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NgR acts as an inhibitor to axonal regeneration in adults

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1. ABSTRACT

After injury to the central nervous system (CNS) in adults, axonal regeneration is confined, in part, by the inhibitory factors in CNS myelin, including Nogo-A protein, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMGP). These proteins, all bind to the same neuronal glycosylphosphatidylinositol-anchored receptors (NgR), and transduce inhibitory signals to cells by the transmembrane co-receptor, p75. The final outcome of this event is inhibition of axonal regeneration and recovery of locomotion. Based on these findings, one avenue for promotion of axonal regeneration is by virtue of blocking the inhibitory effects of NgR.

2. INTRODUCTION

In contrast to neurons in the peripheral or embryonic nervous system, lesioned axons in the adult mammal CNS are unable to regenerate spontaneously after injury, and result in permanent functional deficits. Several reasons have been illustrated to account for such a failure, such as a decline in the intrinsic growth environment in mature neurons (1), the physical barrier formed with the astrocytic scar tissue containing chondroitin sulfate proteoglycans secreted by reactive glial cells around the lesioned site (2), and the presence of inhibitory factors associated with CNS myelin (3). The latter has been believed to be the main reason for the failure of regeneration. To date, three major inhibitory factors in CNS

myelin have been characterized, including Nogo-A, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMGP) (4). In addition, Tanascin-R (TN-R), which is not extensively studied previously, is also being reported to involve in inhibiting axonal regeneration (5). Most studies have been focusing on MAG and Nogo, especially the latter, since it was first found as an inhibitory factor of axonal regeneration.

Nogo contains three forms, Nogo-A, Nogo-B and Nogo-C, all of which share a c-terminal sequence of 188 amino acids homologous to the reticulon protein family (6). Limited study has been done on Nogo-B and Nogo-C, even though Nogo-C has been shown to be able of delaying axonal regeneration in the peripheral nervous system (PNS) *in vivo* (7). Nogo-A is predominantly expressed in the endoplasmic reticulum and the plasma membrane of oligodendrocytes producing myelin, which appears to have two inhibitory domains comprising an amino terminal domain (amino-Nogo) that acts on many cell types and an extracellular domain flanked by two hydrophobic segments, termed Nogo-66, which selectively inhibits postnatal axon outgrowth by binding with Nogo-66 receptor (NgR) (8). NgR expressed at cell surface renders cell Nogo-66 responsive *in vitro*. The amino-Nogo domain functions through unknown NgR-independent mechanisms (9). The two other axonal outgrowth inhibitors, MAG and OMgp, also bind to NgR despite of their lack of sequence similar with Nogo-66 (10,11,12). Thus, these three inhibitory factors associated with axonal regeneration in CNS appear to utilize one common receptor-NgR.

TN-R is a large multidomain protein that forms either dimmers (the 160 KDa isoform) or trimers (the 180 KDa isoform) and is secreted into the extracellular matrix by oligodendrocytes and their precursors (13). It is composed sequentially of a cysteine-rich N-terminal domain, followed by 4.5 epidermal growth factor-like (EGFL) repeats, eight fibronectin (FN) type III like domains and a c-terminal region that bears homology to the fibrinogen calcium-binding sequence. TN-R modulates neuronal survival and axonal regeneration by microglia and macrophage (14,15).

To date, many reviews have targeted on several aspects of inhibition of anxonal regeneration, including Nogo-A, MAG, OMgp as well as other molecules involved in inhibiting signaling pathways, however, few reviews summarized the NgR as a common receptor of the three myelin associated inhibitors. This review focuses on the NgR, including its identification, structure, distribution, functional properties, and molecular mediating mechanism, with an emphasis on the manipulation of NgR to improve axonal regeneration.

3. ROLE OF NGR IN NEUROLOGICAL INJURY

3.1. Identification and structure of NgR

After the findings of those three anxonal regeneration inhibitory factors, their molecular mechanisms in inhibiting axonal regeneration were investigated. By means of the placental alkaline phosphatase (AP) fusion protein approach and an expression-cloning strategy, Strimatter *et al* identified a GPI-linked axonal surface protein, termed Nogo-66 receptor (NgR) that can bind Nogo-66 with a high affinity (8). The protein is predicted to contain 473 amino acids, with a conventional amino-terminal membrane translocation signal sequence. This is followed by eight leucine-rich-repeat (LRR) domains, one LRR carboxy-terminal flanking domain that is cysteine, one unique region and one glycosylphosphatidylinositol (GPI) anchorage site. Enzymatic cleavage of NgR renders neurons unresponsive to inhibition by Nogo-66. In addition, forced expression of NgR is sufficient to impart Nogo-66 axon regeneration to unresponsive neurons. Surprisingly, by the same expression-cloning strategy, the same NgR molecule was found to bind OMgp with a high affinity (12). Both loss of function and gain of function experiments were similar to those for NgR/Nogo-66 interaction (8), thus NgR has also been implicated as a required receptor component for OMgp in neurite outgrowth inhibition (12). Although the c-terminal serine/threonine repeat-containing region can also bind weakly to NgR, the LRR containing domain of OMgp appears to be sufficient for binding NgR to inhibit axonal regeneration. Two independent studies suggested that NgR was also involved in the inhibitory activity of MAG for axons (11). In 2002, Liu *et al* identified that MAG binds to NgR directly with a high affinity (10). Cleavage of GPI-linked protein from axon protects growth cones from MAG-induced collapse, and dominant-negative expression of NgR eliminates the MAG-induced inhibition of axonal regeneration, providing substantial evidence that MAG requires NgR in the inhibition for neurite outgrowth.

