Novel Therapeutic Targets for Axonal Degeneration in Multiple Sclerosis

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Abstract
Multiple sclerosis (MS) is a devastating neurological condition that mainly affects young adults and is associated with long-standing morbidity. The pathophysiology of MS is believed to involve immune-mediated multifocal lesions in the CNS that are characterized by inflammation, demyelination, and axonal injury. Most research efforts to date have concentrated on the mechanisms of immune-mediated demyelination, whereas mechanisms of axonal injury, the major determinant of neurological deficits in MS patients, have been elusive beyond observational analyses. This review discusses current understanding of the pathology and novel clinical investigations of axonal injury in MS and the commonly used MS animal model, experimental autoimmune encephalomyelitis. The review focuses on the etiology and the induction of axonal degeneration through molecular signaling cascades downstream of myelin-associated inhibitory factors. Defining and eventually elucidating the signaling pathways elicited during the onset and progression of MS may provide novel therapeutic strategies to limit axonal degeneration in the acute phase of the disease. Furthermore, blocking or potentiating specific signaling pathways, particularly those that mediate axon retraction and promote disassembly of the tubulin network, may promote regrowth of damaged axons in CNS regions affected by many acute and chronic disease processes.

Key Words: Axonal degeneration, CRMP-2, Experimental autoimmune encephalomyelitis, Multiple sclerosis, Myelin-associated inhibitory factors, Nogo-A, RhoA.

INTRODUCTION
Multiple sclerosis (MS) is a major neurological disorder in which there is inflammation, demyelination, and axonal damage in the brain and spinal cord. Approximately 2.5 million people currently live with MS globally, and this causes a significant health burden; more than 400,000 people in the United States alone are diagnosed with the disease. Women are at least 2 to 3 times more likely to develop the disease than men, and the disease onset is most often between the ages of 20 and 50 years (1). Although MS occurs in most ethnic groups (some more than others, particularly individuals from northern Europe or their direct descendants), strong genetic or environmental risk factors are still to be elucidated (2). The histopathologic patterns of MS have been well characterized, with much emphasis on the destructive immunologic component of the disease in the brain and spinal cord (3). Currently, the mechanisms underlying axonal damage in MS demonstrable in these histopathologic patterns are being defined. Axonal damage can either develop secondary to white matter damage, or, as recently postulated, may be a primary or precipitating event in MS. A more plausible scenario would be that axonal degeneration occurs concomitantly with demyelination (4). Trapp et al (5) reported the common appearance of newly transected axons in active and hypocellular chronic active MS lesions in brains obtained at autopsy, corroborating such a hypothesis. Importantly, it is now recognized that permanent neurological impairment is a consequence of the level of axonal loss (4). Thus, understanding the molecular mechanisms leading to axonal degeneration is of critical importance for the development of novel therapeutic strategies aimed at limiting the neurological decline during MS.

Experimental autoimmune encephalomyelitis (EAE) is an animal model that mimics the clinical course as well as the pathophysiological hallmarks of MS. The EAE model has thus been instrumental for the development of current and novel MS therapeutics (6). The induction of EAE by immunization with the myelin oligodendrocyte glycoprotein (MOG) peptide spanning the 35 to 55 amino acid sequence (MOG35-55) is one of the most commonly used animal models to study MS (7). It has long been believed that axonal damage and loss is secondary to demyelination, commonly referred to as Wallerian degeneration, and readily seen in the MOG35-55 model of MS (8). On the other hand, it has recently been demonstrated that axonal degeneration can precede demyelination in EAE (9). Therefore, the MOG35-55 EAE model of

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Steven Petratos is supported by Australia Project Grant ID No. 384157 from the National Health and Medical Research Council (NHMRC); Michael Azari is supported by the NHMRC Health Professional Research Training Fellowship; Ezgi Ozturk is supported by the Faculty of Medicine Postgraduate Award, Monash University Postgraduate Scholarship; Claude Bernard is supported by the NHMRC Project Grant ID No. 380832, the National Multiple Sclerosis Society, and The Baker Foundation.

J Neuropathol Exp Neurol • Volume 69, Number 4, April 2010

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MS can be used to help define the pathogenetic mechanisms of axonal degeneration.

The normal neurobiological response to axonal injury is an initial attempt at regrowing the distal end of the axon. Local neurons surrounding the lesion also attempt to compensate for the injury by “sprouting” or extending new neurites. However, there are factors within the CNS environment that inhibit these compensatory responses and cause failure of regeneration/sprouting. Chief among these factors are components of myelin or the myelin-associated inhibitory factors (MAIFs), such as the potentiator of neurite outgrowth, Nogo-A (Fig. 1). Expression of Nogo-A in oligodendrocytes has been reported to be upregulated in chronic active demyelinating lesions of MS (10). In addition, immunization against Nogo-A or deletion of the nogo gene has been shown to ameliorate the pathophysiological hallmarks of EAE in C57Bl/6 mice (11). Strong evidence supporting the Nogo-A signaling pathway regulating axonal injury in EAE derives from recent studies showing that deletion of the coreceptor for Nogo-A, LINGO-1 (which can signal through the downstream cytoskeletal regulator, RhoA-GTP), improves axonal integrity and protects against neurological decline in MOG-induced EAE mice (12). This raises the question of whether Nogo-A can signal axonal degeneration in EAE and, by implication, in MS.

