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Nogo and axon regeneration

Martin E Schwab

Nogo-A is one of several neurite growth inhibitory components present in oligodendrocytes and CNS myelin membranes. Nogo has a crucial role in restricting axonal regeneration and compensatory fibre growth in the injured adult mammalian CNS. Recent studies have shown that *in vivo* applications of Nogo neutralizing antibodies, peptides blocking the Nogo receptor subunit NgR, or blockers of the postreceptor components Rho-A and ROCK induce long-distance axonal regeneration and compensatory sprouting, accompanied by an impressive enhancement of functional recovery, in the rat and mouse spinal cord.

Addresses
Brain Research Institute University of Zurich, and Department of Biology, Swiss Federal Institute of Technology, Winterthurerstrasse 190, 8057 Zurich, Switzerland
e-mail: schwab@hifo.unizh.ch

Current Opinion in Neurobiology 2004, **14**:118–124

This review comes from a themed issue on
Development
Edited by Barry Dickson and Christopher A Walsh

0959-4388/$ – see front matter

DOI 10.1016/j.conb.2004.01.004

Abbreviations

CST	corticospinal tract
mAb	monoclonal antibody
MAG	myelin-associated glycoprotein
OMgp	oligodendrocyte myelin glycoprotein
RTN	reticulon

Introduction

''The lame shall walk.'' (Matthew chapter 11, verse 5)

Paraplegia has been a model of human suffering since ancient times. The crucial medical issues are management of the paralysed bladder, prevention of decubitus and wound infections, assuring respiration for individuals with high-level injuries, stabilizing the spinal column, and finally training for a life in a wheelchair. Traditionally, neurology has had almost no role in this field: spinal cord damage has been considered to be incurable and out of the reach of medical intervention.

Over the past 15 years, however, spinal cord injury research has become a focus of neuroscience research. Important new knowledge has been gained, and there is a strong push towards clinical application. The next few years will tell us whether we will be able ''to make the lame walk'', to stand-up and move around for short distances from the bed to the kitchen or bathroom, to control their bladder and to breathe autonomously.

Human spinal cord injuries are usually contusions or partial, rarely complete, transections. The primary mechanical damage is followed by a complex process of secondary damage, in which ischaemic and inflammatory processes have a major role. Inflammation seems to have both damaging and tissue-protective effects. Massive axotomies of descending and ascending tracts, local loss of neuronal elements and glial cells, myelin damage and the formation of cysts and scars characterize the pathophysiological evolution of spinal cord injuries [1–3].

Many injured CNS fibre tracts react to the lesion with a clear, but only short-lasting repair response: they produce sprouts from the cut ends or as collaterals, and the respective cell bodies upregulate growth proteins, such as GAP-43. Sprouting turns into long-distance regeneration in a peripheral nerve environment (e.g. in a nerve graft [4]), but not in CNS tissue, which seems actively to inhibit neurite growth [5].

Three lines of evidence support the crucial role of myelin-associated neurite growth inhibitors in preventing CNS regeneration. First, deleting oligodendrocytes or myelin enhances the regeneration of descending tracts in the differentiated cord of rats, mice and chicken (reviewed in [1]). Second, antibodies against Nogo-A (also called NI-220/250 or IN-1 antigen) applied via the cerebrospinal fluid (from antibody-producing hybridoma implants or pumps) enhance regenerative sprouting and long-distance elongation [6,7]. Third, autoimmunization of mice or rats with myelin or spinal cord homogenates allows regenerative sprouting and growth after spinal cord lesions [8].

Nogo A was first purified as a high molecular weight, highly inhibitory novel membrane protein of spinal cord myelin [9,10], and its cDNA was cloned in 2000 [11–13]. The myelin proteins myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) and the proteoglycans V2 and brevican are additional neurite growth inhibitory components found in CNS white matter [14–16]; however, their roles in regeneration and repair *in vivo* are still largely unknown. In the present review I summarize the current evidence for the role of Nogo-A in axonal regeneration mechanisms of functional recovery after CNS injury.

