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Multiple Signals Regulate Axon Regeneration Through the Nogo Receptor Complex

Toshihide Yamashita,* Masashi Fujitani, Satoru Yamagishi, Katsuhiko Hata, and Fumiaki Mimura

Department of Neurobiology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

Abstract

Several myelin-derived proteins have been identified as components of central nervous system (CNS) myelin, which prevents axonal regeneration in the adult vertebrate CNS. The discovery of the receptor for these proteins was a major step toward understanding the failure of axon regeneration. The receptor complex consists of at least three elements: the p75 receptor ($p75^{NTR}$), the Nogo receptor and LINGO-1. Downstream from the receptor complex, RhoA activation has been shown to be a key element of the signaling mechanism of these proteins. Rho activation arrests axon growth, and blocking Rho activation promotes axon regeneration in vivo. Recent studies have identified conventional protein kinase C as an additional necessary component for axon growth inhibition. Possible crosstalk downstream of these signals should be explored to clarify all the inhibitory signals and may provide an efficient molecular target against injuries to the CNS.

Index Entries: Myelin; p75; Rho; regeneration; central nervous system (CNS).

Regeneration Inability of the Adult Central Nervous System

It is well-established that axons of the adult central nervous system (CNS) are capable of only limited regrowth following injury and that an unfavorable growth environment significantly contributes to the lack of regeneration. In the early 1980s, Aguayo's group *(1)* demonstrated that many neurons could regenerate over long distances if they were offered a peripheral nerve as a substrate.

Berry *(2)* discovered that if damage did not occur to nearby myelinated fibers, nonmyelinated axons in the CNS regenerated following chemical axotomy but not following mechanical axotomy, which damaged myelinated fibers. Because damage to myelinated fibers leads to the release of degeneration products of

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*Author to whom correspondence and reprint requests should be addressed. E-mail: t-yamashita@faculty.chiba-u.jp

CNS myelin, it was proposed that this damage is inhibitory to axonal growth. Subsequently, this hypothesis was tested by exposing perinatal dorsal root ganglion (DRG) or sympathetic neurons to optic and sciatic nerve explants of adult rats in the presence of nerve growth factor. Few or no axons in the optic nerves were observed during 2 wk of culture, whereas abundant nerve fibers invaded the sciatic nerves *(3)*. The absence of neurite outgrowth in the adult optic nerve explants was considered to result from an intrinsic property of adult CNS tissue, rather than from reactions to the lesion or the culture condition. It has been suggested that myelin from the adult CNS is an inhibitory substrate for neurite outgrowth.

Three Myelin-Derived Inhibitors of Axon Regeneration

One monoclonal antibody, inhibitor-neutralizing antibody (mAB IN-1) was obtained and used extensively for subsequent in vitro and in vivo experiments. The inhibitory activity of a crude myelin extract decreased by approx 50% with this antibody, and the inhibitory activity of purified bovine NI-220 (the homolog of rat NI-250) decreased with the antibody *(4)*.

Schwab et al. succeeded in purifying the bovine homolog bNI-220 by using large amounts of bovine spinal cord. The corresponding cloned complementaty DNA is derived from a gene, designated *Nogo*, which gives rise to three messenger RNAs *(5–7)*. The three splice variants of Nogo are *Nogo-A, Nogo-B* and *Nogo-C,* and the latter two are widely expressed outside the CNS. *Nogo-A* possesses a unique amino-terminal region not shared by *Nogo-B* and *Nogo-C.* The two most strongly predicted transmembrane domains are separated by the 66-residue extracellular or lumenal loop, Nogo-66, which causes growth cone collapse *(8)*. The *Nogo-A*-specific amino-terminal region is also inhibitory for neurite outgrowth and prevents the spreading of fibroblasts. *Nogo-A* is most strongly expressed in oligodendrocytes in the white matter, although it has also been detected in neuronal perikarya, including those in the cerebral cortex, spinal motor neurons, and DRG neurons as well as in axons *(9)*.