It is unusual that three different inhibitory molecules bind to the same NgR receptor molecule. However, it is possible that some of the biophysical properties of NgR may allow it to bind different proteins easily, for example, the charged residues enriched in the NgR surface might account for such binding. Many available structural and functional data show that both the N-terminal region of NgR which harbors eight LRR motifs (LRRNT) and the LRR c-terminal region (LRRCT) are capable of binding different ligands (16). Although the precise binding sites for these ligands have not been identified, it is likely that these ligands bind to overlapping but distinct regions.

3.2. Distribution of NgR

The distribution of NgR mRNA expression is consistent with the function for the protein in regulating axonal regeneration and plasticity in adult CNS (8). A high level of NgR mRNA expression is observed in adult brain by Northern analysis, while a low level of its mRNA expression is observed in heart and kidney. There is no expression in peripheral tissues. In brain, NgR expression is widespread and richest especially in gray matter. *In situ* hybridization analysis shows that NgR expresses in cerebral cortical neurons, hippocampal neurons, cerebellar purkinje cells and pontin neurons. Our preliminary observation demonstrates that the expression levels of NgR

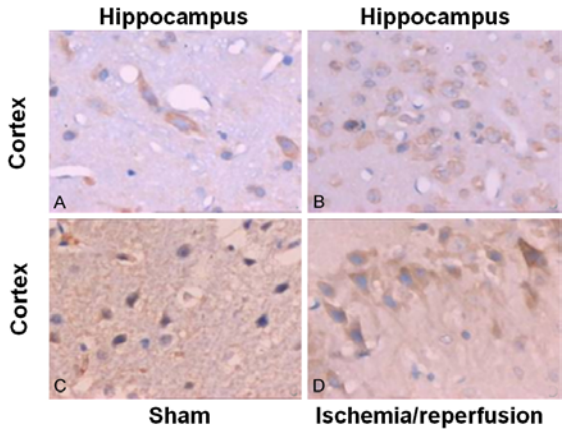

Figure 1. In the sham group a few NgR-positive immunoreactive cells are detected and NgR protein distributes on the membrane and their processes of the neurons in cortex and hippocampus. At 24h after ischemia-reperfusion NgR positive cells were increased most significantly. A,B: cortex; C,D: hippocampus.

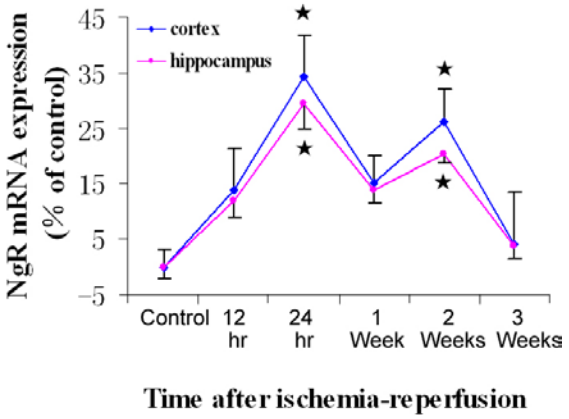

Figure 2. RT-PCR analysis demonstrates demonstrated the expression of NgR mRNA is was significantly increased in the infracted cortex and hippocampus at 24h and 2w after ischemia-reperfusion. ($p<0.01$ compared to control group in the same region, one-way ANOVA).

mRNA and protein in the infarted cortex and hippocampus were significantly increased by RT-PCR and immunohistochemistry analysis, especially at 24 hours and 2 weeks after cerebral ischemia-reperfusion (Figure 1-2). NgR is expressed in cerebral cortex pyramidal neurons whose regeneration is enhanced by IN-1 treatment (17), as well as in cerebellar pukinje neurons which sprouting increased by anti-Nogo antibody (18). NgR mRNA is not detected in white matter, where Nogo-A is expressed in oligodendrocytes.

However, some studies suggested that NgR may not be expressed in all neurons in the adult CNS. Hunt *et al* found that high level of NgR expression could be detected in only 20-25% of dorsal root ganglion neurons by situ hybridization analysis (19).

