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No Nogo: Now Where to Go?

Minireview

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Nogo-A, a reticulon protein expressed by oligodendrocytes, contributes to the axonal growth inhibitory action of central myelin in growth cone collapse and neurite outgrowth in vitro assays, and antibody and inhibitor studies have implicated a role for Nogo in regeneration in the adult CNS in vivo. Three independent labs have now produced Nogo knockout mice with, quite unexpectedly, three different regeneration phenotypes.

Injuries to the adult mammalian central nervous system (CNS) are characterized by a failure of regrowth of transected axons. The lack of regeneration is due to a combination of factors: death of injured neurons, reduced capacity of adult neurons to grow when injured, lack of the necessary trophic molecules to support growth, and the presence of an environment hostile for any growth (Schwab and Bartholdi, 1996). That the adult CNS is nonpermissive for axonal growth has been recognized since the seminal work of Cajal. Replacing this environment with peripheral nerve grafts, Schwann cells, or olfactory ensheathing glia does encourage axonal growth (Behar et al., 2000). What is responsible then for the nonpermissive nature of the environment in the CNS for axonal growth? The axonal growth cone formed after the resealing of injured axons will interact with soluble secreted factors, the extracellular matrix, and cells in close proximity, all of which could abort axonal growth by either activating growth cone collapse signaling in the growth cone or by physically impeding axon extension. Prevention of growth may be due to expression of growth cone collapsing guidance cues normally involved in development, such as the semaphorins, production of proteoglycans at the glial scar that may act as a "glue" adhering to the growth cone preventing its extension, and presence in central myelin of axonal growth inhibitory proteins.

Central myelin, produced by oligodendrocytes, is undoubtedly a powerful suppressor of axonal growth. Axons of cultured neurons do not grow into explants of adult CNS tissue, although they will grow in peripheral nerve explants containing Schwann cell-produced myelin, and neurite outgrowth is massively reduced and growth cones collapse when neurons are grown on or exposed to central myelin extracts (Schwab and Thoenen, 1985). What molecules are responsible for myelin's inhibitory actions, and would neutralizing them be sufficient to promote regeneration? Three myelin inhibitors have been identified to date: Myelin-Associated Glycoprotein (MAG) (McKerracher et al., 1994), Nogo-A (Chen et al., 2000; GrandPré et al., 2000; Prinjha et al., 2000), and Oligodendrocyte-Myelin glycoprotein (OMgp) (Wang et al., 2002a). Heterologous expression of each independently produces potent inhibition of axonal growth in in vitro assays. Lack of MAG in MAG knockout mice results though in minimal regeneration (Bartsch et al., 1995). The consequences of a loss of OMgp on axonal growth in vivo have not yet been examined. What of Nogo-A? Based on in vitro growth assays and in vivo Nogo neutralizing/antagonism studies (Bregman et al., 1995; Liu et al., 2002), the expectation was very high that lack of Nogo would very substantially improve axonal growth and, in this way, promote functional recovery after spinal cord lesions.

Three papers in the present issue of *Neuron* describe Nogo-deficient mice (Kim et al., 2003; Simonen et al., 2003; Zheng et al., 2003). Substantially improved regeneration was found only in the young mice lacking Nogo-A/B used by Strittmatter and colleagues (Kim et al., 2003). Tessier-Lavigne's group studied two lines of mutant mice, one lacking all three Nogo proteins, Nogo-A, -B, and -C, and one, like the mouse used by the Strittmatter group, lacking Nogo-A and -B. In neither mouse line, however, did they detect any evidence of increased axonal growth (Zheng et al., 2003). Schwab's group produced a mouse lacking Nogo-A, which has a compensatory increase in Nogo-B in oligodendrocytes. These animals display a statistically significant improvement in axonal growth, but the numbers of axons that grow are few, and no improvement in functional recovery is reported (Simonen et al., 2003).

The three isoforms of Nogo, A, B, and C, arise by alternative splicing (Nogo-A/B) and promoter usage (Nogo-C). All isoforms share a common C-terminal domain of 188 amino acids (Chen et al., 2000; GrandPré et al., 2000; Prinjha et al., 2000). A 66 amino acid subdomain within the common C-terminal domain is expressed on the surface and binds to a GPI-linked Nogo-66 receptor, NgR, expressed by neurons (Fournier et al., 2001). Recently, MAG and OMgp have been shown also to bind with high affinity (nM) to NgR, which is a coreceptor with the neurotrophin p75 receptor (Liu et al., 2002; Wang et al., 2002b; Wang et al., 2002a; Wong et al., 2002). p75 has no affinity, however, for NgR ligands, it is the ectodomain of NgR which binds to the three ligands, and signal transduction is mediated by the intracellular domain of p75 through Rho GTPases (Woolf and Bloechlinger, 2002). The three ligands for NgR are structurally quite unrelated; what is the basis then for the extraordinary ligand promiscuity of NgR? Do all three ligands bind to the same site on NgR due to a degenerate recognition code on its ectodomain, or is the ligand binding promiscuity due to binding of differently shaped ligands to multiple separate binding spots on NgR? A paper in this issue of *Neuron* reports on the crystal structure of the ligand binding (leucine-rich repeat) domain of the human Nogo receptor (He et al., 2003). The

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authors propose from their structural analysis that quite different ligand architectures—Ig-like domains for MAG, a curved LRR array for OMgp, and a compact globule for Nogo-66—all converge on a selection of distinct binding hotspots on NgR. The presence of conserved aromatic residues in the curved ectodomain of NgR could provide for a la carte binding with each ligand choosing the binding site it prefers.