The neurite growth inhibitory protein Nogo-A

Nogo-A is a membrane protein of 1163 amino acids (rat sequence, apparent molecular weight 200 kDa) that is expressed in the adult mammalian CNS mainly by oligodendrocytes [11–13,17•,18•]. The neuronal expression of Nogo-A is pronounced during development but low in the adult nervous system [17•,18•,19]. The splice form Nogo-B (360 aa, 55 kDa) is found in many tissues and cell types including adult neurons, whereas Nogo-C (190 amino acids, 25 kDa) is expressed mainly in muscle [17•]. The functions of Nogo-B and Nogo-C are currently unknown. All three main products of the gene encoding Nogo share a sequence of 188 amino acids at their carboxy (C) terminus. This sequence shows the only detectable homology to other proteins — namely, the small family of reticulon (RTN) proteins (Nogo would be RTN4). Some of the RTNs are expressed in the nervous system, but they are also expressed in other tissues (reviewed in [20]).

Most of the known RTN proteins have a relatively short amino (N)-terminal sequence, which is similar to that of Nogo-B and Nogo-C. The RTN/Nogo family is evolutionarily very old and occurs in all eukaryotes including plants and fungi [21]. The very long N-terminal sequence of Nogo-A appears very late in evolution — at the frog level — and suggests that Nogo-A is the result of a fusion between an ancient RTN homology domain at the C terminus and 2–3 open reading frame sequences at the N terminus, whereby the protein may have adopted a new function — that of a neurite growth inhibitor in oligodendrocytes [20,21]. This would be in line with the well-known, high regeneration potential of the spinal cord after lesion in fish and salamanders, which lack Nogo-A.

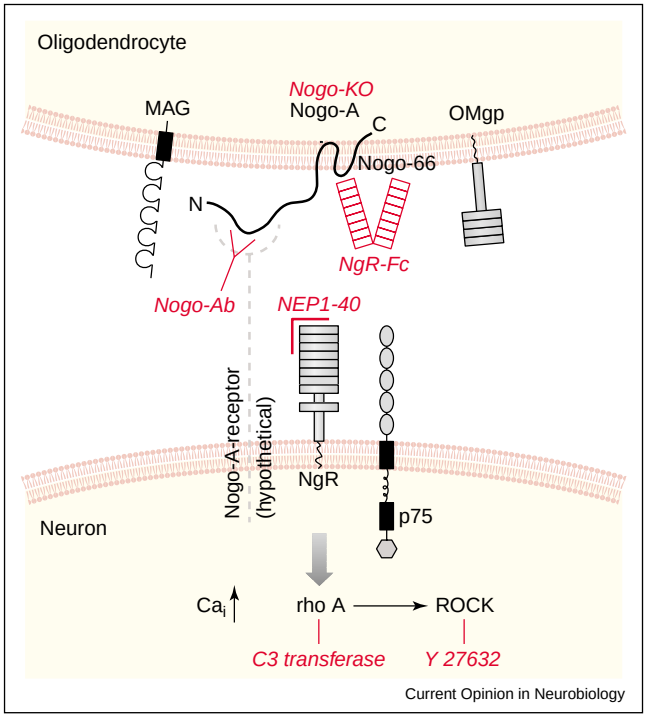
Peptide fragment analysis of Nogo-A has shown that neurite growth inhibition, growth cone collapse and inhibition of fibroblast spreading are associated with 2–3 distinct regions of the molecule [13,22•,23••]. A principal inhibitory region is found in the middle of the Nogo-A-specific sequence (amino acids 544–725) [22•]. The 66-residue loop between the two hydrophobic regions in the RTN homology domain ('Nogo-66') is also able to inhibit neurite growth and to induce growth cone collapse [12,23••]. All of the active sites of Nogo-A are exposed extracellularly on the cell surface of cultured oligodendrocytes [22•].

Neurite growth inhibition by Nogo-A: receptors and intracellular messengers

So far, only one binding site/receptor subunit for Nogo-A has been characterized: the 443-residue glycosyl-phosphatidylinositol-linked, leucine-rich repeat glycoprotein NgR [24–26]. This receptor binds to the region of 66 amino acids in the C-terminal domain that is common to Nogo-A, -B and -C. Interestingly, NgR also binds to the neurite growth inhibitory myelin proteins MAG and OMgp [15,27]. It is complexed with the low-affinity p75 nerve growth factor (NGF) receptor, which may act as a signal transducing subunit [15,27,28]. Although high-affinity binding to an inhibitory site that is specific to Nogo-A has been shown [22•], the receptors that bind to this site remain to be characterized. Similar to Semaphorins, Netrins and many neurotrophic factors, Nogo-A may interact with a multisubunit receptor complex, and a similar situation may exist for MAG and OMgp (Figure 1; [15,27]).

