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Glia-derived Inhibitors of Axonal Regrowth: Implication for the Molecular Strategy of Axonal Regeneration

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Summary. **Axonal injury causes fatal damage to the neuron because of the poor regrowth of the injured axon. This phenomenon is one of the most critical reasons why neurological diseases are currently intractable. Recent studies have revealed that a part of the poor axonal regeneration is caused by the presence of the inhibitors to axonal regrowth, derived from glial cells surrounding the axon. Known molecules such as chondroitin sulfate proteoglycan and myelin-associated glycoprotein (MAG), derived from the astrocyte and the oligodendrocyte, respectively, are thought to be involved in the inhibition of the axonal regeneration. Here, we summarize the current findings on regarding the actions of these molecules, and survey strategies for treating the axonal injury from clinical aspects.**

Key words — growth cone, chondroitin sulfate, nogo, myelin-associated glycoprotein (MAG), nogo receptor (NgR).

Introduction

Higher organisms are relatively less potent it to regenerating axons than the lower organisms[1]. In particular, the central nervous system (CNS) neuron of the higher organisms, such as human beings, has difficultly in axonal regeneration after nerve injury. This is the major reason why neurological diseases – including brain damage- are generally intractable. Since Santigo Ramon y Cajal postulated the principle that the mature CNS neuron poorly regenerates, most neuroscientists have believed this intrinsic property to be irreversible. One decade ago, however, new light came to shed on this area, based upon results of developmental neurobiology, i.e., the discovery of the adult neural stem cell, and the inhibitors for axonal growth.

There are two different aspects to neural regeneration. One is the differentiation of the neural stem cell in the mature CNS to the neuron; the other is the regrowth of the injured axon [2]. We focus on the latter in this review. The axon is a major output of the neuron, and the neuron cannot live unless the regrowth of the injured axon occurs. Without regrowth, the dying back phenomenon – known as the Wallerian degeneration, must occur. Since ramon y Cajal, axon degeneration was also believed to be inevitable; however, the current standard is that axonal regrowth in the mature CNS is attenuated by the inhibitors of the axonal growth, expressed on the glial cells around the neuron.[3,4,5] Thus, the removal of the inhibitors theoretically helps with axonal regeneration. The identification and characterization of several such inhibitors have already been computed. In this review, while bearing in mind that the mature axon rather than the immature one has the intrinsic lower activity to grow. We focus on the molecular properties of these inhibitors as well as the principles of axonal growth, and survey the perspectives of strategies for axonal regeneration.

1. Axonal growth and the neuronal growth cone

Axonal growth of the developing neuron is mediated

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Abbreviations– CSPG, chondroitin sulfate proteoglycans; MAG, myelin-associated glycoprotein; NgR, nogo receptor; OMgp, oligodendrocyte-myelin glycoprotein.

by the activity of the growth cone. The growth cone is the motile tip of the extending axon and directs the movement, causing the axon to grow in a specific direction determined by the axon guidance curs. The growth cone also has an ability to recognize the target for synaptogenesis, and, on reaching the proper target, it stops growing and changes into the presynaptic terminal for synaptic transmission in the future. Thus, the growth cone is extremely important for axonal growth. When the mature injured axon regrows, the growth cone must emerge and be maintained at the leading edge.

The axonal inhibitor can be identified by monitoring its activity to inhibit the axonal growth, so called the growth cone collapse assay[6]. The growth cone of the cultured neuron changes its shape, and when this cone meets with the potent inhibitory activity, its exhibits a club-like structure with few filopodia[6]. Each inhibitor of axonal growth, which we discuss later, was discovered using this assay.

2. Reactive astrocytes

When nervous tissues are suffered from an injury, the astrocytes are differentiated into the reactive astrocytes, which limit the damage[7-11]. The reactive astrocyte itself is believed to be essential to CNS repair[12]. In transgenic mice lacking-glial fibrillary acidic protein(GFAP), the differentiation to the reactive astrocyte is suppressed, resulting in the CNS volume being greatly reduced after the CNS injury[13,14,15]. Reactive astrocytes are also known to release several trophic factors that promote axonal growth[8]. However, currently, the reactive astrocyte is generally believed to inhibit the axonal regrowth because this cell produces a larger amount of proteoglycans inhibiting axonal regeneration[16]. These proteoglycans are composed of core proteins and glycosaminoglycan (GAG) sugar chains, and the latter components have a strong activity to inhibit the growth cone activity in both *in vitro* and *in vivo* experiments. Among the neural proteoglycans, heparan sulfate (HSPC) is a positive regulator of the growth cone activity; however, chondroitin sulfate proteoglycans (CSPG), keratin sulfate (KSPG), and dermatan sulfate(DSPG) act generally as the negative regulators.