3.3. Molecular mechanism of NgR

NgR is a GPI-linked protein bound to cell surface and lacks an intracellular component, suggesting that it depend on additional transmembrane proteins as co-receptors for inhibitory signaling transduction to inhibit axonal regeneration. One study showed that the overexpression of a truncated form of NgR lacking its unique c-terminal regions (CT) allowed extensive neurite outgrowth on myelin substrates, indicating that c-terminal region of NgR may be involved in binding the co-receptors (11). Later on, p75, a low-infinity neurotrophin receptor and a member of the TNF receptor (TNFR) family, was found to serve as a co-receptor for NgR and probably acted on the signaling pathway in this receptor complex (20 21). The interaction of p75 with NgR is mediated through its extracellular domain, but the intracellular domain of p75 is necessary for inhibitory activity of axonal regeneration. Yamashita *et al* reported that mutant mice lacking p75 protein were less sensitive to MAG inhibitory activity (23). It is further demonstrated that p75 is necessary for mediating the inhibitory activity of all three myelin-associated inhibitory factors (20). Binding studies using purified protein also demonstrated that the full-length NgR protein is necessary for its binding to p75, which suggests that the c-terminal of NgR is involved in the co-receptor (20 21). Although there is a relationship between signaling pathway of axonal regeneration and neutrophins, both *in vitro* and *in vivo* experiments suggest that p75 functions as a growth inhibitory molecule (24,25). In cultured sympathetic neurons that normally express TrkA (not TrkB) and p75 nerve growth factor (NGF) p75 could promote neurite outgrowth via TrkA while brain-derived neurotrophic factor inhibited axonal regeneration via p75, suggesting NGF and brain-derived neurotrophic factor function antagonistically in which p75 acts as an inhibitory molecule.

Recently, LINGO, which is a neuronal transmembrane protein and contains 614 amino acids, was found to play an important role in binding of NgR and p75. LINGO makes OMgp responsive to RhoA activation in cos-7 cells transfected with NgR, p75 and LINGO-1, but not in the cell transfected with only two of these three components, indicating that it is necessary for the formation of functional receptor complex for NgR and p75. LINGO is found to be selectively expressed in brain, with the highest level in cortex and lowest level in spinal cord, which is similar to the expression pattern of NgR (27).

In contrast, p75 expression is much more restricted. P75 is highly expressed in the developing CNS, during the periods of axonal outgrowth and dendritic arborization, and p75 decreases over the postnatal period and adult period (28,29). Thus, p75 expression is only detected in some subpopulations of mature neurons, suggesting that additional proteins exist as the co-receptor of NgR (30,31). p75 homologue was reported to be found in Xenopus (32). In addition to p75, other transmembrane molecules may interact with NgR and act in transducing the inhibitory signaling, such as GT1b. Anti-GT1b antibody is reported to be able to neutralize the inhibitory activity of MAG (33) and p75 associates with GT1b (22). Other

ganglioside-binding proteins may function in independent of p75 in at least some types of neurons in the adult CNS.

TROY (also known as TAJ) is also reported to form a functional receptor complex with NgR and LINGO-1 which may mediates axonal outgrowth inhibition and is involved in signaling pathway triggered by myelin inhibitors (36). Meanwhile, it has been demonstrated that less expression of TROY or the presence of a soluble TROY protein can efficiently block inhibitory activity of myelin inhibitors. This implicates a key role of TROY as another co-receptor in mediating the inhibitory effect of myelin components on axonal regeneration and offers new insights into the molecular mechanisms of axonal regeneration failure in the adult CNS system.

Binding of NgR and its co-receptor can trigger small GTPases of the Rho family, In Rho family the most widely expressed members includes RhoA, Rac1 and cdc42 which are known to be regulators of the actin cytoskeleton in all eukaryotic cells. GTP makes these regulators active and GDP makes them inactive through the binding of guanine nucleotides. The function of Rho-GTPases is also regulated by either guanine nucleotide exchange factors (GEFS) which can enhance GTP-binding activity or GTPase-activating proteins that can increase GTP hydrolysis. In neuronal cells activation of RhoA stimulates actinomyosin contractility and stress fiber formation, resulting in growth cones collapse, whereas the induction of cdc42 and Rac1 leads to the extension of filopodia and lamellipodia respectively (37).

It has also been suggested that C3 transferase can inactivate RhoA and induce axonal regeneration (38). Rho-associated kinase (ROCK), a major RhoA effector, is implicated in the myelin-mediated reorganization of cytoskeleton. Y27632, a synthetic inhibitor of ROCK, can block the inhibitory signaling pathway (39,40). Researchers did *in vivo* studies on mice and rats and found that inactivation of RhoA and ROCK could promote axonal regeneration and functional recovery after spinal cord injury, indicating the important roles of RhoA and ROCK in inhibiting axonal regeneration (36,37,41). Cai *et al* found that pretreatment of neurons with neurotrophins could block the inhibitory effects of MAG with elevation of the level of cAMP and activation of PKA suggesting that cAMP-dependent PKA phosphorylate p75 and affect its translocation into lipid rafts, which provides a possible mechanism that intracellular cAMP level may mediate myelin inhibition (42,43).

Ca2+ is also reported to play a role in inhibiting axonal regeneration. Significant increasing in Ca2+ may cause its binding to calmodulin, which can activate proteinase leading to ROCK phosphorylation. Inactivated RhoA induced by phosphorylated ROCK can inhibit axonal regeneration (44).

3.4. Biological function of NgR

NgR which functions as a common receptor to those three myelin-associated inhibitors can induce growth cones collapse by triggering inhibitory signaling pathway, leading to the inhibition of axonal regeneration.