Two intracellular components of Nogo signalling have been identified so far: calcium, and the Rho-A/Rho kinase (ROCK) pathway [15,27–31]. How these messengers are linked is still unknown. Importantly, the inhibition of each one of these components by appropriate blockers can prevent myelin- or Nogo-A-induced growth cone collapse and growth inhibition [29–31].

Figure 1



Nogo-A, MAG and OMgp, the principal inhibitors of neurite growth in CNS myelin, interact with a receptor complex comprising NgR, p75 and additional components. Methods of blocking Nogo and its actions are shown in red. As well as gene deletions (Nogo-KO), Nogo-A, which is shown with its two main active sites facing the extracellular space, can be neutralized by specific antibodies or by a soluble Nogo-66-binding fusion protein comprising domains of the receptor subunit NgR. The NgR subunit itself can be blocked by the NEP1–40 peptide derived from the first 40 amino acids of the Nogo-66 region of Nogo-A. As MAG and OMgp also bind to NgR, NEP1–40 may be a particularly potent reagent. Nogo-A and Nogo-66 activate Rho-A and its downstream target ROCK, the activity of which can be blocked by C3 transferase and the inhibitor Y27632, respectively.

Nogo inactivation: *in vitro* results

The strong inhibitory activity of CNS myelin or CNS tissue extracts *in vitro* can be partially neutralized by antibodies against Nogo-A, Nogo gene deletions, soluble NgR fragments, NgR blocking peptides, inhibition of Rho-A or ROCK, inhibition of the intracellular calcium rise, or high concentrations of cAMP (Figure 1).

Neutralizing Nogo-A antibodies have been found to significantly decrease the inhibitory activity of CNS myelin [7,9,11,32]. These findings formed the basis of early crucial steps towards developing the concept of myelin-associated neurite growth inhibition. In knockout mice for Nogo-A, Nogo-A/-B or Nogo-A/-B/-C from three different laboratories, the inhibitory activity of CNS myelin for *in vitro* neurite outgrowth is about half of the level of wild-type myelin [33•–35•]. Double knockouts of Nogo and other myelin inhibitory constituents, such as MAG, have not been analysed as yet.

Both soluble Fc fusion proteins of the Nogo receptor subunit NgR, which blocks Nogo, and a 40-residue fragment ('NEP1–40') of Nogo-66, which blocks NgR, significantly reduce the inhibitory activity of myelin [23••,36]. A similar reduction can be obtained by blocking the small GTPase Rho-A by ribosylation with the bacterial enzyme C3 transferase or by blocking the Rho effector ROCK pharmacologically [30,31,37,38]. Preventing the intracellular calcium release that occurs in response to NI-35 (which is probably a fragment of Nogo-A) prevents growth cone collapse [29]. Finally, several studies have shown that elevated levels of cAMP can cancel the repulsive and inhibitory effects of several inhibitors, including MAG and Nogo [15,39].

The convergence of similar results from all of these different experiments is remarkable. A similar phenomenon is also seen in the lesioned spinal cord *in vivo*.

Nogo inactivation *in vivo* in the lesioned spinal cord: anatomical results

The adult rat (or mouse) corticospinal tract (CST) has been the system of choice for many recent studies: the CST is the largest descending tract, which carries myelinated and unmyelinated sensory system and motor fibres. Descending rubrospinal, vestibulospinal and reticulospinal tracts and the monoaminergic systems are, however, of greater importance than the CST for most locomotor and basic vital functions. Their responses to regeneration enhancing treatments, as well as the responses of propriospinal and ascending fibres, need to be studied.