Current treatment of MS mostly targets the inflammatory nature of the disease to prevent the presumed autoimmune attack on the CNS myelin and hence limit the devastating neurological complications that ensue. It is possible, however, that by limiting Nogo receptor 1 (NgR1)–dependent signaling, axonal degeneration and hence neurological impairment during EAE might be diminished. The precise dissection of the NgR1 signaling pathways eliciting axonal degeneration during neuroinflammatory diseases such as MS may open novel avenues for therapeutic intervention to limit the neurological decline that accompanies the disease.

**MAIFs AND THEIR ROLE IN MS/EAE**

Although lesioned CNS axons can form growth cones in response to injury, this regenerative attempt is transitory, and no significant regrowth occurs over long distances (13). The reason for the aborted axonal growth in the CNS is not caused by the absence of growth-promoting molecules, but rather by the presence of axon outgrowth inhibitors, including components of CNS myelin (14). This notion is attested to by the extensive axonal regeneration that is observed when mice are immunized with purified CNS myelin before spinal cord injury (SCI) (15). To date, 5 MAIFs have been identified: myelin-associated glycoprotein (MAG) (16, 17); Nogo-A (18–20); oligodendrocyte myelin glycoprotein (OMgp) (21); semaphorin-4D/CD100 (22); and ephrin-B3 (23). Of these, MAG and Nogo-A have been most intensively studied. Biochemical and structural biological reviews of these molecules have been previously described and will not be discussed here (24).

Myelin-associated glycoprotein (17), Nogo-A (18, 20), and OMgp (25) are all normally localized on the innermost lamella of the myelin sheath in direct contact with axons, preventing aberrant sprouting (26). Of these 3 inhibitors, Nogo-A (1,192 amino acids), a Reticulon family member (Reticulon 4A), has the most interesting structure because it has at least 2 domains that can individually inhibit axonal growth (24). The binding of Nogo-A with its cognate receptor, NgR1 (Fig. 1), is a trivalent interaction involving the Nogo-66, Nogo-A-24, and Nogo-C39 domains, with the core of the binding domain centered in the middle of the concave surface of NgR1 leucine-rich repeat domain and surrounded by differentially used residues (27).

The nogo gene is differentially spliced to generate 3 isoforms, Nogo-A, Nogo-B, and Nogo-C. Nogo-A is the longest; it is CNS specific and contains a unique N-terminal sequence (Amino-Nogo). The C-terminal portion of the Nogo-A protein contains 2 transmembrane domains that are separated by a short extracellular domain (Nogo-66) (Fig. 1). Nogo-66 acts via the Nogo-66 receptor (NgR1) (28) in conjunction with 2 coreceptors: the leucine-rich repeat and immunoglobulin (Ig) domain–containing Nogo-receptor interacting protein 1 (LINGO-1) receptor, and either the low-affinity neurotrophin receptor (p75NTR) or a receptor named TROY (29). Activation of this receptor complex mediates inhibition of neurite growth by regulation of intracellular signaling through RhoA GTPase (30), a regulator of actin cytoskeletal changes in neurons (31). The receptor through which Amino-Nogo acts is not yet characterized, but a recent report suggests that Amino-Nogo can bind and inhibit signaling by α5 and αv integrins and inhibit activation of focal adhesion kinase, thereby modulating fibronectin-dependent adhesion of the growth cone to the extracellular matrix (32). Amino-Nogo also increases the affinity of the

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**FIGURE 1.** Oligodendrocyte myelin membrane localized Nogo-A and its interaction with its cognate receptors. (A) Alternatively spliced isoforms of Nogo (Reticulon 4). Nogo A is 200 kD, Nogo B is 55 kD, and Nogo C is 25 kD. Despite the evolutionary conservation of the C-terminus among these isoforms, the N-terminus is longer in Nogo A than the other 2 isoforms, with limited conservation. (B) Full-length Nogo A is 1,192 amino acids (AA) in length and consists of 2 transmembrane domains, a C-terminus extracellular Nogo-66 domain (ligand binding domain), and 2 intracellular tails (one at the N-terminus, one at the C-terminus in the cis configuration). Nogo A in the trans configuration has its N-terminus in the extracellular compartment, which is able to interact with integrin receptors, commonly referred to as Amino-Nogo. (C) The Nogo-66 domain interaction with the leucine rich repeat ectodomain of Nogo receptor 1 (NgR1), is a high affinity binding, at a nanomolar range. This produces conformational changes of the NgR1 coreceptors to signal downstream, modifying the axonal cytoskeleton and favoring retraction of the distal growth end of the injured axon. Microfilament and microtubule dynamics are modified through the activation of RhoA (RhoA-GTP) and its effector kinase, Rho kinase (ROCKII). GDI, guanine dissociation inhibitor; LINGO-1, LRR and immunoglobulin domain–containing Nogo receptor–interacting protein 1 receptor; p75, low-affinity neurotrophin receptor (p75NTR).
Nogo-A/NgR1 interaction (33) and can act through the Rho GTPases (30, 34). A third segment of Nogo-A, a C-terminal domain, can also interact specifically with NgR1 (35).