Several years before Nogo was discovered to be an inhibitor of axon regeneration, two groups identified myelin-associated glycoprotein (MAG) as the first myelin-derived growth inhibitory protein. MAG is a transmembrane protein of the immunoglobulin superfamily that is found in both peripheral and CNS myelin. McKerracher et al. *(10)* detected MAG inhibitory activity in myelin after extraction with octylglucoside, fractionation by ion exchange chromatography, and screening for inhibitory activity. Filbin et al. *(11)* demonstrated that MAG, which was ectopically expressed in Chinese hamster ovary (CHO) cells, inhibited neurite outgrowth. Interestingly, MAG is a bifunctional regulator of axon growth, because it can stimulate the neurite outgrowth of young neurons *(12)*.

Oligodendrocyte-myelin glycoprotein (OMgp) is the most recently identified protein to be an inhibitory component of myelin. In the course of isolating MAG as an inhibitory protein, Braun et al. *(13)* observed two peaks of inhibitory activity, and MAG was present in the first peak. They separated the inhibitory protein in the second peak and identified OMgp. He et al. *(14)* found OMgp as an inhibitor using the hypothesis that any glycosylphosphatidylinositol-anchored myelin protein acts as a regeneration inhibitor. OMgp, which is abundant in myelin, has potent collapse of the growth cone as well as neurite outgrowth inhibitory activity.

Nogo–Receptor Complex

A protein that binds Nogo-66 was identified with high affinity using an alkalin–phosphatase fusion, protein-expression-screening strategy *(15)*. The receptor for Nogo-66 (NgR; Fig. 1) is a glycosyl-phosphatidylinositol (GPI) anchor protein that attaches to the outer leaflet of the plasma membrane and is expressed in CNS neurons as well as their axons *(16,17)*. Because the release of GPI-anchored proteins

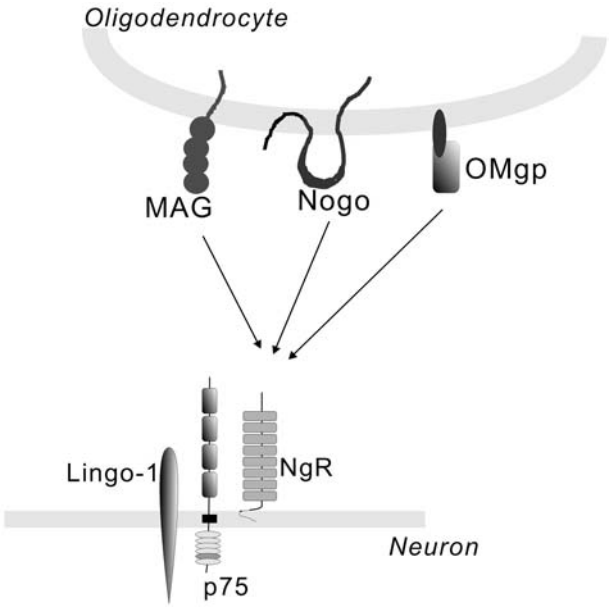

Fig. 1. Signal transduction by a complex of NgR, $p75^{NTR}$, and LINGO-1. MAG, Nogo-66, and OMgp are ligands of NgR and are expressed by oligodendrocytes. These ligands do not share any recognized protein domains. $p75^{NTR}$ interacts with NgR as well as LINGO-1 and mediates the inhibitory signaling of these myelin-derived proteins by activating RhoA.

by phosphatidylinositol-specific phospholipase C from embryonic DRG results in the elimination of growth-cone collapse in response to Nogo-66, NgR mediates the signal from Nogo-66 in at least these neurons. This discovery was a major step toward understanding the inhibitory signal of CNS regeneration, because two other inhibitory components (MAG *[18,19]* and OMgp *[14]*) also bind to NgR. The ectopic expression of NgR induces insensitive neurons to become sensitive to these myelin-derived proteins. These findings intersect these various molecules at the level of NgR. From the perspective of developing a therapeutic approach, it is important to note that a fragment of Nogo-66 binds to NgR as a high-affinity antagonist *(20)*. The antagonist peptide NEP1-40 reduces endogenous inhibitory activity to promote the sprouting of corticospinal tract axons, long distance growth, and functional recovery.