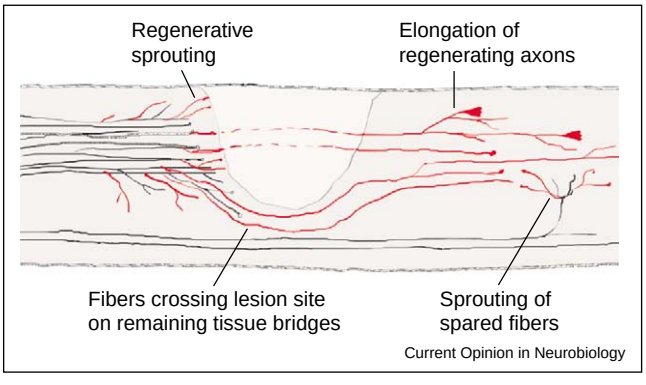
Nogo-A neutralizing antibodies

The most frequently used neutralizing antibody against Nogo has been the monoclonal antibody (mAb) IN-1, which was raised against the Nogo-A band from SDS-polyacrylamide gel electrophoresis separations of rat spinal cord myelin [9]. IN-1 is an IgM that recognizes the region specific to Nogo-A [32]. Because it is conformation-specific, its monospecificity cannot be fully proven. However, crucial *in vitro* and *in vivo* results obtained with IN-1 mAb have now been reproduced by defined antibodies against Nogo-A [11,40–42].

Injection of the IN-1 Fab′ fragments or new purified IgGs against the Nogo-A-specific active site into the intact adult rat cerebellum induces collateral sprouting and an upregulation of several immediate early and growth-related genes in Purkinje cells — a cell type that is known to be extremely resilient with regard to lesions and repair responses [40,43]. The intraventricular application of IN-1 mAb in intact adult rats for 7 days leads to an upregulation of GAP-43 mRNA and protein in the brain and spinal cord and to transitory sprouting of corticospinal axons [44]. These results suggest that neutralizing Nogo antibodies can induce a growth response in the intact adult CNS.

Interruption of the main dorsal corticospinal tract, as well as its minor dorsolateral components, leads to a weak but detectable spontaneous regenerative sprouting from the lesioned axons within a few days. But the presence of IN-1 mAb has been found to result in a very different picture: the sprouting increased and some 5–20% of the labelled fibres grew around the lesion site and down the spinal cord for more than 10 mm (Figure 2; [6,7]). Clear differences between the effects of control antibody and the active IN-1 mAb (rats were number-coded and randomly mixed throughout the experiment) and the reconstruction

Figure 2



Longitudinal sagittal view of a rat or mouse dorsal spinal cord lesion, transecting the main corticospinal tract, that shows minor spontaneous sprouting (black). Nogo neutralizing antibodies, Nogo gene deletions, the NgR blocking peptide NEP1–40, and Rho-A or ROCK inactivation can lead to enhanced sprouting of fibres rostral to the lesion and to fibres crossing the lesion on remaining tissue bridges into the caudal spinal cord and growing down the spinal cord over long distances (drawn in red). Spared fibres also show enhanced sprouting in the caudal spinal cord.

of the labelled fibres on their course around the lesion were crucial criteria in these experiments. This is particularly important in view of the fact that dorsal lesions leave intact the small ventral CST components — fibres that can also sprout caudal to the lesion. The IN-1 mAb also led to a higher density of serotonergic fibres in the caudal spinal cord, but it is unclear whether these were truly regenerating or whether they were sprouting across the midline from spared ventral fibres [45].

Results obtained from the intraventricular application of antibody to hybridoma grafts resemble those obtained from local intrathecal pump infusions of recombinant IN-1 Fab′ or novel Nogo-A IgGs [7,42]. In rat models of stroke with large motor cortex lesions, two different Nogo antibodies were found to induce compensatory growth of the spared CST [41,46]. Increased CST sprouting and regeneration were also found in spinal injured rats immunized with Nogo-66 and MAG [47•]. In all of these studies, a possible role played by the low levels of neuronal Nogo-A remains unknown.

Nogo knockout mice

Three papers describing different lines of knockout mice for Nogo-A, Nogo-A/-B and Nogo-A/-B/-C have been published recently [33•–35•]. In addition to the different constructs used, the three laboratories used S129 ES cells in C57/BL6 foster mice and analysed the genetic hybrids at an early F_2 or F_3 generation. These knockout lines therefore contained different, unknown proportions of the S129 or C57/BL6 genetic backgrounds. It should be noted that the two mouse strains differ greatly in various relevant respects, such as neuroinflammatory response, cell death at lesions sites, scarring response and overall behaviour [33•].