A peptide fragment of Nogo-66 (NEP 1-40), which antagonizes Nogo-A binding to NgR, eliminates much of the axonal growth inhibitory action of central myelin in vitro and promotes axonal growth after spinal cord injury (GrandPré et al., 2002). Nogo-A uniquely among the three Nogo proteins has a long N-terminal extension that contains a domain that also contributes to axonal outgrowth inhibition in vitro, although by an as of yet undefined receptor (Chen et al., 2000). An antibody produced by Schwab and colleagues against purified Nogo-A (NI-250, at the time), IN-1, that has a regeneration promoting activity in vivo (Bregman et al., 1995) recognizes, among others, an epitope on the amino-terminal domain of Nogo-A (Chen et al., 2000). The precise topology of Nogo-A remains undetermined though, and it is not clear whether the growth inhibitory amino-terminal domain is exposed on the surface of oligodendrocytes or is intracytoplasmic.

The three papers reporting Nogo deletions use different strategies, and each is individually compelling. The problem is the stark divergence in regeneration phenotype reported by each group.

The Strittmatter group used a mutant mouse (Nogo-A/B mutant) derived from an embryonic stem cell clone generated by Lexicon Genetics, where the Nogo gene was disrupted as a consequence of random insertion of a gene trap vector containing a retroviral promoter, a strong splice acceptor, and the βgeo sequence. Although the trapping vector was inserted into the large Nogo-A-specific exon (exon 3), both Nogo-A and -B expression is disrupted, presumably due to reduced *nogo* transcription or *nogo*-β*geo* fusion mRNA stability. Nogo-C expression appears unchanged in the gene trap line.

The Tessier-Lavigne group generated several Nogo mutant mice, two of which were studied for a regeneration phenotype. One (Nogo-A/B mutant) has deletion of an amino-terminal genomic fragment including the entire coding region of exon 1 downstream of the ATG start codon. Exon 1 is shared by Nogo-A and -B, and these were disrupted, leaving Nogo-C unaffected. A second mouse deleting the C-terminal region common to all three Nogo isoforms (Nogo-A/B/C) was generated by replacing exon 4 with a floxed neomycin resistance gene, resulting in the absence of all Nogo isoforms.

The Schwab group generated a mutant (Nogo-A) where the large Nogo-A-specific exon (exon 3) was disrupted by conventional gene targeting. Although this mutation disrupts the same exon as that by the gene trap insertion and eliminated Nogo-A, a very substantial compensatory increase in Nogo-B in oligodendrocytes was detected.

The Nogo-A and two Nogo-A/B mutant mice were viable and fertile. The Nogo-A/B/C line was embryonic lethal, except for a single escaper, which was used to derive a line of Nogo-A/B/C mutant viable mice. Collectively, these mice enable the following questions to be addressed. Does Nogo-A have a role in the postnatal formation of highly ordered axon fiber tracts during the time that myelination is established, is it involved in the maintenance of the stability of connectivty by preventing collateral axonal sprouting, and does it prevent central regeneration? In all the different mutant mice, the axonal growth inhibitory action of extracted central myelin was reduced in in vitro assays, indicating that Nogo-A contributes to this activity. Although the analysis must be considered preliminary, none of the Nogo mutant mice have any detectable neurological phenotype or obvious neuroanatomical deficits. Myelin appears normal, as do axons and oligodendrocytes. This suggests that Nogo-A is not involved in either the development of the CNS or in the maintenance of axonal pathways, at least in the absence of injury.