Although NgR is a binding partner for MAG, Nogo-66, and OMgp, the GPI-linked nature of NgR suggests that a second receptor subunit spans the plasma membrane and mediates signal transduction. To date, one such signal transducer, the p75 receptor ($p75^{NTR}$; known as a receptor for neurotrophins) has been identified (Fig. 1). MAG activates RhoA in the cells only in the presence of the receptor *(21)*. Postnatal cerebellar granule neurons from mice carrying a mutation in the $p75^{NTR}$ gene are not responsive to MAG, whereas neurite growth from wild-type mice was significantly inhibited by MAG.

Extrapolating the observations that MAG is a ligand for NgR, we tested the possibility that $p75^{NTR}$ associates with NgR to form a receptor complex for MAG, Nogo, and OMgp *(22,23)*. At least a fraction of $p75^{NTR}$ binds with NgR using co-immunoprecipitation (IP) experiments. Postnatal cerebellar neurons from mice carrying a mutation in the $p75^{NTR}$ gene are insensitive to glutathione s-transferase–Nogo-66 and OMgp–alkaline phosphatase *(22)*. The inhibitory activity of these proteins in cerebellar granule neurons is decreased by the ectopic expression of a dominant-negative form of $p75^{NTR}$ that lacks a cytoplasmic domain. Soluble $p75^{NTR}$-Fc fusion protein also attenuates these effects. These observations suggest that not only is $p75^{NTR}$ required for the inhibitory activity of these myelin-derived proteins but that it also provides a potent molecular target for developing therapeutic agents against injuries to the CNS.

Unexpectedly, mice carrying a mutation in the $p75^{NTR}$ gene did not demonstrate better functional recovery and axon regeneration following spinal cord injury *(24)*. One possibility is that other signal transducers are important for the inhibitory effect in vivo. However, it should be noted that $p75^{NTR}$ has diverse functions, including bidirectional signals regulating axon growth, which suggests that blocking the functions of $p75^{NTR}$ is a less attractive treatment strategy (e.g., neurotrophin binding to $p75^{NTR}$ promotes axon outgrowth; ref. *25*).

It was recently demonstrated that the Nogo receptor complex is comprised of at least three

elements: $p75^{NTR}$, NgR, and LINGO-1. LINGO-1 is involved in the signal transduction of the myelin-derived inhibitors *(26)*, although the precise mechanism of the action remains to be determined (Fig. 1).

Rho: A Key Signal for Axon Growth Inhibition

Downstream from the receptor complex of $p75^{NTR}$, NgR, and LINGO-1, Rho appears to be a key intracellular effector for growth inhibitory signaling by myelin. In neurons, myelin and MAG inhibit growth, and this is abolished by the botulinus toxin C3, which inactivates Rho *(27)*. More specifically, RhoA is activated by MAG-Fc in the cerebellar granule neurons *(21)*. The molecular action mechanism of $p75^{NTR}$ is suggested by the finding that $p75^{NTR}$ releases Rho from Rho guanine nucleotide-dissociation inhibitor (Rho-GDI; Fig. 2; ref. *28*), thereby inducing the activation of Rho. Rho-GDI is an essential part of the signaling mechanism that suppresses the activity of Rho. Rho proteins are regulated either by enzymes that enhance guanine triphosphate (GTP) binding and activity (guanine nucleotide exchange factors) or by proteins that increase the hydrolysis of GTP (GTPase-activating proteins) and, therefore, decrease activity. Rho is maintained in an inactive state in cells by Rho-GDI *(29)*, which inhibits the activity of Rho by binding to and sequestering Rho in the cytoplasm, by inhibiting the formation of active RhoGTP, and by blocking the binding of Rho to its effectors.

These findings have established Rho as a key player in inhibiting CNS regeneration and have launched a new wave of studies aiming to promote the regeneration of injured axons by modulating this inhibitory pathway. For example, Y-27632 (an inhibitor of Rho kinase and a downstream effector of Rho) has been used to probe the role of Rho in growth-inhibiting signaling *(30,31)*. Treating neurons with C3 transferase (a bacterial endotoxin that inactivates Rho) or with Y-27632 promotes growth on inhibitory substrates.

