A Go on NOGO: Promising Therapy for CNS Disease and Injury

Cecilia Reyes and Yaroslav Voronin
Department of Biology
Lake Forest College
Lake Forest, Illinois 60045

Abstract

Mammals have evolved with a limited capacity to regenerate neurons in the CNS. Damage to the CNS by traumatic injury, stroke and neurodegenerative disorders can result in permanent loss of sensory, motor, and cognitive functions. Fifteen years ago, my lab began studying the inhibitory mechanisms in damaged CNS. We have identified the myelin-associated protein Nogo-A as a key player in sprouting inhibition. Nogo-A, as well as two other inhibitory proteins, MAG and OMgp, bind to the nogo-66 receptor (NgR) to inhibit axonal regeneration in the CNS. We identified two mechanisms with neurons that promote Nogo-based CNS inhibition: the rho-ROCK kinase pathway that is selectively activated by NgR, and the integrin-actin pathway that is activated by a 66-amino-acid residue on NgR-A. While genetic and chemical disruption of NgR ligands (nogo-A, MAG and OMgp) has resulted in poor regeneration after injury, manipulation of NgR has shown promising therapeutic value in both in vivo and in vitro. Therapeutic administration of NgR(310)ecto-Fc protein, an NgR antagonist, in tissue and mouse models can neutralize the inhibitory effects of the three NgR ligands and has proven beneficial in promoting motor function after spinal cord injury and stroke. Finally, we have found that inhibiting Nogo-A in ALS and Alzheimer’s disease models reduces pathological characteristics, indicating that manipulating Nogo-NgR based inhibition holds great promise for CNS injury and neurodegenerative disease.

Introduction

Injury to the central nervous system (CNS) from spinal cord injury (SCI), stroke and neurodegenerative disorders often result in permanent loss of sensory, motor, and cognitive functions, contributing to the rising number of disabilities in the United States. In 2014, it was estimated that over 276,000 Americans live disabled, to a degree as a result of a SCI, with an estimated 12,500 new cases occurring yearly (1). Stroke is the third leading cause of death and long-term disability in the U.S., with more than 795,000 cases reported each year (2). Neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and Alzheimer’s disease (AD) are also major contributors to the rising number of disabled Americans. ALS patients suffer from progressive degeneration of motor cells in the CNS resulting in complete loss of motor function. It is estimated to affect as many as 30,000 Americans, with over 5,000 new cases reported yearly (3). AD is clinically characterized by a progressive loss of memory and cognitive functions. Fifteen years ago, my lab began studying the molecular mechanisms that impair neurological recovery in the CNS. Our aim is to understand the mechanisms behind the lack of axonal sprouting at CNS sites of injury and develop therapeutics that can aid in not just SCI but a wide range of CNS degenerative diseases such as AD, ALS, stroke, prion disease, among others.

When I entered the field, little was known about the microenvironmental factors that hindered regeneration in the CNS. Early studies using transplants containing central glia (CNS tissue) and peripheral nerve segments led to the hypothesis that it was microenvironmental differences between the CNS and PNS that result in different neuronal sprouting behaviors in response to injury. Extensive elongation of axons from rat brains can be achieved by inducing changes in CNS microenvironment through implantation of PNS grafts (7). Further research lent support to this hypothesis; Schwab et al. found that CNS tissue in culture were not permissive for the growth of sensory and sympathetic axons even in the presence of NGF, a trophic growth factor. Both sensory and sympathetic axonal growth was observed in sciatric nerve explants in culture (8). These nonpermissive qualities in the CNS tissue were linked to two membrane bound proteins with a protein fraction of 35-kDa and 250-kDa (NI-35 and NI250) that showed inhibitory qualities. The purification of these two proteins led to the discovery of an antibody (IN-1) capable of neutralizing these inhibitory proteins, resulting in a reversal of inhibitory function in damaged tissue (9).

Discovery of Nogo

Nearly twelve years after the discovery of the IN-1 antibody, my lab began studying the two inhibitory molecules found exclusively in CNS tissue: NI35 and NI250. In 2000, my lab was one of three labs that identified the gene that coded for the NI250 inhibitory protein (10, 11, 12). Purification of the bovine homologue of rat NI-250 protein and microsequencing to identify cDNA clones led to the identification of the gene Nogo that encodes for NI-250 (10). The Nogo gene codes for three major protein products: Nogo-A, -B, and –C. We identified Nogo as a member of the Reticulon family, Reticulin 4-A and identified that Nogo is expressed selectively by oligodendrocytes in the CNS and not by Schwann cells in the PNS (10). Nogo-A is the only member of the group recognized by the IN-1 antibody (10). Further studies show that recombinant Nogo-A inhibits neurite outgrowth from the dorsal ganglia and the spreading of 3T3 fibroblast (11). This data identified Nogo-A as a potent neurite growth inhibitor and a potential player in limiting axonal regeneration in damaged CNS.

