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focus

Nogo, myelin and axonal regeneration

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Abstract

Adult mammalian central nervous system (CNS) axons have very limited capacity of regrowth after injury. In recent years, advances in the field of axonal regeneration have proved that neurons do not regenerate, mainly because of the presence of inhibitory molecules. Myelin-associated proteins limit axonal outgrowth and their blockage improves the regeneration of damaged fiber tracts. Three of these proteins, Nogo, MAG and OMgp, share a common neuronal receptor (NgR), and together represent one of the main hindrances to neuronal regeneration. The recent molecular cloning of Nogo and its receptors opened a new door to the study of axon regeneration. However, many of the elements involved in the myelin inhibitory pathway are still unknown, and the preliminary experiments with knockout mice are rather contradictory. Because of this complexity, Nogo and NgR need to be characterized before precise strategies to promote axon regeneration in the CNS can be designed.

Keywords: Myelin-associated protein, neurite growth inhibitor, regeneration, Nogo, NgR.

Resum

El sistema nerviós dels mamífers té una baixa capacitat de reparació axonal després d'una lesió. En els últims anys, diversos estudis han demostrat que els axons lesionats no poden recréixer a causa de la presència d'un gran nombre de molècules inhibitòries. Les molècules associades a la mielina limiten el creixement axonal i el seu bloqueig afavoreix la regeneració de diverses connexions. Tres d'aquestes proteïnes, Nogo, MAG i OMgp, comparteixen un mateix receptor: NgR. El clonatge recent de Nogo ha obert noves vies per estudiar la regeneració axonal. No obstant això, molts dels elements involucrats en la via inhibitòria de la mielina són desconeguts, i els primers estudis amb animals *knockout* són, a més, contradictoris. Per aquesta raó, Nogo i el seu receptor han de caracteritzar-se abans de desenvolupar noves tècniques per promoure regeneració axonal.

In the adult mammalian CNS, axons have very limited capacity to regrow after an injury. Shortly after lesion, axons form a new growth cone and begin to regenerate for a short time. However, their growth cone soon collapses and sprouting is aborted [73]. Several hypotheses have been put forward to account for this failure of regeneration: mature neurons lose their capacity of regeneration, the adult environment does not provide neurotrophic support to foster regeneration or, lastly, several inhibitory molecules limit axonal regeneration. Early experiments with transplants of CNS grafts into peripheral nerves proved that mature neurons could regenerate if provided with the right environment [17], thus, the presence of inhibitory molecules is thought to be the main impediment to axonal regrowth, rather than the lack of growth-promoting factors [80]. Molecules associated with the glial scar or with CNS myelin (such as Nogo-A) are the principal obstacle to regrowth of axons after lesion. In this review, we summarize current knowledge of Nogo-A and the other inhibitors associated with myelin, and discuss the importance of Nogo-A and NgR in the failure of CNS neurons to regenerate.

CNS lesions are generally followed by the formation of the glial scar, a structure formed mainly by astrocytes, although it also recruits microglia, oligodendrocyte precursors, and meningeal cells [23]. The glial scar is both a physical and a biochemical barrier. However, the importance of this mechanical impediment is not clear, since in several lesions, even in the absence of glial scar, axons fail to cross the lesion site. The presence of inhibitory proteins, mainly expressed by oligodendrocytes and astrocytes (but also by meningeal and microglial cells), is thought to be the main impediment to axonal regrowth. Astrocytes and oligodendrocyte precursors produce chondroitin sulfate proteoglycans (CSPGs),

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and their expression is upregulated in the glial scar after lesion together with other inhibitory proteins of the extracellular matrix such as Semaphorins (secreted by meningeal cells). In addition, myelinating oligodendrocytes produce inhibitory molecules such as Nogo-A or Tenascin-R [23]. CNS myelin inhibits the regrowth of injured axons; this is mainly due to myelin-associated proteins. Three of these have been identified: Nogo-A, the myelin-associated glycoprotein (MAG) and the oligodendrocyte-myelin glycoprotein (OMgp) [16, 43, 51, 57]. The current model involves a neuronal GPI-linked receptor (the Nogo receptor, NgR), three co-receptors ($p75^{NTR}$, Troy and Lingo), and three ligands expressed in association with myelin: Nogo-A, MAG and OMgp.

Myelin-derived proteins: the ligands

Nogo-A (NI-250)

Two decades ago, Schwab's group identified two proteins in myelin, called NI-250 and NI-35, as potent inhibitors of axonal growth [14]. A monoclonal antibody, termed IN-1, was raised against NI-250 (although it recognized both NI-250 and NI-35), and blocked the inhibition of myelin, allowing axons to regrow [15]. IN-1 promoted axonal regeneration *in vivo* in several injury models [8, 77, 90], but the identity of the antigen NI-250 remained unknown until three groups independently identified it as Nogo-A [16, 28, 68].

Nogo-A is the major transcript of the *nogo* gene (RTN4), which gives rise to three isoforms called Nogo-A, Nogo-B and Nogo-C according to their different promoter usage and alternative splicing (Figure 1) [16]. Nogo (RTN4), together with RTN1, RTN2 and RTN3, is a member of the Reticulon family of proteins, a recently described family whose functions remain unknown [62]. The products of the four reticulon family genes share a common C-terminus that encodes the reticulon-homology domain (RHD) [62]. This conserved 3' sequence contains two predicted transmembrane domains and a dilysine endopasmic reticulum (ER) retention motif, where these proteins are mainly retained. Nogo-A (NI-250), Nogo-B and Nogo-C appear in SDS-PAGE as ~200-250kDa, ~55kDa and ~25kDa bands, respectively. Nogo-A and Nogo-B share a 172 aa N-terminus domain, followed in Nogo-A by a central region (NiG) that is missing in Nogo-B and –C. Nogo-C has a short 11 aa N-terminus domain and all Nogo isoforms have the reticulon-homology domain at the C-terminus (see below) (see Figure 1).