The action of the MAIFs on severed axons attempting regeneration after injury or disease has been determined to be one of the most critical inhibitory events that limits CNS repair. The molecular interaction of the MAIFs with NgR1 is a high-affinity ligand-receptor interaction that initiates intracellular signaling in affected neurons, converging on the Rho-GTPases to inhibit axonal regrowth (Fig. 1). The downstream effectors of Nogo-A/NgR1 signaling are RhoA-GTP and the collapsin response mediator protein 2 (CRMP-2) that regulates actin and tubulin dynamics after injury (36). We hypothesize that by reducing the capacity of the myelin-derived deposits to signal through their cognate receptor in EAE or in MS, it may be possible to limit the activation of the RhoA/phosphorylated CRMP-2 pathway in the axonal cytoskeleton, thereby preventing degeneration.

On the other hand, MAG can exert its inhibitory effects on neurite outgrowth through either the NgR or gangliosides, depending on the neuronal type. Using primary rat neuronal cultures, the inhibitory effects of MAG were shown to be mediated predominantly by NgRs in dorsal root ganglion (DRG) neurons, mainly by gangliosides in hippocampal neurons and exclusively by gangliosides (GD1a and GT1b) in cerebellar granule neurons (37).

Receptor systems other than through NgR may also contribute to the inhibitory effects of the MAIFs on neurites. For example, paired Ig-like receptor B (PirB), which has been previously implicated in visual system plasticity (38), now seems to be important in MAIF signaling for neurite outgrowth inhibition. Atwal et al (39) reported that MAG, OMgp, and Nogo-66 bind with high affinity to PirB. Moreover, blocking this high-affinity interaction with the use of a His-tagged fusion PirB ectodomain peptide, an anti-PirB antibody, or the PirB genetic mutant, produces a restoration of maximal neurite length of cerebellar granule neurons in the presence of the MAIFs. These investigators did show, however, that there was a requirement for both NgR and PirB to potentiate MAIF-mediated growth cone collapse of DRG neurons, suggesting complexity to the axonal degeneration system mediated through the myelin proteins (39). It may be worthwhile to investigate the effect of combinatorial treatments for inhibition of the NgR and PirB ligand interactions in the context of axonal degeneration in MS.

Given the apparent redundancy in signaling axonal growth inhibition and the tendency of the organism to compensate for genetic deletion of any of these specific ligands, it is not surprising that studies of axonal regeneration in 3 different strains of nogo-deficient mice have shown variable degrees of regeneration (40–42). An additional reason for this variability may be strain-specific differences between the mice used in these studies (43). Recently, the relevance of Nogo-A in neuroinflammation was demonstrated by its direct role in the pathogenesis of EAE (11). Furthermore, immunization against Nogo-A ameliorated EAE in mice and prevented axonal injury (11). It is therefore plausible that abrogation of Nogo-A signaling by deletion of the cognate receptor for Nogo-A may provide resistance to EAE. The mechanisms by which Nogo-A contributes to the pathophysiology of EAE are still unclear, however.

Despite the ambiguity of the role of Nogo-A in the induction of EAE (either immune or neurobiological or both), Mi et al (12) defined a neurobiological role for LINGO-1, the signaling coreceptor for the MAIFs (including Nogo-A) during the course of EAE. These investigators showed that Lingo1-knockout mice or mice treated with an anti–LINGO-1 function–blocking antibody had a reduced locomotor deficit throughout the course of the disease. These results were independent of the activity of immune cell infiltrates, suggesting a neurobiologically specific role for LINGO-1 in the induc- tion of the neurological complications of EAE. They demonstrated this specifically by testing the proliferative indices of knockout versus wild-type littermate–isolated T-cells after MOG35–55 immunization and showing that their MOG responsiveness was almost identical (12). Along the same lines, the cytokine profiles of isolated T-cells from wild-type littermates with EAE were not different from those of Lingo1-knockout mice. Moreover, EAE could be transferred to naive wild-type littermates with encephalitogenic T-cells isolated from Lingo1-knockout mice. By contrast, encephalitogenic T-cells from wild-type mice could not adoptively transfer EAE into knockout littermates, clearly indicating an endogenous role for LINGO-1 in the CNS during the neurological decline associated with EAE.