In particular, CSPG plays the most important role in the axonal growth inhibition as its expression is highest in the injured neural tissue.

3. CSPG

Chondroitin sulfate (CS) is bound to various core proteins and is formed as CSPGs.

In the developing brain, CS is specifically expressed in the various brain regions and is generally thought to form the barrier to axon –axon interactions. Namely, the area where CSPG is highly expressed prevents the axons from entering; thus, the axons avoid such an area and turn in another direction. Neurons, not only glial cells, also produce CSPGs in the developing CNS [16]. Taken together, these facts indicate that CSPG is one of the key components to operate the neuronal connection in the CNS.

1) Structure and synthesis of CS

The backbone of glycosaminoglycan protein in the CSPG is the repetitive structure of disaccharide [GalNAc-GluUA], and sulfation occurs at several positions of these sugars. Four sugar residues consist of Xylose Galactose-Glucuronic acid as the linker, which connects the protein at serine or threonine residues. The extended sugar chains are composed of the repetitive structure of disaccharide [GalNAc-GluUA], and sulfation occurs at several positions of these sugars. Interestingly, HSPG also has the same linker as CSPG, but the repetitive structure differs, i.e.,[GlcNAc-GlcUA/IdUA]. Sufation occurs at the 4-and/or 2-position of GalNAc. Since the CS chain has heterogeneity, at the least five different chains are known: 4S (CS-A), 6S(CS-C), 6di-S(CS-E), and GlcUA(2S) -6S(CS-D). Recent studies have revealed that specific CS chains inhibit the axonal growth in particular although the different chains are more effective in the different systems examined.

Currently, most of the glycosyltransferases involved in the synsthesis of the CS chain have been characterized, which CS chain is most critical to the inhibitory activity has been gradually elucidated. Using *in vitro* model systems, the DNA enzyme inhibiting the xylose transferasre I, the catalyzing enzyme of CSPG at the first step, caused the increase in number of the re-grown axons[17].

The CSPG sugar chain is produced by the reactive astrocytes, and the synthesis of CS is up-regulated on the first to the seventh days after injury. In the area where CS is highly concentrated, the tip of the regenerating axon is degenerated and collapsed[18].

The enzymatic breakdown of CS by the bacterial chondroitinase ABC enhances the axonal regrowh[19].

There are the several hypotheses regarding this: 1) sequestration of the neurotrophic factors; 2) interference of the binding between the neurotrophic

factors and their receptors; and 3) activation or inactivation of CSPG-specific membrane receptors; however, why CSPG act as an inhibitory factor is not clear.

4. Oligodendrocytes

The CNS axon of the higher organisms-including human beings – poorly regenerates; however, the injured PNS axon generally shows good recovery. This had led to an idea that a component specific to the CNS renders axonal regrowth difficult. The myelin sheath, surrounding the axon, differs completely between the CNS and the PNS. The CNS myelin is derived from the oligodendroyte , which is the special component of the CNS. Approximately two decades ago, it was shown that the oligodendrocyte itself is inhibitory to axonal growth, based upon the observation of the growth cone collapse[20]. Currently, three components of the CNS myelin, i.e., myelin-associated glycoprotein (MAG), Nogo-A, and oligodendrocyte-myelin glycoprotein (OMgp), have been characterized as the inhibitors of the axonal regrowth[21] .

5. Myelin-associated inhibitors and their receptors (Fig. 1)

Several myelin/oligodendrocyte components have been characterized as inhibitors of neurite outgrowth. One extracelluar domain of Nogo-A (Nogo-66), oligodendrocyte / myelin glycoprotein (OMgp), and myelin-associated glycoprotein (MAG) are such proteins. Very interestingly, these proteins act as inhibitors of axon regrowth mediated by the same membrane receptor, i.e., Nogo-66 recepor (NgR).