Nogo-A is mainly expressed in the CNS (Figure 2), but also in testis and heart at low levels [35]. Nogo-B has a widespread distribution and Nogo-C is predominantly expressed in skeletal muscle [35]. All Nogo isoforms are expressed in the CNS [62]. At the cellular level, Nogo-A is expressed by oligodendrocytes, as was expected from its association with myelin [16, 28]. Surprisingly, Nogo-A was also found to be expressed by neurons [35, 36, 38, 54, 91] (Figure 2). Neuronal Nogo-A mRNA expression is strong in spinal cord motor neurons, DRG, hippocampus, neocortex, cerebellar cortex, habenular nuclei, piriform cortex, red nucleus, oculomotor nucleus and pontine trigeminal nucleus [35, 36, 38]. Interestingly, developing fiber tracts also express Nogo-A [54, 91], indicating that this protein could have functions other than axonal growth inhibition during development. In addition, we have reported a transitory expression of Nogo by reactive astrocytes after lesion [54].

The predicted topology of Nogo-A is such that the two transmembrane domains situated in the C-terminus (the RTN domain), leave a 66-aminoacid loop into the luminal or extracellular space, while the N-terminus and C-terminus remain on the cytosolic side [16, 28, 68]. This loop, called Nogo-66, induces growth-cone collapse and is exposed extracellularly, at least in oligodendrocytes [28]. This implies that the three Nogo isoforms have potentially inhibitory properties. A second inhibitory domain, situated in the Nogo-A specific region (NiG), inhibits neurite outgrowth and prevents fibroblast spreading [28]. It was hypothesized that this domain could remain intracellular and be released to extracellular space after disruption of the membrane in case of injury. However, NiG has been detected at the cell surface of living oligodendrocytes [63], indicating a second topology in which both Nogo-A active domains were exposed at the surface. Recently, a third inhibitory domain for Nogo was described. This domain is located at the N-terminus of Nogo-A/B and can inhibit 3T3 cell spreading, but has little effect on neurons [63]. Therefore, current research is focused on the characterization of the mechanisms involving Nogo-66 and NiG activity.

As well as its function as a cell-surface signaling molecule, Nogo might have intracellular functions like the other

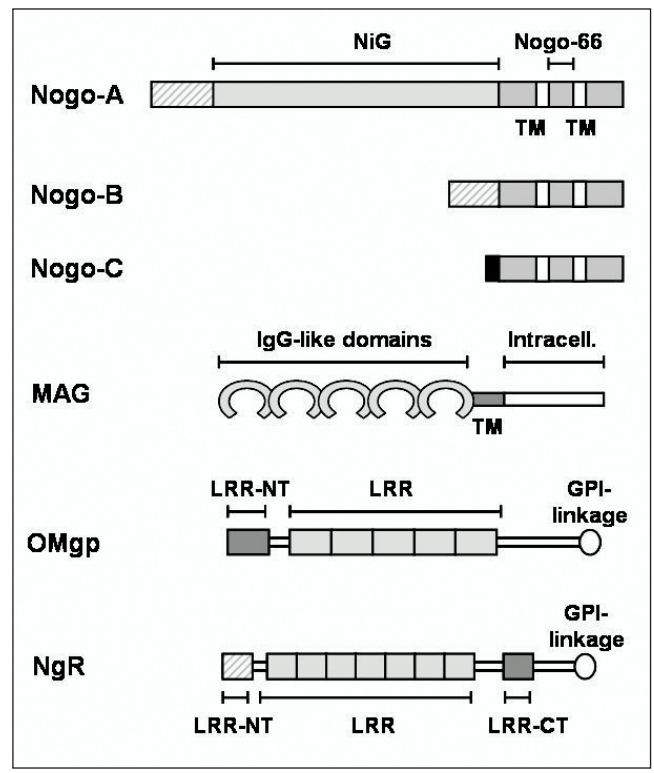

Figure 1. Structure of Nogo proteins, MAG, OMgp and NgR. The different domains are represented. Abbreviations: LRR, leucine-reach repeat; LRR-CT, leucine-reach repeat C-terminal; LRR-NT, leucine-reach repeat N-terminal; TM, transmembrane domain.

reticulon proteins [55, 62]. Cell-surface Nogo accounts for only 1% of total cellular protein. Various intracellular proteins interact with Nogo, such as a novel mitochondrial protein, designated NIMP, which interacts with Nogo-66 in neurons [33], and alpha-tubulin and myelin basic protein in oligodendrocytes [86]. In addition, Nogo-B is proapoptotic [45]. Since Nogo-B is a short form of Nogo-A, lacking the NiG domain, Nogo-A may bind to the same molecules and have a function related to apoptosis. A recent study reports that oligodendrocytic Nogo-A could interact in trans with axonal contactin-associated protein (Caspr) at CNS paranodes, and help modulate axon-glial junction architecture [55, 59].

Myelin-associated glycoprotein (MAG)

The myelin-associated glycoprotein (MAG)/Singlec-4a is expressed by both oligodendrocytes and Schwann cells (CNS and peripheral myelin) [50], and participates in the formation and maintenance of the myelin sheath [49]. MAG has also been suggested as an early mediator of axo-glial interactions [75]. Examination of MAG's inhibitory properties showed that it promoted the outgrowth of young neurons (neonatal DRG), but inhibited adult DRG neurites (and other neuronal populations) [51, 57]. This switch in neuronal responsiveness to MAG (and myelin in general) during development may be determined by changes in the endogenous levels of cAMP [13]. Inhibition of Xenopus growth cones by soluble MAG can be converted to attraction by adding cAMP agonist to the culture media [85], and artificial rising of endogenous cAMP blocks both MAG and myelin inhibition [12].

There are two isoforms of MAG formed as a result of alternative splicing [74], S-MAG and L-MAG, of 67 and 71kDa respectively in SDS-PAGE after deglycosylation. Both proteins share an N-terminus extracellular region formed by five IgG-like domains and a transmembrane domain, but differ in their intracellular domains (Figure 1). MAG is expressed by oligodendrocytes and Schwann cells but not by neurons [50]. L-MAG is the main isoform expressed in developing CNS, whereas S-MAG is the predominant isoform in adult CNS [67]. MAG has been reported to bind to gangliosides GD1a and GT1b, proposed as MAG receptors [89, 93]. However, the implication of gangliosids in MAG-mediated neurite outgrowth inhibition is not clear, and two related proteins have been found to be functional receptors for MAG: NgR and NgR2 [92, 100]. While the binding to the first seems to be sialic acid-independent, at least in non-neuronal cells, NgR2 has been recently identified as a high-affinity and sialic acid-dependent neuronal receptor for MAG [92]. Soluble MAG is released from damaged white matter, inhibiting neurite outgrowth [87, 88], and a soluble chimerical form of MAG (MAG-Fc) inhibited neurite outgrowth of P6 DRG neurons [88]. These observations suggest that MAG could be the main inhibitory diffusible molecule released after injury in the CNS.