The neurobiological role of LINGO1 during EAE was eventually attributed to the function of the receptor in remyelinating oligodendrocyte precursor cells (12). Lower EAE scores of locomotor deficit in the Lingo1-knockout group were correlated with a lower extent of oligodendrogliopathy compared with EAE induced in wild-type littermates, as demonstrated by electron microscopy. Oligodendrogliopathy is characteristic of an “inside-out” degeneration by which the loss of the axon initiates oligodendrocyte and myelin degeneration. Furthermore, when EAE was induced in the Lingo1-deficient mice, there was an abundance of remyelinated axons and an increased number of myelinated axons compared with those in the wild-type littermates. Likewise, treatment of rats with EAE with an antagonistic anti–LINGO-1 antibody led to more myelinated and remyelinated fibers in the spinal cord compared with those in rats treated with control IgG (12). The contention that LINGO-1 plays an important role in remyelination is further supported by the demonstration that LINGO-1 plays a dominant role in the inhibition of oligodendrocyte differentiation and myelination when it is expressed either on the oligodendrocyte precursor cell (44) or on the axon that the pre–myelinating oligodendrocyte eventually myelinates (45). These data suggest that therapeutic targeting of LINGO-1 may be a viable mechanism for repair in demyelinating diseases.

MODELS OF AXONAL INJURY AND THE GENETIC EVIDENCE FOR NgR1-MEDIATED AXONAL DEGENERATION

Nogo-A–knockout mice, including nogo-abrap/rap (40) and nogo-de/-elle/-elle (41, 43) mice, have been reported to show enhanced growth of corticospinal tract axons after SCI,
whereas one of the mutants, termed nogo-ab^{Tg/ab}, failed to show enhanced axonal regrowth (42). These conflicting observations after injury have been mimicked in NgR1 mutant mice after dorsal hemisection (46, 47), but the ngr1^{+/−} mice did recover hindlimb function after SCI and showed regrowth of rubrospinal and raphespinal axons (46). In a selective pyramidotomy paradigm, there is pronounced collateral sprouting of intact corticospinal tract fibers across the midline and into injured spinal cord, with a return of preferred forelimb usage in both ngr1^{−/−} and nogo-ab^{Tg/ab} mice (48). These data confirm a role for Nogo-A and NgR1 in limiting functional recovery through axonal growth within the injured CNS, but these in vivo models do not provide molecular downstream signaling evidence of axonal regrowth. In view of the growing body of pharmacological studies that show enhanced regrowth of axons after injury and disease in the CNS by blocking the binding of the MAIFs with the ectodomain of NgR1 (49), it is imperative to identify the signaling cascade that promotes axonal degeneration to provide therapeutic targets for CNS repair. The role of NgR1 in axonal regrowth related to a neuroinflammatory-induced disease such as EAE is now awaiting investigation.

THE ROLE OF NOGO-A SIGNALING IN THE INDUCTION OF EAE

It has recently been suggested that Nogo-A is involved in autoimmune-mediated demyelination (50). Accordingly, it is important to determine whether the role of Nogo-A in EAE is related to axonal signaling through NgR1 initiating degeneration or through initiating the autoimmune response. Karnezis et al (11) demonstrated that blocking Nogo-A interaction with its cognate receptor limits axonal degeneration in EAE. Interestingly, in that study, nogo-a^{−/−} mice or mice that were vaccinated with the Nogo-623–640 peptide exhibited reduced CNS inflammatory infiltrates, increased anti- and immunomodulatory cytokines such as transforming growth factor-beta and interleukin-19 (IL-19), and enhanced proinflammatory cytokines. These data indicate modulation of inflammatory mechanisms. It is plausible that this dampening of the neuroinflammatory response in these mice may be caused by the reduced axonal pathology also manifested in these mice, but whether the reduction in axonal damage occurs before the appearance of the inflammatory component of the disease or is a consequence of the reduced immune response remains unresolved.

THE ROLE OF NOGO-A AND NgR IN THE IMMUNE RESPONSE IN MS AND EAE

Anti-Nogo-A autoantibodies have been reported in the serum and cerebrospinal fluid of MS patients (51). Notably, these antibodies seem to be more frequently present in relapsing-remitting, rather than in the chronic progressive forms of MS, as well as being more frequent in younger patients (51). It is plausible that this antibody response may play a role in clinical remissions of the disease. Neutralizing the potent MAIF effect on neurite outgrowth inhibition was first demonstrated against Nogo-A in culture using the monoclonal antibody IN-1 (52). Whether blocking antibodies such as IN-1 produce similar blockade of neurite outgrowth inhibition in vivo, particularly in models of MS, has yet to be defined. Interestingly, expression of Nogo-A in oligodendrocytes is upregulated in chronic active demyelinating lesions of MS (10). Fry et al (53) recently demonstrated that NgR induces efflux of macrophages from the injured peripheral nerve to terminate inflammation at the end of Wallerian degeneration. This effect is postulated to contribute to the regenerative capacity of peripheral nerves. Given that depletion of peripheral macrophages reduces the severity of EAE (54), it is possible that NgR may also play a role in the efflux of macrophages from the CNS after injury or inflammatory insult. It is intriguing that when SJL mice were injected with peptides from different regions of Nogo-66, T- and B-cell immune responses were stimulated and EAE was either induced or suppressed depending on the specific epitope of Nogo-66 to which the peptide corresponded (50). The investigators further showed that Nogo-66–specific T-cell lines could ameliorate EAE in this mouse model through a Th2-dependent mechanism. Despite these data, they showed increased spreading of the antibody response against other myelin antigens after immunization with the Nogo-45–66 peptide and enhanced demyelination and paralysis in the mice. Recent data using DNA vaccination for the generation of antibodies against the full-length Nogo-A protein did not induce demyelination or inflammatory disease in naive mice nor did it exacerbate the EAE in mice immunized with myelin proteolipid protein (55). Interpretation of these data may not advocate Nogo-A as a potent encephalitogen.