1) Nogo-A

Since 1988, Martin Schwab and its colleagues have been attempting to characterize the CNS-myelin

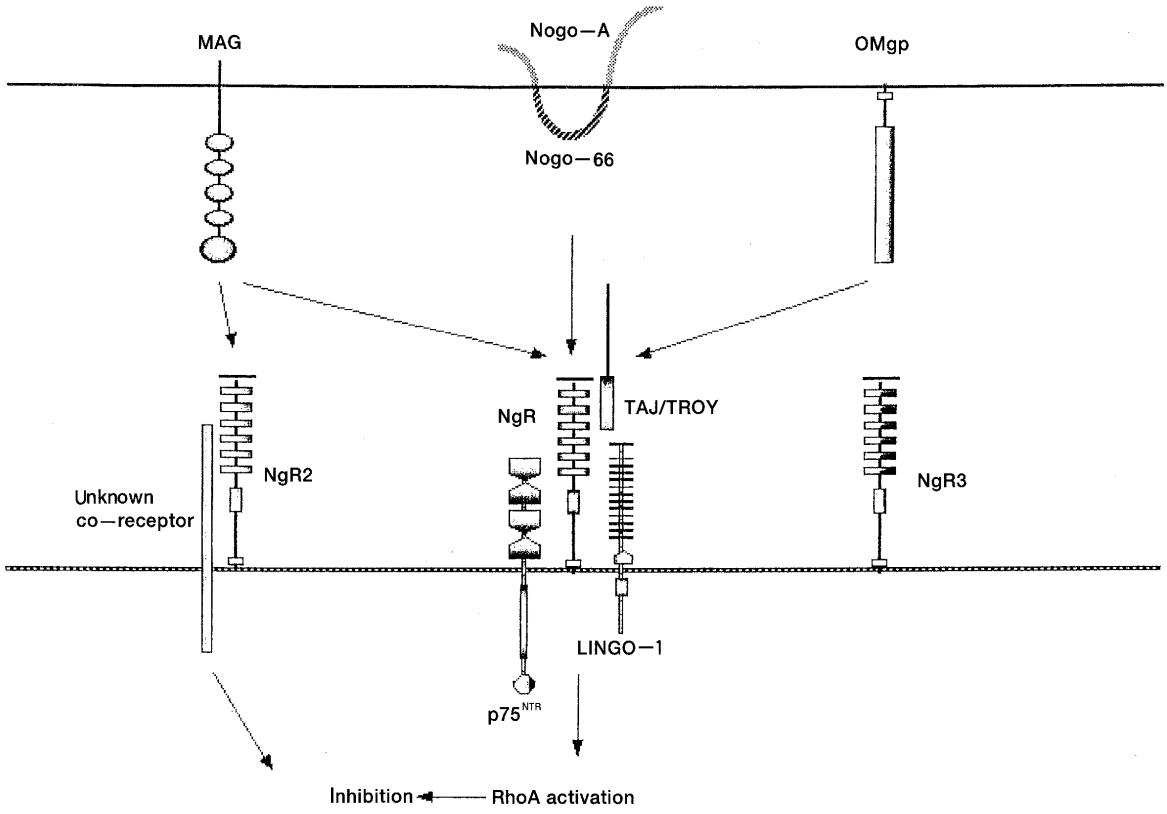

Fig. 1. The inhibitory molecules for axonal regrowth and their neuronal receptors. MAG, Nogo-A, and OMgp are expressed in the oligodendrocyte membrane and are the components of the CNS myelin. NgR is the receptor for these three components; however, it cannot transfer their signals since NgR is lacking in the cytoplasmic protein. Thus, the co-receptors such as $p75^{NTR}$, LINGO-1 or TAJ/TROY are necessary. NgR has two homologues, i.e., NgR2 and NgR3. Rercently, NgR2 has been demonstrated to be a MAG receptor although no specific ligands for NgR3 have been characterized.

derived axonal inhibitor, which is neutralized by the IN-1 antibody[3,22]. The discovery of IN-1 was the first indication that specific molecules are involved in the axonal inhibition in the CNS. Eventually, his group succeeded in identifying its antigen protein as Nogo-A[23]. This protein has two transmembrane protein domains, and belongs to the reticulon family; thus, this protein is also called reticulon-4. Each member of this family is localized to the ER of neurosecretory cells, and Nogo-A is localized to the ER membrane;[24] however, it is currently thought to be also localized to the plasma membrane (see below). These two forms probably have a different confirmation and therefore should remove the distinct signaling pathways, including their receptors. Nogo was first reported to be expressed specifically in the CNS myelin, but this protein has been demonstrated to be expressed in the neuron as well as in the oligodendrocyte. Damage to the neural tissue causes the fragmentation of the plasma membrane and the release of the Nogo-bearing ER to the extracellular space, causing the exposure of Nogo to the tip of the damaged axon. Nogo has three isoforms: Nogo-A, -B, and –C. The latter two, *i.e.,* the short forms of Nogo-A, do not have the axonal inhibitory activity[24].