Oligodendrocyte-myelin glycoprotein (OMgp)

The oligodendrocyte myelin glycoprotein (OMgp) is a GPI-anchored leucine-reach repeat (LRR) protein which has

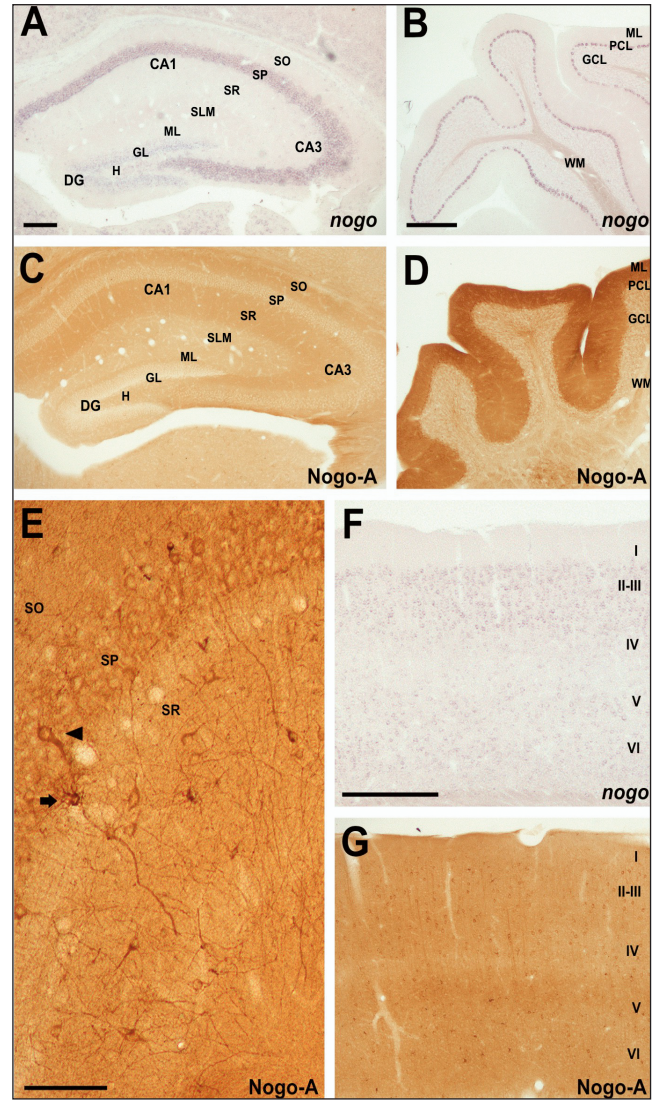

Figure 2. Pattern of expression of nogo and Nogo-A in the CNS. Low power view of the hippocampal region illustrating the distribution of nogo mRNA and Nogo-A protein. A) At P21, *nogo* mRNA is located in the pyramidal layer of the hippocampus proper and, to a lesser extent, in the granule cell layer of the dentate gyrus. B) In the cerebellum, *nogo* expression is restricted to the Purkinje cell layer. C) Immunolocalization of Nogo-A in the hippocampus. At P21, Nogo-A immunostaining is high in the pyramidal layer of the hippocampus proper and also present, but lower, in the granule cell layer of the dentate gyrus. A conspicuous band of immunoreactivity is located in the inner molecular layer of the dentate gyrus. D) Nogo-A protein level is high in the molecular layer of the cerebellum, corresponding to the dendritic trees of the Purkinje cells. E) High-magnification photomicrographs to illustrate details of Nogo-A-immunoreactivity in the CA3 region. Neurons showed multipolar shapes in the stratum radiatum and pyramidal layer (arrowhead). Small immunoreactive cells with multipolar morphologies were also observed in the pyramidal layer (arrow). F) *nogo* mRNA pattern in the cerebral cortex. The different cortical layers are indicated. G) Nogo-A is located in the in the bodies and processes of the principal neurons in the cortex. Some oligodendrocytes are also immunostained. Scale bars: A= 200 µm, pertains to C; B= 100 µm, pertains to D; E= 50 µm= F= 500 µm, pertains to G. Abbreviations: CA1-3, hippocampal fields; DG, dentate gyrus; GCL, granular cell layer; GL, granular layer; H, hilus; ML, molecular layer; PCL, Purkinje cell layer; SLM; stratum lacunosum-moleculare; SO, stratum oriens; SP, stratum pyramidale, SR, stratum radiatum; WM, white matter.

been recently found to inhibit neurite outgrowth in culture [43, 99]. OMgp has 440 aa and migrates in SDS-PAGE gels as a 110-120 kDa band. It is linked by a GPI group to the cell membrane, and lacks transmembrane and intracellular domains. Both mouse and human OMgp have a series of leucine-reach repeats, common in binding proteins (Figure 1) [43]. Deletion mutagenesis shows that OMgp inhibitory activity is independent of the GPI anchor and that deletions in the LRR domains cause loss of function [95]. Screening an expression library, Wang *et al.* [99] identified the Nogo receptor (NgR) as a high-affinity OMgp-binding protein. Additional experiments demonstrated that NgR not only binds OMgp, but is also the functional receptor [20, 48, 99].

OMgp was initially described in myelinating oligodendrocytes, but was subsequently found to be expressed predominantly by neurons [30]. OMgp is present in large projection neurons such as the pyramidal cells of the hippocampus, the Purkinje cells of the cerebellum, motoneurons in the brainstem, and anterior horn cells of the spinal cord [30]. During development, OMgp expression is upregulated, and has a peak of expression in the late stages of myelination [96].

It is interesting that Nogo-A, OMgp and NgR are expressed by neurons. Neuronal OMgp could associate with NgR on the surface of neurons, although this possibility has not yet been tested. OMgp is a potent inhibitor of axonal growth *in vitro*, but its normal physiological role in the CNS remains unclear.