Recently, the complexity of the NgR1 signaling system in vivo has been established with the identification of the expression of the receptor in human T-cells and monocytes from both healthy individuals and MS patients (56). Moreover, Zhang et al (57) demonstrated that a tumor necrosis factor family protein involved during B-cell development, known as B-lymphocyte stimulator, can act as a high-affinity ligand for NgR1 signaling independent of the MAIFs. It would, therefore, be imperative to dissect out the effects of pharmacological antagonists to NgR1 in the context of heightened neuroinflammation for the future application of therapeutic manipulation of this pathway in patients at different stages of MS.

NgR1 SIGNALING AND CYTOSKELETAL ORGANIZATION: TRANSDUCTION OF THE EXTRACELLULAR SIGNAL INTO THE AXOPLASM

Rhoa-GTP (active Rhoa) has been implicated in neurological disease paradigms as an intracellular effector of neurite retraction, primarily involving the MAIFs (58, 59). Inhibition of neurite outgrowth by the MAIFs occurs by using the receptor cluster composed of NgR1, the low-affinity neurotrophin receptor, p75^{NTR}, LINGO-1, and TROY to activate endogenously bound Rhoa (Fig. 1) (29). Downstream, Rhoa-GTP can mediate the reorganization of the microfilament and microtubule networks forming the neuronal cytoskeleton (59). In the neuron, this can include contraction between myosin head groups on actin filaments at filopodia pulling actin
filaments away from the area of directional growth (Fig. 1). Furthermore, RhoA-GTP activates the serine-threonine kinase, Rho kinase (ROCKII), which then proceeds to phosphorylate Lin-11, Isl-1, and Mec-3 kinase (LIMK). The LIMK can itself then impede (by phosphorylation) the ability of the actin-depolymerizing factor/cofilin by phosphorylation, to provide actin monomers for the extension of microfilaments to ensue, thus initiating growth cone collapse (59).

Cytoskeletal rearrangement initiated from the growth cone eventually becomes transferred retrogradely to the larger neuronal microtubule network at the core of the axon/neurite. This larger cytoskeletal network is assembled from a 100-kD α and β tubulin heterodimer and transported to the plus ends of microtubules to potentiate neurite outgrowth (60). Rapid microtubule assembly from the core (C-domain) of the neurite toward the actively growing peripheral domain (P-domain) occurs where there is substantial actin rearrangement (60). Therefore, dynamic growth cone regions provide an ideal platform for microtubule assembly and neurite outgrowth. This is an important event that regulates the synaptic contact of various neurons with each other through long-range and short-range cues during CNS development (61). Assembly/polymerization of this intricate neuronal cytoskeletal network, which during development provides growth, can also be accompanied by negative chemical and contact cues that prevent aberrant or inappropriate growth to specific CNS regions (62). This is achieved through transduction of inhibitory extracellular cues to intracellular signals that promote contraction and disassembly of the neuronal cytoskeleton in an opposing direction to the signal. In the adult CNS, this important signaling mechanism prevents aberrant growth of axons and dendrites and the formation of pathophysiological connections. The MAIFs, such as Nogo-A, are major extracellular inhibitory molecular cues in the adult CNS. After injury, deposits of these myelin proteins at lesion sites prevent endogenous axonal regrowth after degeneration of axons; they may also initiate axonal degeneration in their own right (63, 64). Because myelin debris is deposited after neuroinflammatory-mediated demyelination in EAE and MS (65), induction of axonal degeneration in these conditions might be potentiated by these MAIFs, thereby prohibiting any endogenous regrowth capacity.

**Rho-GTPases and Their Role in Axonal Damage in Eae**

Rho-GTPases are key switches that transduce MAIF-inhibitory signals from the axonal membrane to the intracellular cytoskeleton. They are ubiquitously expressed and are involved in diverse cellular processes including cell division and morphogenesis (66) and migration (67). Of particular significance is that they represent the point of convergence of multiple signaling pathways involved in inhibition of neurite outgrowth. These include signals mediated by MAIFs, Ephrins, Semaphorin 3A, and chondroitin sulfate proteoglycans.

Stimulation of the Nogo receptor signaling complex dissociates RhoA guanine diphosphate dissociation inhibitor α from the small GTPase RhoA, resulting in RhoA activation (21, 59) (Fig. 1). Activated RhoA integrates with the plasma membrane through isoprenylation, allowing ROCKII to dock with it, resulting in activation of its kinase domain (59). Active ROCKII can phosphorylate CRMP-2 at its threonine 555 site to confer decreased association with tubulin heterodimers, thereby decreasing microtubule polymerization and in turn reducing neurite growth (68). The ROCKII can also phosphorylate LIM kinase-1 (LIMK1) (59) and the regulatory light chain of myosin II (MLC) (70). These cumulative phosphorylation events that are instigated by the activation of RhoA and ROCKII promote the contraction of microfilaments and destabilization and disassembly of microtubules, thereby resulting in neurite retraction from the growth cone, that is, growth cone collapse. Both Amino-Nogo and Nogo-66 have been reported to be inhibitory in vitro.