After spinal cord lesion, the Nogo-A-specific knockout mice showed a moderate but clearly detectable increase in regenerative sprouting and elongation [33•]. The same phenotype was quantitatively enhanced in one of the Nogo-A/-B knockout lines [34•]. Because Nogo-B was greatly upregulated in the Nogo-A knockout, the Nogo-66 site seemed to compensate partially for the absent Nogo-A-specific active site. At odds with these observations are the results from the third laboratory, in which neither the Nogo-A/-B knockout lines, nor the survivors from a single Nogo-A/-B/-C knockout mouse that escaped lethality, showed major enhancement of sprouting or regeneration of the lesioned CST [35•]. The analysis of backcrossed strains on a pure genetic background and conditional knockout mice will hopefully help to resolve these interesting discrepancies in the future.

NgR-blocking peptides

Analysis of the 66-residue active site in the Nogo C-terminal region has shown that a peptide comprising the first 40 of these 66 residues binds to NgR without activating it [23••]. This NEP1–40 peptide reduces the inhibitory activity of CNS myelin *in vitro*. When infused locally over a spinal cord lesion site or when applied systemically, NEP1–40 was found to enhance CST sprouting and regeneration, as well as the density of serotonergic fibres in the caudal spinal cord. Such results could even be obtained with a delayed application of NEP1–40 (7 days after lesioning) [48•]. NEP1-40 may block inhibitory responses to Nogo as well as to MAG and OMgp.

Second messenger level: Rho-A, ROCK, cAMP and p75 manipulations

Both the Nogo-66 region and Nogo-A-specific active fragments induce activation of the small GTPase Rho-A in neurons [30,31]. The Rho-inactivating enzyme C3 transferase has been applied to optic nerve and spinal cord lesion sites [31,37,49], resulting in enhanced regenerative sprouting of retinal axons and CST fibres, and in more axons located distally from the lesion. On the one hand, because Rho-A has been shown to be a common signal transducer for several different repulsive and inhibitory factors [15,27], inhibition of this crucial switch would seem to be a particularly attractive approach. On the other hand, Rho-A inhibition also has significant neuroprotective effects in the spinal cord [50].

A similarly complex picture may arise from inhibiting an important downstream Rho effector, ROCK. The local infusion of a small-molecule inhibitor of ROCK (Y27632) over spinal cord lesion sites leads to CST sprouting similar to that observed after inactivation of Nogo or its receptor [31]. A more detailed analysis, including possible tissue-sparing and inflammation-related effects, may be required, however.

Increasing the levels of cAMP can counteract various inhibitory signals, including those triggered by Nogo and MAG [15]. An *in vivo* infusion of cAMP analogues has indeed been shown to result in increased sprouting and regeneration of ascending sensory fibres in the spinal cord [51,52]. cAMP may act locally on growth cones, as well as on neuronal cell bodies, to mediate growth-enhancing effects [15,39].

The jury is still out on the role of p75 as a mediator of Nogo and myelin-induced growth inhibition in the spinal cord *in vivo*. In intact p75 knockout/NGF transgenic mice, however, a massive growth of sympathetic axons into the white matter of the cerebellum has been observed [53].

The convergence of the anatomical results of various ways of Nogo-A inactivation suggests that this molecule has a crucial role in inhibiting spontaneous axonal repair processes in the injured spinal cord. This conclusion is supported by the observations that the ectopic expression of Nogo-A and, to a lesser degree, Nogo-C in mouse peripheral nerve myelin (Nogo-A is not expressed in

Schwann cells in wild types) impairs the normally very efficient axonal regeneration and functional repair after sciatic nerve crush [54••,55•].

Movement control: functional consequences of Nogo inactivation

The current results of experiments in which Nogo has been suppressed in rats or mice with spinal cord injuries are best understood against a background of the physiological concepts underlying movement control. Movements are controlled on different levels in the mammalian CNS: local spinal circuits can generate simple components of movement and rhythmic movements. These circuits are controlled and modified by sensory input (reflexes) and by descending central pathways. Brainstem serotonergic and catecholaminergic fibres set the level of excitation of spinal neurons. Specific descending commands from the brainstem (posture and movements) and the motor cortex (voluntary and skilled movements) reach the spinal cord through four major tract systems: reticulospinal, vestibulospinal and rubrospinal tracts from the brainstem, and the CST. Early experiments on partial lesions, transplantations of serotonergic cells and pharmacological experiments have shown that monoaminergic drive and a few descending motor tract fibres allow a high degree of recovery of overground locomotion after spinal cord injury [56].