To identify the indispensability of NgR in inhibiting and restricting axonal regeneration in the adult CNS, Kim *et al* generated ngr-/- mice (45). Their result shows that mice lacking NgR protein are viable but display hypoactivity and motor impairment. DRG neurons lacking NgR protein do not bind Nogo-66 and their growth cones are not collapsed by Nogo-66. The recovery of motor function after dorsal hemisection or complete transaction of spinal cord is improved in mice without NgR. However, not all fiber systems can regenerate in the adult spinal injury when NgR protein is absent. Regeneration happens in raphespinal and rubrospinal fibers, but not in corticospinal tracts after spinal injury despite regeneration of these tracts was observed in some stains of Nogo-A mice and treated with NgR antagonists, suggesting that NgR protein represents just one of several components preventing significant regenerative axon growth of certain fiber systems in the adult CNS (45). Other studies demonstrate that there can be two proteins most closely related to NgR protein, namely NgR2 and NgR3. Both of them do not bind to Nogo-66, MAG and OMgp in *in vitro* experiment (16). Thus null mutation of ngr gene may be predicted to delineate the role of all three myelin-associated inhibitory factors separately from amino-nogo. Amino-nogo, an amino terminal domain, same as the other inhibitory domain for axonal regeneration in CNS in Nogo-66, acts on many cell types to inhibit axonal regeneration, indicating that in addition to NgR, there may be other receptors to bind amino-Nogo to function as axonal inhibition. However, it is found that the improvement of behavior and electrophysiology after complete spinal transaction was observed only in mice lacking NgR protein.

Similar studies in mice lacking Nogo with NEP1-40 treatment did not reveal a same obvious level of improvement. Therefore, the regenerative capacity of fiber systems descending from the brainstem to the spinal cord seems mostly to depend on NgR and its ligands, rather than on Amino-nogo or other not identified factors. Meanwhile, a study has shown that mice lacking NgR grow with normal body weight. Both males and females are fertile. There are no unexpected deaths in homozygous up to 13 months of age (45). In histology, brain size and gross anatomy are normal in adult mice lacking NgR. There is no gross abnormality in the white matter tracts of the brain and spinal cord assessed by Luxol fast blue staining for total myelin content and location. By hematoxylin and eosin staining, the major brain, nuclei and neuronal layers of cerebral cortex and cerebellum are all indistinguishable from those of wide-type mice. The data indicate that neuronal placement and survival are normal in adult mice lacking NgR (45).

Despite of the normal histology appearances, the absence of NgR might cause changes in neuronal function and mouse behavior through the effects on axonal regeneration. The deficits in function are suggested by neurological examination on mice lacking NgR. These mice display normal health and fertility; however, their

behaviors are altered in the open field and rotorod tests compared to wild type mice. Mice lacking NgR make fewer movements, avoid the center of arena and fell off of a spinning rod early. Despite of the subnormal performance on rotorod test, in the test of Basso-Beattie-Bresnahan (BBB), which is another measure of to evaluate locomotor function in the open field and is commonly used to assess deficits after spinal cord injury (46), mice lacking NgR exhibit a full range score similar to wild type mice. Overall, these anatomic and locomotor changes in the mice lacking NgR are not lethal. At present, the reason for these anatomic and behavior changes is not clear. The presence of these changes suggests that NgR contributes to the formation, refinement or maintenance of some pathways required for normal mouse behavior. While NgR may be negative to axon regeneration after adult CNS injury, it appears somehow to be beneficial to animal behaviors in intact mice.

To determine whether disinhibition of Nogo-NgR activity might induce increased axon sprouting after stroke and promote the recovery of locomotor behavior, Lee *et al* used the genetic method to assess the role of Nogo-NgR system played in axonal plasticity and behavioral recovery after ischemic stroke (47). Transgenc ngr-/- mice were generated to be investigated in stroke model. These mice have a target mutation on ngr gene and lack immunoreactive NgR protein. Investigators compared the response of ngr-/- mice versus heterozyous littermate wild type mice after a photothrombotic lesion of the left sensorimotor cortex. Rose Bangal was infused systemically, and then the cortex was illuminated at one focus to produce a discrete stroke based on light absorption by the dye. Results showed that the lesion volumes at 1 month after injury were identical in both ngr-/- and ngr+/- mice. Despite that limb movement and open field lomotion returned promptly to normality after the lesion in both mice, a persistent deficit in motor performance was detected in wild type mice compared to ngr-/-mice, In stair test of skilled forelimb reaching ability, photothrombotic lesions reduced the number of pellets (the food to evaluate forelimb function) retrieved with the contralateral forepaw in both mice, but significantly greater recovery was observed in the ngr-/-mice. In behavioral test, before stroke ngr-/- mice was indistinguishable from wildtype mice, however, after stroke the recovery performance was significantly greater in ngr-/- mice. Axonal plasticity was also evaluated in ngr-/- mice. An axonal tracer, BDA, was injected into the intact (non-stroke) motor cortex and those fibers were traced into the midbrain at the level of the red nucleus. Before a stroke, the pattern of labeling in ngr-/- mice and wildtye mice was identical, however, after a stroke, the stroke-induced plasticity in ngr-/- mice was doubled in comparison with wildtye mice, suggesting that the lack of NgR protein promoted a greater number of stroke-induced corticorubral fiber sprouting. Stroke can greatly increase the number of corticofugal fiber sprouting (47), and the lack of a functional NgR protein may account for the improved recovery of locomotion by enhancing axonal plasticity in the stoke model, which provides an effective method for the functional recovery after stroke.