These share a conserved common carboxyl (C)-terminal structure having two transmembrane domains. GrandPre and Strittmatter argued that 66 amino acids (Nogo-66), lying between the two C-terminus transmembrane domains, mainly inhibit neurite outgrowth[25]. In contrast, Schwab and his colleagues claimed that a unique amino-terminal domain of Nogo-A (Amino-Nogo) mainly contributes to the inhibitory activity[26]. One explanation for these controversial results is that there are two different topologies predicted for Nogo-A. The most common topology is that Nogo-66 is outside the cells, and the N-and C-terminus are present in the cytoplasmic structure. The other topology is that Nogo-66 is in the cytoplasmic area, and ***N***-and ***C***-terminus are located in the extracellular space[27]. These two configurations also appear in the different intracellular localization. The first structure is present in the plasma membrane, and the second one is localized to the endoplasmic reticulum (ER) membrane.

Three groups have produced Nogo-A lacking mice using different strategies[28,29,30]. These results are different from one another: in brief, the Nogo-lacking mice partially showed better results after the axonal injury, but the effects were not so much as they expected in *in vitro* experiments[31]. Thus, the simple deletion of Nogo-A is insufficient to improve poor regeneration of the axon.

2) MAG

MAG is a membrane glycoprotein with one transmembrane domain specific to the CNS myelin. MAG belongs to the immunoglobulin superfamily (IgSF) and is classified as a cell adhesion molecule via homophilic binding; it has five extracellular immunoglobulin (Ig)-like domains, a terminal variable-like domain, and four C2-type Ig domains. MAG is known to be a bifunctional molecule in that it promotes axonal growth in young neurons; however, after maturation, the neuronal response to MAG switches to axonal inhibition[32]. Each IgSF member usually has different roles from the cell adhesion through the heterophilic binding, and the MAG inhibitory activity to axonal growth is currently via NgR. Before the discovery of the interaction between with NgR, MAG was characterized as a myelin-derived inhibitory protein to axonal growth, which is the protein distinct from Nogo. MAG is not only associated with NgR but interacts with ganglioside GT1b[33].

Before the finding that NgR is a receptor of MAG, the signaling of MAG for the inhibition of axonal growth was characterized; namely, the second messenger of MAG's action is cAMP, snd the elevation of cAMP is associated with the MAG inhibitory action[34]. In addition, MAG activates *rho*, a small GTP-binding protein regulating F-actin. Yamashita and his colleagues[35] found that MAG acts through rhoGDI, which links $p75^{NTR}$, a co-receptor, with NgR. In addition, the action of MAG to the growth cone also involves an alteration in Ca2+ levels. The biological significance of MAG to axonal growth inhibition was explained as follows: myelination must carried out after the axonal growth has ceased, and so MAG, which plays a role in myelination, may prevent the overgrowth of axon.

3) OMgp

OMgp was first characterized as a CNS myelin-specific glycoprotein; however, an immunohistochemical study showed that this protein is expressed in the neuron as well as the CNS myelin[36]. Currently, OMgp is known to be localized on the surfaces of oligodendrocytes and the axon-adjacent myelin layer next to the Node of Ranvier. This protein is a GPI-anchored protein with leucine-rich repeats, so its molecular structure is related to NgR itself. OMgp has been shown as a potent inhibitor of neurite outgrowth in the cultured neuron[37,38]. Although the significance of this molecule was not found until NgR was identified, OMgp is currently regarded as one of the inhibitory molecules for axonal growth through NgR[37].

4) Receptors for myelin-associated inhibitors

Strittmmatter and his colleagues identified a protein having the high binding afiinity to the recombinant soluble Nogo-66, and termed it the Nogo-66 receptor (NgR);[25]. NgR contains the *N*-terminus signal sequence, eight leucine-rich repeats (LRR) domains, a LRR carboxy-terminal flanking domain (LRRCT) enriched in cysteine residues, and a C-terminus glycophosphatidylinositol (GPI) anchorage site. *In vitro* experiments, revealed that NgR mediates Nogo-66-dependent inhibition of axon growth. However, recent results have revealed that NgR is not a receptor for amino-Nogo, nor does NgR mediate the amino-Nogo inhibitory activity of axonal growth. Thus, there should be another unknown receptor for amino-Nogo. The Nogo-66 antagonist peptide (NEP1-40) blocks the Nogo-66 inhibition of axonal outgrowth *in vitro*. Intrathecal infusion of NEP1-40 to rats with thoracic spinal cord hemisection results in axon growth of the corticospinal tract and improves functional recovery[39], and truncated NgR competitively inhibiting the Nogo-NgR interaction partially prevents the action of Nogo-A[40], indicating a possibility that the inhibition of the Nogo-NgR interaction is effective to stimulate axonal regeneration.