Before discussing the effects of the different Nogo gene deletions on axonal growth, it is worth considering exactly what changes in axonal growth may be expected, and what outcome measures are appropriate to detect them. Three forms of axonal growth can occur in the adult nervous system after injury: collateral sprouting (a form of plasticity or growth of the axons of intact neurons typically into denervated areas), regenerative growth, growth of injured axons originating close to the site of injury and continuing beyond the lesion to reestablish disrupted synaptic connections, and finally, regenerative sprouting, which occurs proximal to a lesion and involves growth from intact sections of injured neurons proximal to the site of the lesion and where the axons do not grow beyond the lesion. These forms may coexist and it is not always easy to differentiate them with bulk labeling techniques. A further problem is that sprouting or regeneration without establishment of working synaptic contacts is of no functional significance. How to show restoration of function? This is extremely difficult. Demonstration of physical contact requires ultrastructural analysis, as light microscopy cannot reveal synapses. Ideally, an electrophysiological demonstration of transmitter release and postsynaptic responsiveness is required to justify a claim for functional connectivity but is rarely provided. The commonest functional outcome used is the BBB open field behavioral score, a measure of restoration of locomotor function. This measure has, however, some important limitations. First, it is nonlinear and subject to observer bias and interpretation. Second, untreated animals with lesions to their corticospinal tracts (in rodents, this lies in the most ventral portion of the dorsal columns) show recovery of spontaneous hindlimb locomotion without any demonstrable regeneration, so the meaning of changes is difficult to ascertain. Third, a wide range of treatment strategies targeted at Nogo, MAG, Rho, and proteoglycans produce effects very early, much earlier than would be expected for regenerative growth of axons. This may reflect short-range collateral growth or promotion of function in the autonomous central pattern generator (CPG) that is present in the spinal cord. The CPG enables synchronized alternating activity in flexor and extensor muscles to be generated below a spinal

cord transection, producing a rhythmic walking-like pattern of locomotion independent of descending input from the brain (Kaegi et al., 2002). The degree to which the CPG operates in mice is not known, and this may confound interpretations of recovery in the BBB, which may not be due to axonal growth. A final consideration is which pathway to study. In spinal cord injury, the descending corticospinal and the ascending dorsal column tracts have been most extensively investigated. The choice of which axonal pathway to study to detect regeneration includes issues such as ease of label and capacity to detect functional recovery. A further key issue is whether the neurons express and continue to express after injury the molecular system targeted. For example, in the case of Nogo, MAG, and OMgp, do pyramidal cells in the motor cortex or large dorsal root ganglion cells coexpress NgR and p75? If they do not, then they might display a lack of responsiveness to these inhibitory factors. This information is not always known for all axonal pathways studied, particularly in mice, an increasingly favored animal model of spinal cord regeneration.

The profound increase in corticospinal sprouting found above spinal cord dorsal hemilesions and the very substantial numbers of corticospinal axons that were observed below the lesion by the Strittmatter group in their Nogo-A/B mutant mice (Kim et al., 2003) appear to indicate that Nogo-A is indeed a major contributor to the failure of regeneration of adult CNS neurons. A critical caveat, however, is that this facilitated regeneration appears to be restricted only to young ($<$9 weeks old) mice. In the three older mice examined (11–14 weeks), the degree of sprouting above the lesion was reduced by a factor of 16. If substantiated, this could mean that neurons in older animals either have a reduced capacity to grow or that some non Nogo growth inhibitory factor is increasingly expressed with age, and the implication of this for human adult CNS lesions is unknown. It is not clear if the results previously obtained by the Strittmatter group on inhibiting Nogo-66 binding to the NgR (Liu et al., 2002) are also age dependent. The much more modest results obtained by the Schwab group (Simonen et al., 2003) could potentially be explained by a gain of function resulting from increased Nogo-B expression in oligodendrocytes. Nogo-B contains the growth cone collapsing Nogo-66 C-terminal domain. If this were correct, then treatment of these mice with the Nogo-66 peptide NgR antagonist (NEP 1-40) should reveal growth equivalent to that produced by the Strittmatter Nogo-A/B mice. However, the in vitro neurite outgrowth activity of central myelin from the Nogo-A knockout mice was substantially reduced, something that would not be expected if Nogo-B overexpression began to inhibit axonal growth. The age of the Nogo-A mice studied (8–17 weeks), considerably older than most of those studied by the Strittmatter group, may be an important factor in reducing the axonal growth observed. The failure by the Tessier-Lavigne group to see any augmentation of sprouting or regeneration in their Nogo-A/B and Nogo-A/B/C mutant mice is very difficult to explain if Nogo-A has a major role in preventing axonal growth after injury (Zheng et al., 2003). Age is unlikely to be a factor, since 80% of the animals they studied were 6–10 weeks old, and technical factors such as the extent/nature of the lesion and the degree of label of corticospinal tracts are most unlikely given the exceptional care taken by this group to exclude this possibility.

What then can explain the divergent regenerative responses found in response to the absence of Nogo-A in these different mutant mice? Differences in genetic background? Unidentified compensatory changes generated by chance in both of the Tessier-Lavigne mutant mice leading to suppression of axonal growth? Loss of another growth inhibitory factor in the Strittmatter mice due to changes in genetic loci neighboring the Nogo gene consequent on the gene trap insertion? We simply do not know. Truth in science is not determined either by a majority vote or even by a veto. Further work is required to define exactly what role Nogo-A plays, and this must involve elucidating the basis for the discrepancies in the mutant mice. In the meantime, conclusions about the role of Nogo-A need to be tentative, and considerable caution is required in even considering therapeutic approaches for patients based on neutralizing or blocking the action of Nogo-A. We are undoubtedly making rapid progress in studying why the adult CNS does not regrow but need now to pause, like a growth cone contacting central myelin, before we overcome the barriers to successful regeneration by gaining more insight into the actions of Nogo and other inhibitory molecules.

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