**The Role of CRMP-2 in Axonal Degeneration and Axonal Transport**

More than a decade ago, a 62-kD cytoplasmic protein was first discovered to mediate the growth cone-inhibitory effects of collapsin (semaphorin-3A) (71), which was later termed CRMP-2. The gene for CRMP-2 was found to be expressed during neuronal differentiation in the rat and to be related to unc-33, a *Caenorhabditis elegans* gene that is also involved in axonal growth (72). Mutations of *unc-33* in *C elegans* produce axonal abnormalities and uncoordinated bodily movements (73). The CRMPs are a family of neuronal phosphoproteins with some sequence homology to dihydroprynidinase (74). The CRMPs regulate microtubule assembly as well as anterograde vesicular transport of important growth-related molecular cargo along neuronal microtubules (75). The CRMPs, particularly CRMP-2, are involved in the formation, outgrowth, and guidance of neurites. The CRMP-2 is phosphorylated by cyclin-dependent kinase 5 at serine 522 (76, 77), glycogen synthase kinase 3β at threonine 555 site to confer decreased association with tubulin heterodimers, thereby inhibiting microtubule polymerization and in turn reducing neurite growth (68). The ROCKII can also phosphorylate LIM kinase-1 (LIMK1) (59) and the regulatory light chain of myosin II (MLC) (70). These cumulative phosphorylation events that are instigated by the activation of RhoA and ROCKII promote the contraction of microfilaments and destabilization and disassembly of microtubules, thereby resulting in neurite retraction from the growth cone, that is, growth cone collapse. Both Amino-Nogo and Nogo-66 have been reported to be inhibitory in vitro.

**FIGURE 2.** The collapsin response mediator protein 2 (CRMP-2) and its role in the axonal cytoskeleton. (A) The alternatively spliced variants of CRMP-2 are CRMP-2A (75 kD) and CRMP-2B (62 kD). Full-length CRMP-2 is 571 amino acids in length with a dihydropyrimidinase (74). The CRMPs regulate microtubule assembly as well as anterograde vesicular transport of important growth-related molecular cargo along neuronal microtubules (75). The CRMPs, particularly CRMP-2, are involved in the formation, outgrowth, and guidance of neurites. The CRMP-2 is phosphorylated by cyclin-dependent kinase 5 at serine 522 (76, 77), glycogen synthase kinase 3β at threonine.
509/514, serine 518 (78, 79), and also Rho kinase at threonine 555 (68, 80); all of these can mediate neurite retraction. Such phosphorylation disrupts the association of CRMP-2 with tubulin heterodimers so that tubulin cannot be transported to the plus ends of microtubules for assembly, thereby impeding directional growth of the neurite (75). Importantly, CRMP-2 phosphorylation also reduces its binding to the microtubule-related motor protein kinesin-1, which is involved in anterograde vesicular axonal transport of molecules that regulate synaptic integrity and plasticity (e.g. brain-derived neurotrophic factor) to the distal ends of axons (81) (Fig. 2).

The overexpression of CRMP-2 in rat cerebellar neurons results in neurite outgrowth even in the presence of MAG or Nogo-66 (36). Using purified tubulin and CRMP-2 purified from bovine brain, Fukata et al (75) found that CRMP-2 directly binds tubulin heterodimers and that CRMP-2 has greater affinity for tubulin heterodimers than microtubules by a factor of 10. Indeed, coinubcation with video microscopy experiments shows that CRMP-2 dramatically promotes microtubule assembly (75). The ROCKII inactivation of CRMP-2 by phosphorylation at the Thr555 residue was demonstrated in rat cerebellar neuron transfection experiments using a mutant form of CRMP-2 with a single point mutation at T555A that rendered the molecule incapable of being phosphorylated by ROCKII (36). Furthermore, DRG neurons transfected with the same construct showed an increase in axonal length similar to DRG neurons transfected with wild-type CRMP-2 (68). Inhibition of neurite outgrowth is also observed with siRNA knockdown of CRMP-2 in cerebellar granule cells or by transfection of these cells with the dominant negative form of CRMP-2, which lacks the microtubule-binding domain (CRMP-2ΔC), thereby demonstrating the central role of CRMP-2 in neurite outgrowth (36). Immunohistochemical studies using antibodies against CRMP-2 and phosphorylated Thr555–CRMP-2 on DRG neurons revealed that CRMP-2 is localized to the growth cone and colocalizes with microtubules and actin at distal parts of the axon (68). Phosphorylated CRMP-2, however, was localized diffusely in the growth cone; it was present as punctate structures and was also localized in the filopodia. Unlike CRMP-2, phosphorylated CRMP-2 was not localized with microtubule bundles. Electron microscopy and freeze-etching replica method using DRG neurons revealed CRMP-2 to be localized on filamentous tubulons, on clathrin-coated pits, and on actin filaments at the plasma membrane of the growth cone. However, phosphorylated CRMP-2 was only localized with actin filaments at the growth cone (68).