The Nogo receptor

The Nogo receptor was first identified as the Nogo-66 receptor, but has turned out to be the common receptor for the three myelin-associated inhibitors discovered so far (see above and Figure 3). Fournier *et al*. obtained a cDNA in a screening for alkaline-phosphatase (AP) Nogo-66 interacting proteins, which encoded a GPI-linked protein that was called the Nogo-66 receptor (NgR) [25]. Cleavage of GPI-linked proteins from axonal surface turned neurons insensitive to Nogo-66, and NgR expression was sufficient to inhibit unresponsive neurons in contact with Nogo-66.

Another screening, using AP-NgR fusion protein, demonstrated that NgR can self-associate and, surprisingly, also binds MAG [48]. A subsequent study identified the ~80kDa protein that immunoprecipitated with MAG as NgR, which confirmed that NgR was MAG is functional receptor [20]. The third myelin-associated inhibitory protein, OMgp, was also shown to be a ligand of NgR, and needed the receptor to induce growth-cone collapse [99].

NgR is a 473 aa protein, detectable in SDS-PAGE as an ~80-85kDa band. This GPI-anchored protein contains eight central LRRs flanked by a cysteine-rich C-terminus (LRR-CT) and by a leucine-rich N-terminal domain (LRR-NT) (see Figure 1) [25]. Different domain-deletion analyses were run to identify the NgR domains involved in ligand binding or co-receptor interaction, with conflicting results. Fournier *et al*. [26] reported that all NgR domains (LRR-NT, LRR and LRR-CT) are required for Nogo-66 binding. However, Wang *et al.* [100] indicated that the LRR-CT domain was necessary and sufficient to bind Nogo-66. A recent study provides evidence that Nogo-66, like MAG and NgR itself, requires only the LRR region of NgR to bind to the receptor at the cell surface [4]. Both the LRR domains and the more carboxyl regions (LRR-CT) are necessary for association with p75 and OMgp [100]. In addition, it is not well established whether MAG and Nogo-66 compete for binding with NgR [20, 48]. More studies are needed to clarify which NgR domains are needed for the interactions with the three ligands or co-receptors, and whether these bindings compete with each other or not.

NgR mRNA is expressed in adults mainly through cerebral cortical neurons, cerebellar Purkinje and granule cells, pontine nucleus, deep nuclei, habenular nucleus and hippocampal neurons [25, 36, 39]. It is pertinent that adult spinal cord downregulates its levels of NgR mRNA, which become barely detectable in both mice and humans [39]. This absence of local expression refers to interneuron somas, since cortical projection neurons do express NgR mRNA, which is detectable in the cortical layers together with the protein [39, 101]. Similarly, DRG cells also downregulate NgR mRNA expression in the adult [36, 39]. Some regions, such as the striatum or medial septal nucleus, do not express NgR at any developmental stage [36, 39].

Neuronal responsiveness to Nogo-A seems to depend on the developmental stage [13, 54]. Embryonic rat dorsal root ganglion and chick retinal ganglion cells are only a little sensitive to Nogo-A, but their adult counterparts collapse and increase intracellular calcium levels when exposed to this inhibitor [2]. These experiments show that neuronal sensitivity to Nogo-A is acquired around the time of myelination, which reinforces the hypothesis that Nogo-A has some function during development unrelated with its inhibition *via* NgR.

The coreceptors

Before the cloning of NgR, Yamashita *et al*. reported that p75, the low-affinity neurotrophin receptor, is necessary to the inhibition of neurite outgrowth by MAG, and that cerebellar and spinal sensory neurons from p75-deficient mice were unresponsive to MAG [105]. They also indicated that MAG's inhibition is mediated by an activation of the GTPase RhoA in the presence of p75 [105]. Subsequently, p75 was shown to be the coreceptor of NgR and to mediate the signal transduction of Nogo, MAG and OMpg [100, 104] (Figure 3). In accordance with this, axonal outgrowth is enhanced in the myelinated portions of the CNS in p75 mutant mice [98]. Priming of neurons with neurotrophins has been reported to reduce myelin and MAG inhibition [12], which could be due to coreceptor competition between Trks and NgR as well as to changes in intracellular cAMP levels.

p75 interacts with NGF and modulates the activity of Trk receptors [18]. It colocatizes with NgR in embryonic neurons [104], but its expression is downregulated during development and adult neurons express background levels of it. It has been recently reported that Troy, a NTF receptor family member, can replace p75 in the adult CNS and activate RhoA in response to myelin-associated inhibitors [65, 81]. Lastly, a LRR protein named Lingo-1 has been shown to form a complex with NgR and either p75 or Troy, and be necessary for the inhibition to occur [53].

Other receptors

The discovery of NgR as a central point of myelin-mediated inhibition could lead us to think that a single receptor is responsible for the failure of CNS axons to regenerate. However, we should be cautious with these observations. As we will discuss at the end of this review, myelin is not the only source of inhibition in the adult CNS, and even for myelin, more than one receptor seems to be involved. For instance, the neuronal receptor for NiG (Nogo-A specific domain) has yet to be identified. NiG not only inhibits neuronal outgrowth, but also prevents non-neuronal cells (e.g. fibroblasts) spreading, so its receptor must be widely expressed [25, 28, 60]. Oertle *et al.* found NiG is exposed at the surface in mature oligodendrocytes and can bind to responsive cells and brain cortical membranes, which indicates the existence of the unknown receptor [63].

In addition, MAG binds to NgR2, a receptor structurally related with NgR [92]. Niederost *et al.* have also reported that phospholipase treatment does not completely block the inhibitory effects of MAG on cerebellar granule cells, suggesting that no GPI-linked protein is needed for MAG mediated inhibition [60], and some other receptors, as the gangliosides GT1b and GD1a could act as MAG receptors [87, 93].