Overall, NgR plays a key role in inhibiting axonal regeneration and blocking functional recovery after CNS injury even though it may be beneficial to behaviors in normal mice. Further studies are needed to determine the other functions of NgR.

3.5. Signaling Pathway of NgR

The binding of NgR to each of the three myelin associate inhibitory factors can induce growth cones collapse through inhibitory signaling pathway and block axonal regeneration, thus, NgR may be regarded as a pivot target in improving axonal regeneration.

One study has shown that NEP1-40, a synthetic Nogo-66 (1-40) peptide (Nogo extracellular peptide, residues1-40), bind NgR, but not GPI, at the cell surface for inhibitory signaling pathway because of characteristics of c-terminal-deleted Nogo-66 deprivation (48). It was found that NEP1-40 did not cause collapse E12 (in embryonic day 12) chick DRG growth cones even at 1um concentration. When NEP1-40 was added into E12 chick DRG explant cultures together with the NgR antagonists glutathione s-transferase (GST)-Nogo-66 and AP-Nogo-66, it blocked growth collapse induced by both GST-Nogo-66 and AP-Nogo-66. The ability of NEP1-40 to neutralize Nogo-66-induced inhibition of neurite outgrowth has also been assessed. Soluble NEP1-40 alone has no obvious effect on neurite outgrowth, but it can reverse the inhibitory effects of bound GST-Nogo-66. A scrambled sequence version of NEP1-40 does not possess NgR antagonist activity. A second domain of Nogo-A and Amino-nogo seems to inhibit axonal regeneration by a mechanism of independence on NgR (48). These data propose Nogo and NgR have important roles in limiting axonal regeneration. Analysis of ligand binding to NgR reveals that a high-affinity binding domain is largely separatable in the amino acid residues involved in activating NgR signaling in neurons. As a competitive antagonist of NgR, the ability of NEP1-40 to attenuate the inhibition for axonal regeneration by CNS myelin demonstrates that NgR is a very important mediator of this inhibition.

The binding of Nogo-66 to NgR requires a soluble N-terminal domain (residues27-310 for human NgR, NTF-NgR) containing the LRRNT, LRR and LRRCT sub-domains of the receptor. The lack of any two of the eight repeats in the LRR domain is insufficient for its binding to ligands (8,49). On the other hand, NgR binding to NgR requires the c-terminal domain (residues311-346, CTF-NgR) (20). Like many other GPI-anchored proteins, CTF-NgR resides with cholesterol-rich regions of cellular membrane known as lipid rafts which serve as platforms for a number of signaling transduction pathways (50). The raft-association of NgR is not necessary for growth cones collapse induced by Nogo-66, although the efficiency of this inhibition is diminished when NgR resides outside rafts (49). In addition, several reports have shown that the soluble full length NgR ectodomain lacking the GPI anchor and an artificially c-terminal truncated NgR ectodomain comprising the ligand binding domain alone are potent antagonists of neurite regeneration inhibition mediated by Nogo-66, MAG, OMgp and whole myelin *in vitro* as they

are proficient in binding ligands but incapable of tranducing inhibitory signaling (11,16,23,49).

The recent study has focused on identifying potential pathway involved in the regulation of NgR in neuronal cells. Adrial *et al* found that human NgR expressed in human neuroblastoma cells was constitutively proteolyted in a post-ER compartment processed by the action of Zinc metalloproteinase to generate a lipid--raft associated C-terminal fragment and a soluble N-terminal fragment. The C-terminal fragment is present on cell surface but not in the medium and its location is consistent with the C-terminal of GPI-anchors of NgR. The N-terminal fragment is released into the medium and is capable of binding the NgR ligands, Nogo-66, MAG and OMgp, but not the NgR co-receptor p75. Mass spectrometric analysis demonstrated that the N-terminal fragment attaches just at the C-terminus of the ligand-binding domain of NgR. Researchers also observed some N-terminal fragment of NgR in the lipid raft fractions with the sucrose gradient. It is possible for this raft location that N-terminal fragment of NgR interacts with lipid raft associated full length NgR. More importantly, the C-terminal fragments and N-terminal fragments of NgR were detected in the human cortex and cerebrospinal fluid (CSF) and were similar to those generated by neuroblastoma, demonstrating that NgR proteolysis occurs also in the human nervous system (51). This provides a potential endogenous mechanism for the regulation of NgR. In other shedding mechanism, the release of the N-terminal fragments of NgR is blocked by a hydroxymate based inhibitor of Zinc metalloproteinase, PKF226-967, but not by other inhibitors of other protease classes and up-regulated by treatment with the cellular cholesterol deleting agent methyl-β-cyclodextrin (MβCD), which induces the ectodomain of another neuronal expressed GPI-anchored protein (51).