Other ligands for NgR, i.e., MAG and OMgp have been identified. These two molecules were also the CNS myelin components, and MAG was known to be an inhibitor of axon regrowth[41,42]. They bind to NgR with a high affinity similar to that of Nogo-66, and – like Nogo-66- it was shown that they mediate neurite outgrowth inhibition by NgR binding. Thus, Nogo-66, MAG, and OMgp share a common receptor, NgR, which mediates the inhibition of axon regrowth.

5) Co-receptor of NgR: the signaling pathway mediated by NgR

Since NgR is lacking in an intracellular signaling domain, it alone cannot transduce the intracellular signal. Thus, the presence of a co-receptor for NgR was anticipated for NgR-mediated signaling. One such NgR-parter was p75^{NTR}, the low-affinity neurotrophin receptor, which acts as a receptor for various cytokines[43,44].

NgR activation strengthens the interaction between p75^{NTR} and a small GTP-binding protein *Rho*. Inactivation of *Rho*, or its downstream target *Rho*-dependent kinase (ROK), stimulates neurite growth *in vivo* and *in vitro*. However, in the reconstituted system, the co-expression of NgR and p75^{NTR} together is unable to activate *Rho*, suggesting that the additional components should be required for the NgR-dependent signaling (LINGO-1 is one such candidate; it binds to both NgR and p75^{NTR}, and has been demonstrated to be a component of the NgR/p75^{NTR} complex[45].
Furthermore, recent studies discovered an additional component of the NgR-receptor complex, TROY/TAJ, in the TNF receptor family[46,47]. Since it was reported that NgR and p75^{NTR} are not always co-localized in the mature CNS, these other receptors are likely to contribute to the NgR-dependent signaling[48].

Gene targeting studies revealed that NgR has a potential role in regeneration and cell survival after the CNS damage, but its importance on the axon regeneration is still controvertial. While the regeneration of some raphespinal and rubrospinal fibers does occur in NgR-knockout mice, the corticospinal fibers do not regenerate[49]. Thus, NgR may be responsible for partially limiting the regeneration of some fiber systems in the adult CNS [50]. These results suggest that other receptors for myelin-derived inhibitors act simultaneously with NgR-dependent signaling to regulate the axonal regeneration[50].

6. Homologues of NgR

Genomic search has revealed that the vertebrate has two other GPI-anchored homologues of NgR, named NgRH1/NgR2 and NgRH2/NgR3, respectively[51,52]. These molecules carry eight LRR repeats as well as NgR motief, and these domains, have high homology with those of NgR. These two are also known to be expressed in the nervous systems, but their brain distributions differ from one another[53,54].

Two groups showed that these two NgR homologues do not bind to the known NgR-ligands, such as Nogo-66, MAG and OMgp[51,53]; however, Venkatesh et al[55]. showed that NgR2 produces receptor mediating MAG inhibitory responses. The ligand for NgR3 has not been identified yet. It will be necessary to study whether NgR3 binds to the NgR ligands. The identification of a receptor and the complex that interacts with myelin inhibitors is an important step for overcoming inhibitors of regeneration. Progress in this field gives real hope for therapeutic interventions to promote neuronal repairs following the CNS injury. More work is needed to understand fully the CNS regeneration.

7. Semaphorins

Semaphorins have several members which have the sema domain. Members of this family generally act as the inhibitory molecules for axon guidance in the

developing CNS; however, the injured neural tissue sometimes produces semaphorins, which prevent the axon from regeneration.

Conclusion

To elucidate how the injured axon of the CNS poorly regenerates is one of the most challenging question for neuroscience in the 21st century. Considerable progress concerning knowledge of the inhibitors for axonal regrowth has been seen over the past many decades. Effective antibodies, peptides, and recombinant proteins neutralizing the inhibitors have been identified and characterized at the level of laboratory experiments using rodents; [56] a vaccination was also attempted.[57]

However, no single molecule of these can explain the poor axon reconstruction and completely repair the axon. Namely, not even model animals such as knockout mice lacking an inhibitory molecule have exhibited favorable recovery of the damaged CNS, indicating that the intractable CNS axon regeneration involves at least several distinct inhibitory molecules[50]. As for human CNS injury, in the brains, are more complicated. It shall take a long time to find complete molecular pathways enabling the injured axon to regrow; nevertheless, investigation of each pathway will provide an important progress toward the therapeutic options for nerve regeneration[7].

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