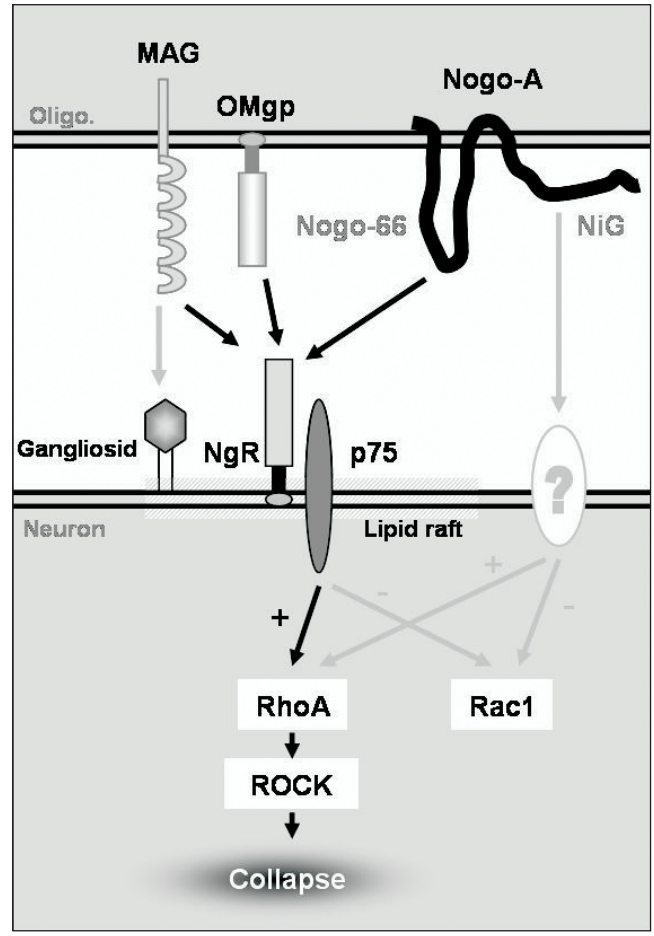

Figure 3. Schematic representation of the proposed interactions between NgR and its ligands. Black arrows symbolize well established interactions; grey arrows correspond to supposed interactions. Nogo-66, MAG and OMpg bind to NgR, and its association with p75 activates Rho and ROCK, leading to the growth cone collapse. In addition, interaction of MAG with gangliosids may facilitate NgR-p75 binding. The putative neuronal NiG receptor and the subsequent activation of Rho and inhibiton of Rac by NiG binding are also represented in this figure. Although the complete receptor complex is formed by NgR, p75 or Troy and Lingo-1, only p75 has been represented. Abbreviations: MAG, myelin-associated glycoprotein; NgR, Nogo receptor; Oligo., oligodendrocyte; OMgp, oligodendrocyte myelin glycoprotein; ROCK, Rho-kinase.

Intracellular signaling

It was suggested that inhibition of axonal outgrowth by Nogo-A and MAG was dependent on Ca^{2+} signaling [1, 104]. However, it remains unclear how cytosolic calcium participates in NgR signal transduction. GTPases are well characterized as mediators of growthcone collapse. p75 modulates RhoA activity after neurotrophin binding [105] and also modulates GTPase activity in response to NgR ligands [102]. Yamashita and Tohyama described how p75 releases RhoA from Rho-GDI (which prevents RhoA activation) after NgR binding to Nogo-66 and MAG, allowing RhoA to become activated, which promotes growth-cone collapse [106]. Consistent with this, Nogo-A and MAG signaling involves the inactivation of Rac1 [60]. Both GTPases have antagonistic effects and are involved in the axonal guidance cues mediated by other molecules such as Ephrin A1, sema3A and Netrin [19]. However, the inhibition of ROCK, a downstream effector of Rho, blocks Nogo and MAG activities. Thus, the NgR intracellular pathway could involve the activation of LIM kinase (a target of ROCK), which inactivates the protein cofilin and promotes growth-cone collapse (Figure 3) [60].

As previously mentioned in this review, treatment with neurotrophins blocks inhibition caused by MAG and myelin [12]. This interaction has been attributed to an elevation of cAMP by neurotrophins. Artificial elevation of cAMP with analogs also blocks inhibition by myelin and is dependent on PKA activation [12]. Therefore, the activation of PKA interferes with some step in the myelin-signaling pathway. A second important kinase regulating myelin-induced inhibition is PKC [31, 84], whose blockade *in vivo*, similarly to elevation of cAMP *in vivo*, promotes spinal cord regeneration, proving that interference with some elements of the NgR intracellular pathway can be useful to try to promote axonal regeneration [70, 84].

Functional studies in KO mice

In vitro outgrowth assays and *in vivo* neutralizing experiments show that the independent elimination of any of the NgR ligands, NgR itself or even p75, substantially improves axonal regeneration. MAG and p75 mutant mice were generated and described before the discovery of their involvement in myelin-mediated inhibition. Recently, three groups have independently produced Nogo deficient mice. However, Nogo and MAG knockout mice lack just one of the NgR ligands, and the p75-deficient mice phenotype is affected by alterations in neurotrophin signaling. Thus, the study of NgR knockout mice could provide us with crucial information about the role of myelin-associated inhibition in the failure of injured CNS axons to regrow. Unfortunately, the OMgp knockout mice phenotype has not yet been reported.

Nogo knockout mice

Three independent groups have generated four lines of mice lacking just Nogo-A, both Nogo-A and Nogo-B and a knockout line lacking Nogo-A, –B and –C [41, 83, 110] (see Table 1). Contrary to what was expected from *in vitro* experiments and antibody blockage of Nogo *in vivo*, there was no clear improvement in the regenerative capacities of Nogo-deficient mice. The first mutant mice, generated by Schwab's group, lack Nogo-A expression due to gene targeting of exon 3 (which encodes the Nogo-A-specific central domain) [83]. In these mice, a compensatory upregulation of nogo-B could be observed in oligodendrocytes (see Table 1). Strittmatter's group produced Nogo-A/B mutant mice by inserting a retroviral trapping vector into the Nogo gene, thus disrupting both Nogo-A and Nogo-B, but not Nogo-C [41]. A second Nogo-A/B knockout line was generated by the Tessier-Lavigne group's deletion of the amino-terminal genomic fragment that includes the entire exon 1, shared by Nogo-A and –B, mutant mice had no behavioral alteration or differences in their brain histology, although little information about their CNS anatomy was reported. Corroborative *in vitro* assays demonstrated that Nogo was a powerful inhibitor of axonal growth, as the inhibitory properties of myelin extracted from all the different Nogo mutant mice were very low. Oligodendrocytes of Nogo mutant mice were indistinguishable from wild-type oligodendrocytes and myelin structure was not altered, indicating that the presence of Nogo in myelin is not related to the maintenance of the myelin sheath.