Recent finding shows p75 can be cleaved proteolytically by TnF-alpha converting enzyme (TACE), one of the main numbers of a disintegin and metalloproteinase (ADAM), resulting in the release of a N-terminal fragment containing the binding domain for nerve growth factor (52). The proteolysis of NgR and its co-receptor highlights a potential role for ectodomain shedding in the regulation of neurite regeneration inhibition mediated by NgR. Investigators also found that the combination of basic fibroblast growth factor, neurotrophin-3 and brain-derived growth factor could promote greater axonal regeneration than NTT alone by regulating intramembraneous proteolysis of p75 and ectodomain shedding of NgR, suggesting that combined modulation of inhibitory signaling elements together with stimulation of growth signaling is a more efficient approach to improves axonal regeneration and functional recovery after adult CNS injury (53).

3.6. Approach for improving axonal regeneration by NgR

To date, researchers have identified some approaches for improving axonal regeneration by both *in vitro* and *in vivo* experiments at different levels, such as antibody, signaling molecule and receptor approaches. Of them, receptor approach appears to have a greater effect on promoting axonal regeneration by some studies.

In vivo studies have reported that the antibody against Nogo could reduce myelin inhibitory capability. Investigators from Schwab's group produced an antibody by a hybridoma that was effective but showed a low affinity. They later produced a high-affinity fragment, which can neutralize Nogo inhibitory activity in cell culture (54). In the *in vivo* experiments researchers administered IgM antibody against Nogo to animals after spinal cord injury, seeing increasing sprouting of axons at the lesioned site. The improvement was also seen in locomotor and reflex functions, demonstrating that Nogo-A antibodies acts effectively after spinal cord injury (55). A partially humanized, recombinant Fab fragment, deriving from the original hybridoma-generated antibody, can also promote long-distance regeneration of injured axons into the spinal cord of adult rats. Following spinal cord injury, the antibodies were delivered into the injury site, and an obvious regeneration of corticospinal tracts was observed after the treatment for two weeks (56).

In addition to spinal cord injury, the monoclonal Nogo antibody also acts effectively in the stroke model induced by permanent middle cerebral artery occlusion (MCAO) indicated by the improvement in the forelimb function (57). Despite that the antibody against Nogo-A does not affect infarct volume or brain atrophy, it can increase the number of midline-crossing corticospinal fibers in the cervical spinal cord, which originate in the unlesioned sensorimotor cortex that is related to behavioral outcome. These data suggest that the Nogo antibody is very beneficial to improve axonal regeneration.

The blockade of intracellular activity of signaling molecules has also been reported to improve axonal regeneration. Three myelin-associate inhibitory proteins induce the activation of RhoA which is one of the key regulators of cytoskeletal organization in some neurons. Inhibition of Rho or Rho-kinase which is a downstream component promotes axonal regeneration *in vivo* (38,41). These finding reveals that Rho and Rho-kinase play important roles in inhibiting axonal outgrowth in adult CNS. The recent finding suggests that several new candidate proteins may be axonal growth inhibitors. These proteins activate not only Rho/Rho-kinase but also other inhibitory signaling molecules *in vitro*. Inhibition of repulsive guidance molecule (RGM), one of inhibitory proteins, is reported to promote neurite outgrowth and functional recovery after spinal cord injury in rats (58,59). These findings reveal that agents that block the multiple signals elicited by these axonal growth inhibitors may provide efficient tools to promote functional recovery following CNS injury. Repulsive guidance molecule (RGM) is a protein implicated in both axonal guidance and neural tube closure. It is reported that RGM is a potent inhibitor of axonal regeneration in adult CNS and it might inhibit mammalian CNS neurite outgrowth by a mechanism dependent on the activation of the RhoA/Rho-kinase pathway. RGM is mainly expressed in oligodendrocytes,

myelinated fibers and neurons of the adult rat spinal cord. An antibody has been developed to efficiently block the effect of RGM *in vitro*. *In vivo*, intrathecal administration of antibody to rats with thoracic spinal cord hemisection resulted in significant axonal regeneration of corticospinal tracts and improved functional recovery (58,59). These data suggest RGM antibody as a possible therapeutic candidate in clinical conditions when failure of CNS regeneration happens.

Y27632 is also reported to inhibit Rho-kinase and promote axonal regeneration. In a recent study, investigator focused on the effect of Y27632 treatment on astrocytes which are a key component in reactive gliosis. *In vitro*, overnight treatment with Y27632 (1 to 50 μM) caused the astrocytes to assume an activated morphology and to upregulate CSPG expression as shown by CS56 immunostaining. Cortical neurite growth decreased on the extracellular matrix (ECM) deposited by Y27632-treated astrocytes compared to that on ECM from nontreated astrocytes. Thus, Y27632-treated astrocytes might be a less permissive substrate for neurite growth than nontreated astrocytes. On the other hand, conditioned medium from drug-treated astrocytes promoted neurite growth. *In vivo*, astrocyte activation and CSPG expression have also been assessed in vehicle- and Y27632- treated rats one week after spinal cord dorsal hemisection injury. The results indicate that at the edge of the lesion cavity, close to the transected dorsal column tract (DCT) (or sensory) tract, glial fibrillary acidic protein (GFAP) immunoreactivity increased in Y27632-treated animals compared to that in vehicle controls. Y27632-treated animals had elevated neurocan immunoreactivity at the lesion edge and along the degenerating DCT. Thus, Y27632 treatment may exert both promoting and inhibiting effects on axonal regeneration *in vivo* (60).