Considering the physiological relevance of CRMP-2 in the regulation of axonal growth, it has recently been shown that there is an increase in the levels of phosphorylated threonine 555 CRMP-2 after axonal transection in a model of SCI (64). These increases correlated with increased levels of Nogo-A deposits at and around the site of injury. When the level of Nogo-A deposits was reduced by daily administration of the neuropoietic cytokine, leukemia inhibitory factor, a corresponding reduction in the levels of phospho-Thr555 CRMP-2 also ensued (64). These data may suggest that an increase in extracellular MAIF deposits after CNS injury can signal CRMP-2 phosphorylation, possibly limiting microtubule assembly and therefore axonal regrowth. The role of MAIF signaling in axonal injury has been shown to be linked with axonal RhoA activation in white matter tracts surrounding a transection SCI lesion in the rat (36). Importantly, these findings also correlated with an increase in phosphorylated CRMP-2 in axons with depolymerizing tubulin (36). Treatment of these rats with the ROCK inhibitor Y-27632 immediately after spinal cord thoracic transection confirmed that phosphorylation of CRMP-2 was dependent on ROCK (36). Inhibition of ROCK rescued levels of polymerized tubulin, and phosphorylated CRMP-2 levels returned to baseline in axons. Collectively, these data raise the important question of whether interference with the Nogo-A signal transduction pathway in neurons would promote axonal integrity in EAE.

**CURRENT TREATMENT OF MS**

Currently, 4 disease-modifying agents have been approved by the US Federal Drug Administration for the treatment of MS. They are interferon-β1a, glatiramer acetate, mitoxantrone, and natalizumab (82). All of these compounds are targeted toward modulating the immune response in an attempt to limit damage that occurs as a consequence of neuroinflammation. Novel experimental therapies that focus on the neurobiological aspects of the disease are now emerging. There has been significant success in blunting neurological complications in EAE models.

**NOVEL THERAPEUTIC TARGETS IN MS**

A number of promising therapeutic strategies tested in EAE have involved inhibition of the Nogo-A/NgR/LINGO-1/Rhoa/ROCKII/CRMP-2 signaling pathways. These strategies include blockade of either Nogo-A/NgR or LINGO-1/NgR/p75(or TROY) interactions, the use of statins or geranylgeranyl transferase inhibitor to block Rhoa-GTP isoprenylation, inhibition of ROCKII by the use of fasudil, Y27632 to bind to and inactivate the coiled-coiled region of the phosphorylating enzyme, and antagonizing the Rho kinase through the use of a therapeutic dose of the *Clostridium botulinum* protein C3 exoenzyme or its recently developed pharmaceutical agent, Cethrin, a recombinant fusion protein of C3 and a membrane transport sequence (BioAxone Therapeutics, Montreal, Canada). The latter is currently in clinical-phase trials for its use in SCI patients (83). An alternative mechanism for limiting axonal degeneration in EAE has also involved the inhibition of calpain, a calcium-dependent cysteine protease highly expressed during neurological disease and injury.

**INHIBITION OF THE NgR COMPLEX**

**Inhibition of Nogo-A**

As previously indicated, passive or active immunization against Nogo-A decreases the clinical and histopathologic manifestations of EAE (11). This may have been caused by either increased clearance of Nogo-A deposits in CNS lesions, thereby decreasing the interaction of Nogo-A with its cognate receptor on injured axons, or reduction in proinflammatory cytokines. Clinical trials are currently being...
performed by Schwab et al in collaboration with Novartis Europharm Ltd, using a recombinant human monoclonal antibody (IgG1κ) directed against the human Nogo-A protein to enhance axonal regrowth after SCI (84). Current data show a significant enhancement of locomotor performance in a rat model of SCI after the intrathecal administration of a rodent version of the anti–Nogo A antibody (11C7) (85). The rigorous behavioral improvements (i.e. hindlimb kinematic patterns) correlated with the promotion of axonal regrowth and neurite sprouting events in the anti–Nogo-A antibody–treated group (85). We await the outcome of the clinical phase trial for the human anti–Nogo-A antibody that may consolidate these exciting results and may have implications for the neurodegenerative phase of MS.

Inhibition of NgR1-Ligand Interaction

Recombinant fusion proteins using the soluble form of the ectodomain of NgR1 and the Fc portion of human IgG1 have been used in cell culture and in vivo to promote axonal regrowth (49, 86). It was recently shown that administration of a fusion peptide corresponding to the ligand-binding domain of the NgR1(310) resulted in neurite outgrowth (axon regrowth and sprouting) after transection of corticospinal tract and raphespinal fibers and improved functional recovery in a mouse model of SCI (49). This may be caused by the fusion peptide competing with endogenous NgR1 for binding to Nogo-A, OMgp, and MAG, thereby reducing NgR1 downstream signaling and promoting axonal regrowth. Recently, the binding of Nogo-66 to NgR1 was found not to be sialic acid dependent, whereas the interaction of the first 3 Ig-like domains of MAG to NgR1 and NgR2 is sufficient to confer sialic acid–dependent binding. These data, along with a definition of molecular specificities for Nogo-A and NgR, allowed for the generation of a receptor variant that exhibited potentiated binding to the MAIFs (86). The investigators constructed a chimeric soluble receptor protein, NgR1GM1, which had an increased binding avidity than the wild-type soluble NgR1. The successful therapeutic administration of the NgR1(310)-Fc fusion protein in SCI models (49, 87) and the development of the NgR1GM1 chimeric protein that provides a highly specific MAIF inhibitor for use in various in vivo models of CNS axonal degeneration point to application in EAE and MS.