Blockage of Nogo-A *in vivo* with antibodies after spinal cord injury has been seen to enhance the regeneration of the descending corticospinal tract (CST) [8, 9], but failed to promote regeneration of ascending sensory axons [64]. Therefore, the regenerative capacities of Nogo-deficient mice were determined by examination of the regeneration of CST axons after dorsal hemisection of the spinal cord. After spinal cord lesion, the CST axons of the young adult Nogo-A/B mutant mice generated by Strittmatter's group sprouted extensively and regenerated into distant cord segments, improving the functional recovery of the animals [41]. However, it should be emphasized that this increased regeneration was restricted to young animals, suggesting that Nogo was not one of the inhibitors in older animals. Simonen *et al.* obtained much more modest results with the Nogo-A transgenic line [83]: their test was qualitatively comparable with the previous example, but quantitatively lower. Though the upregulation of Nogo-B in these mice could attenuate the deficiency of Nogo-A, Nogo-B was not present on the surface of oligodendrocytes and there was little *in vitro* neurite outgrowth inhibitory activity of central myelin from these mutant mice, which did not support a compensatory inhibitory effect of Nogo-B. The third group did not report evidence that any of their Nogo mutant mice (Nogo-A/B knockout and the Nogo-A/B/C deficient line) in-

Table 1. Nogo-deficient mice characteristics.
Summary of the main characteristic presented by the different Nogo-deficient mice. Groups that generated the different lines and mice genotypes are indicated. Y and N refer to the presence or absence of the characteristic indicated.

	Schwab group[a]	*Strittmatter group*[b]	*Tessier-Lavigne group*[c]	*Tessier-Lavigne group*[d]
	Nogo A	*Nogo A/B*	*Nogo A/B*	*Nogo A/B/C*
Changes is viability, fertility or behavior	N	N	N	N
Changes in CNS anatomy	N	N	N	N
Changes in myelin structure	N	N	N	N
Nogo-B expression	upregulated	–	–	–
Nogo-C expression	unchanged	unchanged	not tested	no functional
Myelin inhibitory activity *in vitro*	N	N	N	N
Enhanced regeneration *in vivo*	modest	Y[e]	N	N

[a] Simonen *et al.* 2003 [83].
[b] Kim *et al.* 2003 [41].
[c] Zheng *et al.* 2003 [110].
[d] Nogo-A/B/C knockout line was originally lethal, except for one mouse, carrying a mutation that lead to the expression of a non functional Nogo-C (see text for more details).
[e] only young mice.

creased regeneration or sprouting. This could indicate that Nogo-A/B contributes to inhibition, but their absence is not enough to permit axonal regeneration. These results are very surprising. As stated above, *in vivo* delivery of antibodies against Nogo-A not only enhanced axonal regeneration, but permitted the regeneration and sprouting of CST axons in the same model that was employed for the characterization of mutant mice. Differences in genetic background, compensatory changes or alteration of other loci as a result of gene-trap insertion might explain these discrepancies. Further studies are needed to determine the causes of the variations in the mutant mice and clarify the function of Nogo in the CNS.

Two additional Nogo mutant mice generated give us important information. These mice expressed Nogo-A or Nogo-C in Schwann peripheral nerve cells [41, 69]. Axonal regeneration after sciatic nerve lesion in these mutant mice was delayed or inhibited, demonstrating that Nogo-66 has a function as an inhibitor of axonal regeneration *in vivo* and is exposed at the cellular surface.

MAG

The first studies of MAG pointed to a relationship of this protein with the formation and maintenance of the myelin sheath, and MAG-deficient mice were generated to verify these roles. In the CNS of MAG-deficient mice, the integrity of myelin is altered, the initiation of myelination is delayed, and the formation of morphologically intact myelin has abnormalities [56, 75]. However, when MAG was found to be a potent inhibitor of axonal growth *in vitro* [51, 57], the regenerative capacities of MAG-deficient mice were tested. Axonal regeneration in the injured optic nerve and corticospinal tract in MAG-deficient mice either was no different from in wild-type animals [5] or enhanced axon regeneration only a little [44]. In addition, myelin from MAG-mutant mice was a potent inhibitor of axonal growth *in vitro* [44, 61], indicating that there had to be other axonal growth inhibitors associated with myelin, as was subsequently shown.

The lack of axonal regeneration in MAG-deficient mice, together with the changes observed in myelin, indicated that MAG is involved in the formation and maintenance of myelin and did not support MAG as a functional inhibitor of axonal growth *in vivo*. However, the number of regenerating axons after sciatic nerve lesion of C57BL/Wlds mice (slow-degenerating mutant mice) was doubled if the MAG gene was eliminated [76], providing some evidence that MAG actually can inhibit axonal growth *in vivo*.

p75

Whereas in Nogo-A and MAG knockout mice, just one of the NgR ligands is missing and their regenerative capacities are conditioned by the presence of the other two NgR ligands; in p75-deficient mice a major part of the NgR signaling system is impaired. Neurotrophin signaling is also affected in these animals, conditioning the final phenotype, however, neither Lingo-1 nor Troy deficient mice regenerative potential has been reported, and p75 knockout mice phenotype is the only data available.

In p75 knockout mice, the lack of the receptor affects various neurotrophic features, such as neuronal size, neurotransmitter synthesis and target innervation [107]. However, there are not many reports about CNS regeneration in these animals. Adult sympathetic neurons from p75-deficient mice can grow in CNS myelin, which inhibits wild-type mice neurons [98]. This provides the best evidence for p75's function in axonal growth inhibition *in vivo,* although it is not clear whether sympathetic neurons express NgR. *In vitro*, the first experiments demonstrating that p75 was necessary for MAG-dependent inhibition also showed that DRG and cerebellar neurons from p75 knockout mice were not inhibited by MAG [105]. Subsequently, when the interaction of p75 with NgR was described, the sensitivity of p75 knockout mice neurons to Nogo and OMgp was also tested, with the finding that they are no longer responsive to myelin or to the known NgR ligands [100]. Recently, the regenerative potential of p75-deficient mice after spinal cord compression injury was tested, but functional recovery in these animals increased no more than in wildtype controls [21]. To develop our understanding of the normal role of p75 in growth inhibition, additional experiments should be performed with p75-knockout mice (such as unilateral pyramidotomy, which gives good results with Nogo and NgR knockout mice).