Receptor-ligand Interaction

Analysis of Nogo-A shows the presence of a 66-amino-acid inhibitory domain (Nogo-66) expressed at the extracellular surface of oligodendrocytes (10, 11, 12). The extracellular inhibitory domain suggests that Nogo-66 may interact with a receptor type protein to cause observed inhibitory effects. We identified a leucine-rich repeat protein, NgR, with high affinity for soluble Nogo-66. Cleavage of the Nogo-66 receptor, NgR, from axons rendered the neurons insensitive to the Nogo-66 inhibitory domain (12). This suggested a nogo-nogo receptor pathway of neuronal inhibition; Nogo-A acts on NgR to later cause a downstream effect of inhibited axonal growth. By disrupting this Nogo-A/NgR, my lab and I hoped to fill in the blank gaps of inhibition in damaged CNS. We hypothesized that interaction between Nogo-A on oligodendrocytes and NgR on axons played a significant role in CNS inhibition after injury. To test this hypothesis, we compared the localization of Nogo-A and NgR using immunohistochemistry. The distribution of NgR and Nogo-A suggested an interaction at contact sites between axons and myelin (13). Nogo-A was primarily detected on oligodendrocytes processes in adult mice CNS, while NgR protein was selectively expressed by axons of adult and maturing CNS neurons. Before axonal myelination, we found minimal expression of NgR. We observed an increase expression of Nogo-A at sites of injury. We found modest interaction between NgR and Nogo-A in uninjured CNS, suggesting that Nogo-A may also play a role in limiting axonal sprouting and plasticity in normal development (13).
Figure 1. Axon generation in PNS vs CNS. Microenvironmental differences promote axonal regeneration in the PNS, while inhibiting regeneration in the CNS. Damage to an axon through traumatic injury results in fragmentation and disintegration of the axon. This axonal debris is rapidly cleared by macrophages in the PNS, supporting spontaneous regeneration. CNS neurons upregulate regeneration-associated genes (RAGs) to promote neurite outgrowth resulting in recovery of function. In contrast, after CNS axonal injury slower debris clearance and scarring induced by astrocytes all result in limited CNS neuronal regeneration. Failure to regenerate axons is also largely contributed to endogenous myelin inhibitory molecules (nogo, MAG, OMgp) present only in the CNS.

Other key player in CNS axonal inhibition: MAG and OMgp

Other key myelin-derived proteins, MAG and OMgp have been shown to limit axonal regeneration after CNS injury. MAG is a member of the immunoglobulin superfamily and is expressed in myelinating cells. In vitro studies have shown that MAG inhibits axonal regeneration similarly to Nogo-A (14). A third myelin-derived axonal outgrowth inhibitor protein, OMgp, discovered in 1990 and it is a glycosylphosphatidylinositol (GPI)-anchored leucine-rich repeat protein (LRR) that does not appear to share sequence similarity with Nogo-A (15). Despite differences in sequences, both MAG and OMgp have a high binding affinity to nogo-66 receptor (NgR). We found that while both Nogo-A and MAG bind to NgR and activate axonal inhibitory pathways, MAG and NgR-B to separate and distinct binding regions on NgR (16). This discovery highlights the importance of NgR, suggesting NgR plays a central role in mediating myelin dependent inhibition of axonal outgrowth, as it is capable of binding three distinct ligands. A genomic search for proteins that share LRR identity with NgR led to the discovery of NgR2 and NgR3 (17). Despite sequence similarities, NgR2 and NgR3 did not bind Nogo-A, MAG or OMgp (14).

Disruption of NgR/Nogo-A

Next, we wanted to disrupt the NgR/Nogo-A interactions to test further its role in CNS inhibition. We hypothesized that the NgR/Nogo-A pathway played an important role in inhibition of CNS repair and that by targeting either component, we could disrupt the pathway. We knocked out the gene encoding Nogo-A/B in young adult mice. After SCI, extensive regeneration was seen in Nogo-A/B deficient mice. However, while our Nogo-A/B deficient mice were recovering well, two other labs at the time had conducted similar experiments with Nogo-A knockouts. Schwab’s lab found limited regeneration in their knockout mice (18). These results partially supported our own observations, but were different enough to raise questions. Zheng’s lab found no regeneration in their Nogo-deficient mice (19). This suggested the existence of a number of variables not yet controlled for.

We hypothesized that injury location played a role in CNS recovery. Instead of a corticospinal hemisection (which previously yielded conflicting results), we decided to cut the pyramidal neurons higher up along the CNS in two test groups: NgR-deficient mice and Nogo-A/B deficient mice. After pyramidotomy, we found both test groups had improved recovery and axonal regeneration (20). This meant that while corticospinal injuries may not heal as well, damage to pyramidal neurons do recover in systems with disrupted Nogo-A/NgR pathways.