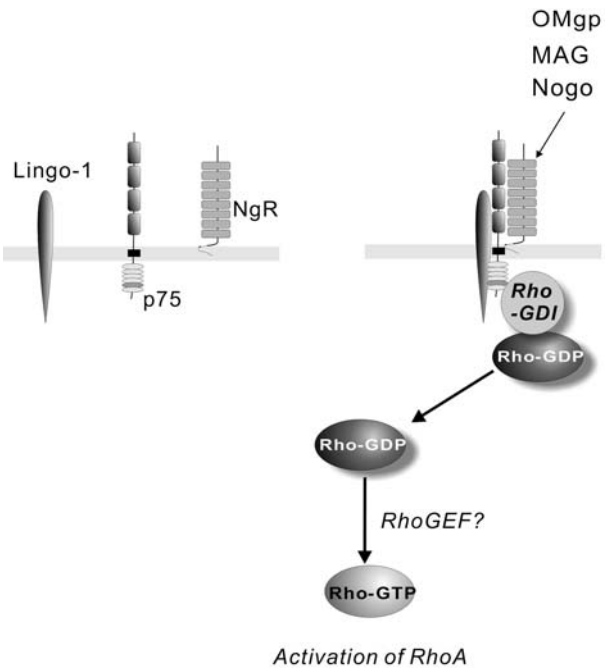

Fig. 2. Mechanisms of axon growth inhibition by $p75^{NTR}$. In the absence of MAG or Nogo-66, growth and regeneration occur as a result of the Rho guanine nucleotide-dissociation inhibitor (Rho-GDI)-induced suppression of Rho activity. Rho-GDI maintains Rho in an inactive state by binding and prevents Rho from interacting with its effectors. $p75^{NTR}$ activation promotes the dissociation of Rho-GDI from RhoA, allowing RhoA to activate through the exchange of guanine diphosphate for guanine triphosphate. Activated RhoA then interacts with its signaling molecules to elicit axon growth inhibition in some neurons.

Intriguingly, a peptide has been identified that blocks the pathway elicited by MAG, Nogo-66, and OMgp *(28)*. The binding region of Rho-GDI on $p75^{NTR}$ was identified as the fifth α-helix in the $p75^{NTR}$ intracellular domain. The short sequence of the fifth helix is similar to mastoparan, a 14-residue peptide of wasp venom known to be capable of activating Rho *(32)*. A peptide ligand to this region was previously reported *(33)* by screening a combinatorial library using a variation of the selectively infective phage method. This peptide, designated Pep5, inhibits the interaction of $p75^{NTR}$

with Rho-GDI in vitro and in vivo. The inhibitory peptide completely eliminates the effects mediated by MAG or Nogo-66 in adult DRG neurons and postnatal cerebellar granule neurons *(28)*, establishing the Rho-GDI–p75^{NTR} association as an important mechanism of the p75^{NTR}-induced suppression of axon growth by myelin proteins. A particularly notable aspect is that the peptide does not inhibit other functions of p75^{NTR}, such as axon elongation or cell death by neurotrophins.

Conventional Protein Kinase Cs Are Involved in Inhibition of Axon Growth

Another important signal, protein kinase C (PKC), was identified in the effects mediated by myelin-derived inhibitors. PKC is a ubiquitously expressed family of kinases that have key roles in regulating multiple cellular activities. The PKC family consists of several groups: conventional, novel, and atypical PKCs.

Conventional PKC, including PKCα, β and γ, was activated by myelin-derived proteins, and the inhibitors of conventional PKC eliminated the protein effect on neurite outgrowth *(34)*. Because RhoA activation by myelin-derived proteins was abolished by blocking PKC activation, PKC is considered to be upstream of RhoA. A later study by Hasegawa et al. *(35)* largely confirmed these findings but disagreed with the suggestion that PKC inhibition did not modify RhoA activation, indicating that these signals are independent. Although the precise up- and downstream signals of PKC remain unknown, PKC was found to be another key molecule regulating axon regeneration.