NgR

During the final stages of writing this review, the first information about NgR knockout mice was made public [42, 111]. Two lines of NgR deficient mice have been generated by independent groups, and a different regenerative potential has been reported for them. Cultured neurons from NgR knockout mice generated by Dr. Strittmatter's lab had low sensitivity to myelin *in vitro*, and the functional recovery of the knockout mice after mid-thoracic dorsal hemisection or complete transection of the spinal cord, improved significantly, although corticospinal fibers did not regenerate [42]. In sharp contrast with this report, neurons from the NgR knockout mice generated by Dr. Tessier-Lavigne's lab are inhibited by myelin (and by purified Nogo-66) in the same extent as wild type neurons, and regeneration after spinal cord hemisection was not improved in vivo [111]. Together, these reports indicate that NgR is only partially responsible for limiting axonal regeneration in the adult CNS.

Therapeutic approaches

NgR and its three ligands are central to restricting axonal regeneration, and represent a promising target for new therapies. The blockage of Nogo inhibitory domains with both antibodies and peptides *in vivo* has resulted in extensive regeneration. In addition, therapeutic vaccination against myelin has achieved encouraging results. This indicates that selective targeting of myelin inhibitors could be a therapeutic tool.

Blocking Nogo in vivo

a) IN-1

The monoclonal antibody IN-1 was used in many *in vitro* and *in vivo* studies prior to the molecular characterization of the Nogo-A antigen. Now we know that IN-1 recognizes the central domain of Nogo-A (exon 3) [24], a region not shared with Nogo-B, which was initially proposed to be the protein NI-35 recognized by IN-1. Since Nogo-B lacks the IN-1 epitope, NI-35 is more likely to correspond to a proteolytic fragment of Nogo-A.

Most of the experiments *in vivo* with IN-1 focused on the corticospinal tract (CST). The cortical neurons express high levels of both NgR and Nogo-A [37]. The first studies, by Schnell and Schwab (see Table 2), used IN-1-producing hybridomas, implanted intracerebrally, to try to promote axonal regeneration after lesion of the corticospinal tract [77, 78]. Treatment with IN-1 increased the length of the CST axons, allowing long-distance regeneration. This length was enhanced by NT-3 treatment and the regenerating axons were mainly found in the CST [79]. Subsequently, IN-1 treatment improved not only regeneration but also functional recovery [8, 72, 94]. However, regenerating axons grew in ectopic areas rather than in the former CST, and avoided the lesion site, extending through the intact tissue. In fact, compensatory sprouting from the uninjured fiber tract was found. Complete transections, in which regeneration could only occur if the lesion site was crossed, would indicate whether the functional recovery was due to regenerating axons or to the sprouting of collateral, intact axons. However, this kind of lesion has not been tested in experiments with IN-1.

Along with the CST axons, other types of CNS axons respond to IN-1 treatments (see Table 2). Treatment with IN-1 enhances the regeneration of the rubrospinal [51], corticofugal [102, 109], and corticostriatal tract [40] (see Table 2). In all these cases, IN-1 induced compensatory sprouting across the spinal cord midline, improving functional recovery. Other fibers such as septohippocampal axons [11], the auditory nerve [89] and the cortico-efferent projections [66] are other examples of fiber tracts that respond to IN-1 treatment. However, administration of IN-1 failed to promote the regeneration of ascending sensory axons across a peripheral nerve bridge back into the adult spinal cord [64]. This experiment suggests that inhibitors other than Nogo-A prevent

Table 2. IN-1 *in vivo* treatments.
Summary of the main *in vivo* experiments performed with IN-1 and their characteristics. References are in chronologic order. “yes” indicates functional recovery. “compensatory sprouting” represents inervation from the unlesioned hemisphere

Reference	*Type of lesion*	*Neuronal population studied*	*Treatment*	*Anatomical Regeneration*	*Functional regeneration*
[77-79]	Bilateral Pyramid.	CST	IN-1 secreting hybridoma intracerebral	Long-distance regeneration	Yes
[11]	Fimbria/fornix	cholinergic Septohippocampal tract	human amnion extracellular matrix material containing NGF and NI-1	Moderate	Non tested
[94]	Chronic injury CST	CST	Delayed IN-1 and NT3 treatment	moderate	Slight
[8, 90, 109]	Unilateral pyramidotomy	CST	IN-1 secreting hybridoma cells into the hippocampal formation	Compensatory sprouting Long-distance regeneration	Yes
[40]	Unilateral aspiration lesion	Corticostriatal tract	IN-1 secreting hybridoma cells into the lesion cavity	Compensatory sprouting	Yes
[9]	Unilateral pyramidotomy	CST	IN-1 Fab into the injury	Long-distance regeneration	Non tested
[64]	Nerve graft paradigm*	Sensory axons	IN-1 secreting hybridoma cells into the cerebral cortex or thoracic cord	None	Yes
[6]	Unilateral pyramidotomy	Corticopontine tract	IN-1 secreting hybridoma cells into the contralateral cerebral cortex or hippocampus	Compensatory sprouting	Non tested
[61-62]	Bilateral Pyramid.	rubrospinal tract and CST	IN-1 secreting hlybridoma cells into the hippocampal formation	Compensatory sprouting	Yes
[3]	Unilat eral pyramidotomy	CST	IN-1 secreting hybridoma cells into the hippocampal formation	Compensatory sprouting	Non tested
[89]	Section of audirory nerve	Cochlear nerve fibers	IN-1 Fab intrathecal	Long-distance regeneration	Yes
[66]	Ischemic lesion	Cortical neurons	IN-1 secreting hybridoma cells posterior to the site	Compensatory sprouting	Yes
[22]	Unilateral SMC lesion	sensorimotor cortex	IN-1 secreting hybridoma cells into the hippocampal formation	functional reorganization of the intact motor cortex	Yes

Abbreviations : CST: corticospinal tract; Pyramid., Pyramidotomy (lesion of the corticospinal tract); SMC, sensorimotor cortex;
* conditioning lesion of the peripheral proyection of the sensory neurons prior to implantation of the nerve graftin the spinal cord.

the regeneration of the central processes of DRG neurons (from the graft) into the spinal cord.

