains the level of p-s6, suggesting

that retaining the mTOR protein translation system active is essential for sustained axon regeneration [138].

In the spinal cord injury model, the intrinsic growth capacity of regenerating axons of the dorsal column can be improved when a simultaneous or preceding sciatic nerve lesion is performed [139]. The molecular basis of this is a peripheral lesion-induced increase in cAMP levels, which also allows regenerating fibers to overcome myelin inhibition [131, 132]. Activating transcription factor-3 (ATF-3) also enhances the intrinsic growth capacity of regenerating dorsal column fibers, but it does not overcome myelin inhibition [140]. A recent study by Dill et al. also points to glycogen synthase kinase-3 β (GSK-3 β) inactivation as increasing the intrinsic growth potential of adult DRG neurons [141]. These findings add to the constant expanding list of genes found that can elicit axonal extension of the DRG neurons such as GAP-34 and CAP-23, two major growth cone proteins [142]. Additionally, IL-1 β has been also shown overcome MAG-induced neurite outgrowth inhibition via p38 MAPK pathway that deactivates RhoA [143].

Concluding Remarks

The molecular mechanisms mediating the inhibition of CNS regeneration appear to be highly redundant. There are upwards of 11 known, or proposed, cell surface receptors on axons interacting with at least seven distinct ligands. Interestingly, many are integral to axonal guidance so that, at least superficially, it appears that the same molecules that initially allow for the systemic generation of complex topographical circuits may be a hindrance to effective regeneration. Yet these molecules can only be part of the conundrum that is CNS regeneration as they exist in a variety of organisms where effective regeneration does occur. Perhaps then it is wise to consider the organism as a whole and the contributions of non-neuronal cells, in particular, on regenerative outcomes.

Acknowledgments We would like to thank Dr. Aaron McGee and Corrie Fox for their comments and suggestions on the manuscript and graphic work, respectively.

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