A previous study suggested that G_i, a G protein, is activated by MAG *(36)*. The elevation of intracellular Ca^{21} concentration induced by MAG *(23)* may result from G_i activation. Because the elevation of intracellular Ca^{21} concentration is abolished when treated with the antibody against p75^{NTR}, p75^{NTR} may be required for signal transduction *(35)*. Some G protein-coupled receptors may be functionally associated with p75^{NTR} to transduce the conventional PKC/IP_3 signals.

p75^{NTR} Is a Bilateral Regulator of Axon Growth

It has long been known that p75^{NTR} is a receptor for neurotrophins that promote survival and differentiation. Consistent with its function in controlling the survival and neurite formation of neurons, p75^{NTR} is expressed during the developmental stages of the nervous system. In contrast, p75 ^{NTR} is re-expressed in various pathological conditions in adults and even acts as an inhibitor of axon regeneration. Diverse effects mediated by p75 ^{NTR} are partly the consequence of the interaction of p75 ^{NTR} with other membrane-associated proteins, such as Trk tyrosine kinases, NgR, and sortilin, as well as multiple intracellular signaling molecules *(37)*.

The signals of the myelin-derived inhibitors of axon regeneration are considered to contribute to the lack of regeneration of the injured CNS. Many of the proposed strategies to treat patients with CNS injuries either block inhibitory proteins or block signaling by inhibitory proteins. However, it should be noted that many of the neurons used in in vitro studies are not relevant for spinal cord injury. Because different neurons have different responses to the inhibitors, it is important to focus on a relevant target population to permit the identification of relevant pathways in future studies. If we can further understand the mechanisms of axon regeneration at the molecular level, it would open the door for the development of new biotechnological strategies to promote CNS regeneration.

References

1. David S. and Aguayo, A. J. (1981) Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. *Science* **214**, 931–933.

2. Berry M. (1982) Post-injury myelin-breakdown products inhibit axonal growth: An hypothesis to explain the failure of axonal regeneration in the mammalian central nervous system. *Bibliotheca Anatomica* **23**, 1–11.
3. Schwab M. E. and Thoenen H. (1985) Dissociated neurons regenerate into sciatic but not optic nerve explants in culture irrespective of neurotrophic factors. *J. Neurosci.* **5**, 2415–2423.
4. Spillmann A. A., Bandtlow C. E., Lottspeich F., Keller F., and Schwab M. E. (1998) Identification and characterization of a bovine neurite growth inhibitor (bNI-220). *J. Biol. Chem.* **273**, 19,283–19,293.
5. Prinjha R., Moore S. E., Vinson M., et al. (2000) Inhibitor of neurite outgrowth in humans. *Nature*, **403**, 383,384.
6. GrandPre T., Nakamura F., Vartanian T., and Strittmatter S. M. (2000) Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. *Nature* **403**, 439–444.
7. Chen M. S., Huber A. B., van der Haar M. E., et al. (2000) Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. *Nature* **403**, 434–439.
8. Fournier A. E., GrandPre T., and Strittmatter S. M. (2001) Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. *Nature* **409**, 341–346.
9. Huber A. B., Weinmann O., Brosamle C., Oertle T., and Schwab M. E. (2002) Patterns of Nogo mRNA and protein expression in the developing and adult rat and after CNS lesions. *J. Neurosci.* **22**, 3553–3567.
10. McKerracher L., David S., Jackson D. L., Kottis V., Dunn R. J., and Braun P. E. (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. *Neuron* **13**, 805–811.
11. Mukhopadhyay G., Doherty P., Walsh F. S., Crocker P. R., and Filbin M. T. (1994) A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron* **13**, 757–767.
12. Cai D., Qiu J., Cao Z., McAtee M., Bregman B. S., and Filbin M. T. (2001) Neuronal cyclic AMP controls the developmental loss in ability of axons to regenerate. *J. Neurosci.* **21**, 4731–4739.
13. Kottis V., Thibault P., Mikol D., et al. (2002) Oligodendrocyte-myelin glycoprotein (OMgp) is an inhibitor of neurite outgrowth. *J. Neurochem.* **82**, 1566–1569.
14. Wang K. C., Koprivica V., Kim J. A., et al. (2002) Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. *Nature* **417**, 941–944.
15. Fournier A. E., GrandPre T., and Strittmatter S. M. (2001) Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. *Nature* **409**, 341–346.
16. Josephson A., Trifunovski A., Widmer H. R., Widenfalk J., Olson L., and Spenger C. (2002) Nogo-receptor gene activity: cellular localization and developmental regulation of mRNA in mice and humans. *J. Comp. Neurol.* **453**, 292–304.
17. Wang X., Chun S. J., Treloar H., Vartanian T., Greer C. A., and Strittmatter S. M. (2002) Localization of Nogo-A and Nogo-66 receptor proteins at sites of axon-myelin and synaptic contact. *J. Neurosci.* **22**, 5505–5515.
18. Liu B. P., Fournier A., GrandPre T., and Strittmatter S. M. (2002) Myelin-associated glycoprotein as a functional ligand for the Nogo-66 receptor. *Science* **297**, 1190–1193.
19. Domeniconi M., Cao Z., Spencer T., et al. (2002) Myelin-associated glycoprotein interacts with the Nogo66 receptor to inhibit neurite outgrowth. *Neuron* **35**, 283–290.
20. GrandPre T., Li S., and Strittmatter S. M. (2002) Nogo-66 receptor antagonist peptide promotes axonal regeneration. *Nature* **417**, 547–551.
21. Yamashita T., Higuchi H., and Tohyama M. (2002) The p75 receptor transduces the signal from myelin-associated glycoprotein to Rho. *J. Cell Biol.* **157**, 565–570.
22. Wang K. C., Kim J. A., Sivasankaran R., Segal R., and He Z. (2002) p75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. *Nature* **420**, 74–78.
23. Wong S. T., Henley J. R., Kanning K. C., Huang K. H., Bothwell M., and Poo M. M. (2002) A p75(NTR) and Nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein. *Nat. Neurosci.* **5**, 1302–1308.
24. Song X. Y., Zhong J. H., Wang X., and Zhou X. F. (2004) Suppression of p75NTR does not promote regeneration of injured spinal cord in mice. *J. Neurosci.* **14**, 542–546.
25. Yamashita T., Tucker K. L., and Barde Y. A. (1999) Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. *Neuron* **24**, 585–593.
26. Mi S., Lee X., Shao Z., et al. (2004) LINGO-1 is a component of the NOGO-66 receptor/p75 signaling complex. *Nat. Neurosci.* **7**, 221–228.
27. Lehmann M., Fournier A., Selles-Navarro I., et al. (1999) Inactivation of Rho signaling pathway