As commented above, IN-1 antibody enhances the sprouting of both injured and intact neurons and increases the expression of growth-associated genes. A recent study aimed to determine the effects of IN-1 on the uninjured hemisphere after unilateral sensorimotor cortex (SMC) lesion [22]. It showed intact neurons sprouting into the injured hemisphere and innervated deafferented subcortical targets, leading to an increase in functional recovery. In similar experiments reporting functional recovery after IN-1 treatment, this was attributed to the plasticity or sprouting induced by the antibody rather than to true regeneration, as observed in cortical projection neurons [3, 40]. In uninjured animals, IN-1 promotes sprouting of corticospinal axons, which grow into abnormal territories [3]. In addition, uninjured Purkinje cells sprout within cortical grey matter when treated with IN-1 [10]. Since sprouting is a necessary step prior to regeneration, these data suggest that IN-1 may disturb some signals controlling this initial process (such as NiG). Afterwards, induced axonal sprouting could lead to regeneration if axons are provided with the necessary signals to regrow in their previous direction. Supporting this theory, IN-1 treatment has been reported as leading to an increase in the expression levels of growth-associated genes, such as GAP-43, in the spinal cord and Purkinje cells [3, 108]. In addition, the transcription factors c-Jun and JunD and NADPH diaphorase are upregulated after treatment with IN-1 in both injured and intact Purkinje cells [108].

b) NEP1-40

IN-1 (both original and its recombinant F_{ab} fragment) has very low affinity and limited specificity for Nogo. This has hampered the use of the monoclonal antibody. An improved therapeutic agent has been developed to provide a specific blockage of Nogo functions: the NEP1-40 peptide [29]. The competitive agonistic peptide of NgR is derived from amino-terminal fragments of Nogo-66 (corresponding to residues 1-40 of the Nogo-66 loop) and inhibits its binding to NgR [29]. NEP1-40 does not compete with MAG [48], indicating that MAG and Nogo-66 bind to different NgR domains.

Treatment with NEP1-40 promoted significant regeneration of the CST in rats with mid-thoracic spinal cord hemisection and increased their functional recovery [29]. Moreover, NEP1-40 administration delayed for up to one week was as effective as immediate treatment [46]. Systemic therapy with subcutaneous NEP1-40 improved sprouting of serotonergic fibers, upregulated axonal growth protein SPRR1A levels and permitted extensive growth of corticospinal axons together with synapse re-formation after thoracic spinal cord injury [46]. These data indicate the central functions of Nogo-66 and NgR in limiting axonal regeneration after spinal cord injury and suggest that NEP1-40 is a therapeutic agent.

In addition to antibodies and peptides, a soluble function-blocking NgR ectodomain (which binds to Nogo-A, MAG and OMgp and prevents them from binding to their receptors) has been administered to spinal cord injured rats obtaining increased axon growth and functional recovery [47].

Vaccination

Vaccination against myelin-inhibitory antigens is a recent therapeutic approach to promote axonal regeneration. Stimulation of the immune system to produce antibodies against myelin-associated inhibitors resulted in a strong regeneration of the CST after dorsal hemisection [34]. Subsequently, more precise vaccination against specific myelin components, such as NgR ligands, was performed with similar results. Nogo-A antibody infusion 24 hours before lesion permitted functional recovery in a stroke model [103]. After partial crush injury, rats' optic nerve recovery was significantly promoted by post-traumatic immunization with a peptide derived from Nogo-A [32], and long-distance axon regeneration and sprouting of the corticospinal tract was seen in myelin and Nogo-66/MAG immunized mice CST [82]. Current studies are trying to make immunotherapy as effective as possible and to minimize the risks of auto-immune diseases in response to immunization [52].

Nogo, NgR ligands, and axonal regeneration

As many molecules are involved in the failure of CNS axons to regenerate, varied therapeutic approaches are being studied, most of them giving encouraging results. The specific blockage of Nogo and vaccination against myelin-associated inhibitors are some of the strategies able to promote neuronal regeneration. Other current strategies are the degradation of CSPGs by Chondroitinase ABC [7], the use of cyclic nucleotide analogs [58, 70], blockade of PKC [84] and the inactivation of Rho GTPase [27]. However, interfering with Nogo signaling, for instance, could have disastrous consequences if axonal inhibition is just a secondary function of the protein. Therefore, the characterization of the normal functions of myelin inhibitors will determine the viability of the blockage of NgR ligands to promote regeneration. Even if the main role of these proteins is related to regeneration, as inhibition of axonal sprouting, their blockage could lead to uncontrolled sprouting of uninjured neurons and result in improper connections (with the consequent abnormal functions). As p75 also has numerous functions, it would not be a good target, and functions of Troy or Lingo-1 are unknown. With the current knowledge about the role of myelin in preventing regeneration (summarized in this review), the use of NgR antagonists seems to be the most promising, and cautious, therapeutic tool. However, fully characterization of NgR-deficient mice will be needed to clarify the contribution of myelin to regenerative failure and allow us to evaluate the therapeutic potential of interference with the shared receptor.

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About the authors

Since 1995 the research group on Regeneration and Repair of the Nervous System of the Department of Cell Biology at the University of Barcelona has focused on the study of the molecular and cellular clues responsible for the development and regeneration of neuronal connections. Using the entorhinal-hippocampal connection as an in vitro model the group has analysed various factors involved in the absence of regeneration of the pathway, such as semaphorins, netrins and, more recently, molecules derived from myelin. Among the most important results of the group, we could highlight the discovery and characterisation of the functions of a transitory population of neurones, the Cajal-Retzius cells, which are responsible for the establishment and maturation of part of the connections in the hippocampus. We also reported the molecular characterisation of the functions of Nogo and its receptor during the development and regeneration of entorhinal-hippocampal connections. For this work the group uses various techniques in vitro and in vivo, including systems for the reconstruction of neuronal cytoarchitecture in organotypic cultures of the nervous system and histological methids. The main results have been published in several international scientific journals and the studies have been financed by national and international grands.