Inhibition of LINGO-1

Inhibition of the function of LINGO-1 by either genetic means or the use of an antibody antagonist can promote axonal integrity and remyelination in MOG-induced EAE (12). Moreover, the function-blocking LINGO-1–Fc fusion protein potentiated oligodendrocyte differentiation in vitro, and an anti–LINGO-1 monoclonal antibody enhanced remyelination and reduced EAE severity in vivo (44). The remyelinating potential of the anti–LINGO-1 antibody was also shown in 2 neurotoxicant models of demyelination (lysophosphatidylcholine or cuprizone), indicating that effects are unrelated to the inflammatory nature of the demyelination (88). These data advocate for the use of LINGO-1 antagonistic therapies (e.g. LINGO-1–Fc or anti–LINGO-1 antibody) in human demyelinating conditions.

ROCK INHIBITION: PREVENTION OF INFLAMMATION AND AXONAL DEGENERATION

Rho kinase (ROCK) inhibitors have already reached the clinic; more than 30,000 patients are receiving the first generation of ROCK inhibitors, fasudil (89). Fasudil is a moderate ROCK inhibitor (Ki, 330 nM) (90), but the modified dimethyl-fasudil is the most potent ROCK inhibitor to date (91). Clinical trials using fasudil in patients after subarachnoid hemorrhage have not produced serious side effects (92). ROCK has recently been implicated in compromising the blood-brain barrier by phosphorylating claudin-5 and occludin, molecules involved in the maintenance of endothelial cell tight junctions (93). Transendothelial migration of immune cells is a key event in the pathophysiology of EAE and MS, and fasudil treatment inhibited T-cell migration into the CNS (94). In addition to reducing CNS inflammation, it also reduced axonal loss in the proteolipid protein model of EAE (95). The investigators attributed these findings to downregulation of IL-17–producing T cells and T11 cells as a result of fasudil treatment, but we hypothesize that the treatment might also preserve axons through inhibition of ROCK1 activity, thereby reducing axonal degeneration.

Intraperitoneal administration of a single dose of fasudil in clip compression SCI in the rat improved locomotor performance up to 4 weeks after injury (96). By contrast, oral administration of the ROCK I and II inhibitor, Y-27632 (Ki, 140 for ROCK I and 300 for ROCK II), did not produce the same positive effects in locomotor performance (97). Nevertheless, direct administration of Y-27632 to the transected spinal cord promoted axonal growth beyond the lesion site, decreased neurodegeneration, and enhanced locomotor performance in rodents up to 30 days postinjury (58). These data suggested that Y-27632 may not be as effective in crossing the blood-spinal cord barrier as fasudil. The C3 transferase–derived synthetic protein Rho antagonist, Cethrin, is more effective at penetrating the lesion when applied subdurally to effect neurological improvement in patients than either compound (98). Cethrin is currently under a multicenter clinical-phase trial for SCI and is awaiting development for clinical use. Despite the robust data pertaining to the use of these ROCK inhibitors in axonal regrowth and locomotor recovery after SCI, their use in animal models of MS has been minimal. Studies have mainly defined the role of ROCK inhibitors in reducing inflammatory infiltrates (95, 99). Appropriate experiments remain to be done in relation to the direct role of ROCK inhibitors in preventing axonal degeneration or alternatively promoting axonal regrowth after axonal transection has occurred as a consequence of neuroinflammatory CNS lesions. In a similar context, ROCK inhibitors may also play a significant role in remyelination because the molecular signaling mechanisms driving this process in the demyelinated adult CNS are very similar to those of axon outgrowth (99, 100).

CONCLUSIONS

The mechanisms of axonal injury and degeneration in MS and its animal models, at both cellular and molecular
levels, need to be investigated thoroughly to identify novel therapeutic targets and limit or prevent axonal injury and the resultant neurological manifestations. It is likely that multiple mechanisms are involved in axonal injury in these disorders. We propose that the molecular machinery that mediates axon retraction, particularly the NgR1–LINGO-1/RhoA/ROCKII/CRMP-2 that promotes disassembly of the tubulin network within the axon, should be a focus of intense investigation. We also postulate that many of the promising experimental therapeutics that have been examined in animal models of MS, at least in part, exert their effects through modulation of this molecular machinery. Because demyelination is a dominant event in limiting regeneration within the mammalian CNS, understanding the control of these complicated molecular events will be of major significance for attenuating the severity of devastating neurological diseases that involve axonal abnormalities such as traumatic spinal cord and brain injury, stroke, and demyelinating diseases.

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