promotes CNS axon regeneration. *J. Neurosci.* **19**, 7537–7547.
28. Yamashita T. and Tohyama M. (2003) The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI. *Nat. Neurosci.* **6**, 461–467.
29. Sasaki T. and Takai Y. (1998) The Rho small G protein family-Rho GDI system as a temporal and spatial determinant for cytoskeletal control. *Biochem. Biophys. Res. Commun.* **245**, 641–645.
30. Dergham P., Ellezam B., Essagian C., Avedissian H., Lubell W. D., and McKerracher L. (2002) Rho signaling pathway targeted to promote spinal cord repair. *J. Neurosci.* **22**, 6570–6577.
31. Fournier A. E., Takizawa B. T., and Strittmatter S. M. (2003) Rho kinase inhibition enhances axonal regeneration in the injured CNS. *J. Neurosci.* **23**, 1416–1423.
32. Koch G., Haberman B., Mohr C., Just I., and Aktories K. (1991) Interaction of mastoparan with the low molecular mass GTP-binding proteins rho/rac. *FEBS Lett.* **291**, 336–340.
33. Ilag L. L., Rottenberger C., Liepinsh E., et al. (1999) Selection of a peptide ligand to the p75 neurotrophin receptor death domain and determination of its binding sites by NMR. *Biochem. Biophys. Res. Commun.* **255**, 104–109.
34. Sivasankaran R., Pei J., Wang K. C., et al. (2004) PKC mediates inhibitory effects of myelin and chondroitin sulfate proteoglycans on axonal regeneration. *Nat. Neurosci.* **7**, 261–268
35. Hasegawa Y., Fujitani M., Hata K., Tohyama M., Yamagishi S., and Yamashita, T. (2004) Promotion of axon regeneration by MAG and Nogo through divergent signals downstream of G_i/G. *J. Neurosci.* **24**, 6826–6832.
36. Cai D., Shen Y., De Bellard M., Tang S., and Filbin M. T. (1999) Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism. *Neuron* **22**, 89–101.
37. Dechant G. and Barde Y. A. (2002) The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. *Nat. Neurosci.* **5**, 1131–1136.