The suppression of Nogo-A by IN-1 mAb, by NgR blockade by NEP1–40, or by inhibition of Rho-A or ROCK activity in adult rats with partial, thoracic spinal cord lesions leads to improvements in hindlimb locomotion, as reflected by a 21 point scoring system, the BBB open field locomotion scale [23••,31,34•,37,48•,57,58]. Improvements in locomotion over grids, in foot placement and in electromyographic activity, as well as in the CST-dependent placing response have been also reported [45,48•,57]. Most importantly, malfunctions such as spasticity or increased pain have not been observed so far [57].

It remains to be seen whether the different ways of inactivating Nogo, Nogo receptors or second messenger pathways produce identical functional outcomes, especially when using more refined outcome measures. The role of activity — that is, rehabilitative training — also remains an important target for future research. It is highly probable that the connections formed by regenerating fibres in the caudal spinal cord will not be totally specific from the start: activity-dependent stabilization and fine tuning can be expected to play a crucial role. An additional important element for functional recovery is enhanced compensatory fibre growth from unlesioned fibres.

Enhancing compensatory mechanisms in the injured CNS

Even large spinal cord lesions that are partial can be followed by a considerable degree of functional recovery in humans and rats [56]. Motor recovery takes a few weeks in the rat and several months in humans, and probably depends on compensatory sprouting of spared fibres. So far, only very few studies document these changes on the anatomical level. Thoracic spinal cord lesions including the CST result in spontaneous sprouting of hindlimb CST axons into the cervical spinal cord: forelimb, whisker, shoulder, but also hindlimb movements can be elicited by stimulating the hindlimb motor cortex, pointing to the formation of various new connections [59]. Transection of the dorsal CST leads to a spontaneous compensatory sprouting of the few ventral CST fibres in the spinal cord [60]. Such sprouting events are greatly enhanced by Nogo neutralizing antibodies and have been also observed in stroke models [41,46,61,62]. An increase in compensatory sprouting of spared ventral CST fibres after spinal cord lesions is also observed in animals where NgR was blocked by the peptide NEP1–40 [23••].

In all of these studies, impressive functional recoveries of skilled movements have been reported. Sprouting and plastic changes can be expected to occur in many parts of the circuitry in the spinal cord and brain. Mechanisms of neuronal target recognition and synapse specification probably persist throughout life, thus assuring that, even under conditions of enhanced axon growth and regeneration, functional networks are formed and chaos is prevented.

Conclusions

The similarity of the results obtained after antibody-mediated neutralization of Nogo-A, Nogo gene deletions, NgR blockade and blockade of the downstream messengers Rho-A and ROCK in rat and mouse models of spinal cord lesion are striking. Enhanced regenerative sprouting and long-distance regeneration (mostly of the CST), as well as an impressive enhancement of functional recovery have been observed. Nogo-A thus seems to be a crucial factor for restricting spontaneous fibre regeneration and repair in the adult CNS.

Many issues still remain, however, including the interplay and detailed roles of the different inhibitory components present in CNS tissue and myelin, their receptors and signalling modes, and the best and safest way in which to neutralize growth inhibition. Detailed functional studies of the time course and mechanisms of recovery, the role of rehabilitative training, and the correlation of function with the underlying anatomy are issues of high neurobiological and clinical interest. Finally, the route to a clinical application of these findings will have to include proof-of-concept studies in primates, a determination of the time window after injury during which treatments can be applied effectively, and the use of combined treatments with scar-reducing agents, neurotrophic factors and neuroprotective drugs.

Acknowledgements

I thank D Dodd, L Dimou and L Schnell for critical comments on the manuscript. Work in my laboratory is supported by grants from the Swiss National Science Foundation (31-63633.00), the Swiss National Science Foundation National Centre for Competence in Research (NCCR) 'Neural Plasticity and Repair' programme and the Christopher Reeve Paralysis Foundation (Springfield, NJ).

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