C3 transferase was reported to induce axonal regeneration by inactivating RhoA. When C3 transferase was administrated in adult mice following dorsal T7 hemisection, it resulted in the improvement of axonal outgrowth which is correlated with recovery of motor function. The study also indicated that C3 transferase seems to be much better than Y27632 in promoting axonal regeneration, suggesting that RhoA might be an effective target than Rock foy reoair in injured CNS (39,41).

Axonal regeneration can also be improved by prevention of intracellular calcium influx and elevation of cAMP. Dantrolene protects growth cones of cultured rat dorsal root ganglion neurons from collapse induced by exposure to a neurite growth inhibitor. Decreasing in internal calcium storages by caffeine largely prevents the effect of the neurite growth inhibitor, suggesting that the action of dantrolene is likely due to its inhibition of calcium release induced by caffeine. The study also demonstrated that inhibition of the synthesis of polyamines inhibits the ability of the increased cAMP concentrations to block myelin inhibitors like MAG (5).

The above approaches for improving axonal regeneration only target a single myelin protein or a signaling molecule, which may not sufficient to facilitate maximal CNS axonal regeneration. NgR, as a common receptor for all three myelin-associated inhibitors, play a pivot role in inhibiting axonal regeneration. Thus, the intervention or blockade of NgR may be regarded as a key target for clinical therapy and pharmaceutical application in CNS diseases, for example, NEP1-40, as a NgR antagonist, has been reported to improve axonal regeneration and promote functional recovery in experimental CNS injuries.

Previous methods used to promote axonal regeneration have been focused mostly on traumatic spinal cord. Blockade of myelin inhibition of axonal growth by myelin immunization promotes axonal regeneration. Anti-Nogo antibodies have been reported to be beneficial to rats with middle cerebral artery occlusion (MCAO), however, these antibodies such as IN-1, only target a second inhibitory domain in Nogo-A and do not directly block NgR protein. Recently, the soluble NgR (310) Ecto-Fc protein, which can bind Nogo-66 because of its containing the ligand-binding domain and which does not transduce inhibitory signaling (49,63), is reported to improve axonal outgrowth and promote locomotor function recovery (47). In this experiment, NgR (310) Ecto-Fc protein was infused 7 days after stroke into the lateral ventricle of the rats opposite to the stroke side and continued for 2 months meanwhile, control animals received rat IgG at the same dose. The histological examination carried out at 11 weeks after MCAO demonstrating reproducible stroke lesions and no significant difference in the size of the stroke between the NgR (310) Ecto-Fc protein-treated group and the control group, which suggested that any improvement in behavioral recovery after this treatment could not be induced by neuroprotection. Behavioral recovery was also evaluated by stair and rotarod tests. In stair test, recovery is significantly much better in the NgR (310) Ecto-Fc protein-treated group. In rotarod test, the performance also improved in a greater extent in NgR(310)Ecto-Fc protein-treated rats, while a persistent deficit was detected in the control group. The researchers provided evidence that rats treated by NgR (310) Ecto-Fc protein have a greater recovery, demonstrating that the NgR (310) Ecto-Fc protein treatment could double corticofugal axonal plasticity response to stroke. It is likely that a combination of NgR (310) Ecto-Fc and anti-Nogo-A antibodies may produce greater effects than either reagent alone. In addition, the Inosine action is also thought to be dependent on the Nogo-NgR pathway, thus it is possible that NgR (310) Ecto-Fc and Inosine treatments can also promote greater axonal plasticity and functional recovery after stroke. Currently NgR (310) Ecto-Fc was administered intracerebroventricularly, which was different for some cases of clinic stroke. NEP1-40 administered intracerebroventricularly has been reported to promote axonal regeneration and locomotor recovery after spinal cord recovery (48). Furthermore, it is believed that NgR(310)Ecto-Fc also access to CNS to promote stroke recovery (47).

To date, pharmacological stroke therapy always focuses on preventing risk, immediate acute thrombolytic and neuroprotective strategies to reduce existed damage in brain, Application of NgR(310)Ecto-Fc offers another

approach to enhance recovery after stroke by promoting axonal regeneration and axonal plasticity in clinic therapy in the future. 7E11, as a specific monoclonal antibody to NgR, is found to block the interactions of Nogo-66, MAG and OMgp with NgR and reverse the inhibitory effects of CNS myelin on axonal regeneration in primary neurons. One study used protein-based ELISA binding assays to test the biochemical effects of 7E11 on the interactions of Nogo-66, MAG and OMgp with NgR. The result demonstrated that 7E11 could dose-dependently inhibit the binding of all three myelin-associated proteins,-Nogo-66, MAG and OMgp to their common receptor NgR, however, it had no effect on the binding of Nogo-66, MAG and OMgp and the IgG protein. Investigators compared the potency of 7E11 to the NgR(310)Ecto-Fc protein in binding with Nogo-66, MAG and OMgp. Their results demonstrated that 7E11 was a more potent inhibitor than NgR(310)Ecto-Fc protein, suggesting that it represents the first specific anti-NgR antibody which inhibits NgR binding to Nogo-66, MAG and OMgp. The ability of 7E11 to prevent CNS myelin-dependent inhibition of neurite outgrowth of DRG neurons has also been evaluated. The result showed that 7E11 significantly attenuate the inhibitory effect of CNS myelin on axonal regeneration and reverse the inhibition of MAG-Fc on DRG neurite outgrowth in a dose-dependent manner, and effectively promote axonal regeneration of rat DRG cultured on a CNS myelin substrate. Thus, 7E11 is also the first neutralizing anti-NgR monoclonal antibody that can promote neurite regeneration against CNS myelin inhibition *in vitro*. Meanwhile, the molecular epitope of 7E11 was defined to be DNAQLR which locates in the third LRR domain (LRR3) of rat NgR. These data demonstrated that anti-NgR antibody 7E11 could recognize this epitope and neutralize CNS myelin-dependent inhibition of neurite outgrowth. The characteristic of the key epitope with LRR3 on NgR should facilitate rational drug development for finding potent and specific NgR antagonists that promote CNS axonal regeneration, which might offer a very useful therapeutic approach for CNS injury (64).

Vaccination against infectious agents has been heralded as a triumph in modern medicine, and more recently cancer vaccines have risen in prominence. Previous study has also shown that a myelin vaccine could prevent induction of experimental autoimmune encephalomyelitis (EAE), lead to generating antimyelin antibody that neutralize the inhibitory activity in myelin *in vitro* and promote neurite outgrowth after spinal injury (65,66). Immunization with two recombinant inhibitors has also been shown to promote axonal regeneration after spinal injury (66). However, it would be hazardous and impractical to immune humans against the whole myelin antigens because some myelin proteins bear the functions of neuprotection. It is also not safe to immunize human against the inhibitory molecules because those inhibitors might be involved in other functions in addition to inhibition of neurite outgrowth (67,68,69). Recently, investigators are interested in the application of a vaccine strategy to circumvent and modulate the obstacles to the repair and regeneration following CNS injury, namely myelin inhibition of axonal outgrowth. To target specifically multiple inhibitory epitope, Xu *et al* generate a recombinant DNA vaccine, encoding Ig1-5 domain of rat MAG, EGF-L domain of human TN-R and N-terminal and 66 amino acid extracellular domains of human Nogo-A, which was used to immunize animals (70). Their result demonstrated that a small but distinct number of regenerated fibers were found in the main CST domain in the vaccinated group, and axonal regeneration was much better than that in the control group. Locomotion was also evaluated in the vaccinated group by BBB score, revealing that DNA vaccine could promote functional recovery in vaccinated animals. Most importantly, immunohistochemical and ultrastructural analysis did not show any evidence of demyelination following the DNA vaccine treatment. Other data further showed that the application of DNA vaccine did not induce experimental autoimmune encephalomyelitis (EAE) (70). The appearance of DNA vaccine has offered a convenient and effective approach of immunization against diseases and it recently has been used in clinical trial stages for several diseases. If a DNA vaccine can be developed, in which the inhibitory and binding domain from NgR in addition to inhibitory factory factors is incorporated in the construct, a better axonal regeneration and locomotion recovery might be achieved, offering a novel strategy in therapy for CNS injury.

Several experimental approaches to improve axonal regeneration have been reviewed above, for example, blocking the activation of RhoA and ROCK with antagonist, C3 transferase and Y276342 respectively, enhancing axonal regeneration on myelin substrates *in vitro* and vivo. However, their effectiveness may be depended on their mode of delivery. To achieve a long-term disinhibition, targeting the genes of inhibitory molecules is likely to be more effective than antagonist treatment. Recent study showed that disinhibition of axonal outgrowth in CNS could be achieved by using short interfering RNA (siRNA), a tool to specifically silence gene expression of targeted mRNA (71). In this experiment, researchers investigated and compared disinhibition of fibroblast growth factor 2 (FGF2)-stimulated dorsal root ganglia neurons (DRGN) neurite regeneration after siRNA-mediated silencing of NgR. Their result demonstrated that FGF2 stimulated DRGN axonal generation. It was consistent with the disinhibition effects found by Fisher *et al*, who used a dominitant-negative NgR mutant to significantly enhance the number of lens injury-stimulated regenerating retinal ganglion cell axons traversing any growing beyond an optic nerve injury site through the putative inhibitory environment (72). The efficient NgR siRNA would be expected to be developed to induce significant regeneration of the corticospinal tracts and become an effective therapeutic strategy for the adult CNS injury.

3.7. Conclusion

There have been some encouraging results in recent studies on NgR. As further studies go on, there will be more approaches appear to improve axonal regeneration and promote locomotion recovery after CNS injury. Some unknown questions will also need to be answered in future

studies. What is the normal function of NgR? How do we manipulate the beneficial effects and adverse effects of NgR in the clinical therapeutic applications? With unfolding these questions, NgR pathway will become a new target in clinical therapy for CNS injury.

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