After observing the conflicted results with our Nogo-A/B knockout mice, next we targeted NgR. We found that NgR had two distinct regions: a receptor-ligand binding and an inhibitory region. We decided to see if it was possible to make copies of the receptor-ligand binding region in order to bind Nogo-A, OMgp, and MAG before they could bind to an intact nogo-66 receptor on the neuron. We found that when injected into COS-7 cells containing wild type NgR and Nogo-A, our soluble binding domain of NgR prevented axonal inhibition. We named this protein NgR(310)-ecto-Fc. NgR ecto’s effect at preventing inhibition in vitro by inhibiting Nogo-A/NgR binding said volumes on the importance of NgR in the lack of regeneration in the CNS (21). If one receptor/ligand pairing could be disrupted and have such a dramatic effect, this suggested that NgR must play a significant role in axonal inhibition. By examining the crystal structure of NgR Ecto, we gained insights on how NgR can associate with ligands and co-receptors (22). Besides NgR Ecto, there was another NgR antagonist we tested. We picked NgR antagonist peptide NEP1-40, an extracellular Nogo peptide containing the first forty amino acid residues of Nogo. NEP1-40 treatment was beneficial in reforming damaged synapses in vitro and locomotor recovery following subcutaneous injection in vivo. Delaying NEP1-40 treatment for a week after injury in rats did not show any decline in locomotor activity. This meant that axons could be regenerated weeks after injury (23).

To further test NgR inhibition, we generated mice lacking NgR. We performed SCI and found that mice lacking NgR had improved motor recovery. Upon closer inspection, we found that corticospinal fibers did not regenerate, but raphespinal and rubrospinal fibers did (24). This supported the idea that NgR played a key role in limiting regeneration. Other studies found that NgR deficient mice were found to have greater motor recovery than control, as did Nogo-A/B deficient mice. NgR Ecto administration after stroke improved recovery. All three methods point to potential therapies for CNS injury to be directed at the Nogo-NgR interface (25). We continued on to see if NgR Ecto would be effective at restoring recovery to mice with SCI. Intrathecal administration of NgR Ecto in SCI mice demonstrated improved spinal cord conduction, locomotion, and axonal sprouting of corticospinal and raphespinal fibers (26). This supported our idea that NgR Ecto was a potential therapeutic for CNS injury. To build upon this idea, we transgenically altered mice astrocytes to excrete NgR Ecto using gene regulatory elements. NgR Ecto levels were not altered in undamaged, transgenic mice. However, after damaging the mice through mid-thoracic SCI, NgR Ecto was secreted by reactive astrocytes around the site of injury. Mice exhibited a regain in function and locomotion, along with improved raphespinal and corticospinal axonal sprouting near the site of injury compared to control (27). This supported our notion that NgR Ecto was a potentially strong therapeutic for SCI.
Pathways of CNS axonal inhibition

While NgR and its ligands were known, little was known about the inhibitory mechanism involved with NgR. We hypothesized that NgR acted upon a neurite outgrowth inhibitor family called Rho. Rho kinases are a family of small GTPases that, when activated, inhibit neurite outgrowth. The Rho family inhibitory mechanism was well known; it was known that Rho was directly linked with a downstream effector known as p160ROCK.

To test our hypothesis, we used a binding assay with Nogo-66 and Rho. Nogo-66 stimulation resulted in increased levels of GTP Rho. We pharmacologically disabled Rho using a C3 transferase and found that neurite outgrowth was promoted in vitro. Inhibition of the downstream effector of Rho, p160ROCK, also promoted neurite outgrowth in vitro and promoted locomotor recovery after CST lesions in adult rats. With this data we concluded that NgR was directly linked to the Rho-Rock inhibitory pathway within the neuron and stimulation of NgR resulted in activation of this particular pathway (28). We later revisited the Rho-Kinase pathway, looking not at p160ROCK but ROCKII. We manipulated ROCKII genetically to give us ROCKII negative mice. After cervical multilevel dorsal rhizotomy or mid thoracic dorsal cord hemisection, we found that mice lacking ROCKII showed growth local to injury of raphespinal axons (29). These findings demonstrate that ROCKII limits axonal growth through the Rho-Rock kinase pathway and is an effective target in limiting axonal inhibition.

A peculiarity regarding Nogo-A/B deficient mice and different results was noted before. The different results caused by different methods of Nogo-A/B deletion. Nogo-A has two main extracellular binding domains. One is the domain that binds to NgR to inhibit axonal sprouting through the described mechanisms above. A second extracellular domain called amino-nogo also inhibits recovery through an unknown mechanism. We found that inhibition by amino-nogo depends entirely on the composition of the extracellular matrix. Amino-nogo inhibition was partly reversed when antibodies that activated integrin were added (30). Integrins are cell surface proteins that mediate cell-to-extracellular matrix and cell-to-cell interaction. Integrin activation is necessary for force generation during growth cone advance and for actin/cytoskeleton coupling during cell spreading. We found that certain integrins specific to CNS neurons, αvβ3, αv, and α4, were sensitive to inhibition by amino-nogo. Integrin activation activates focal adhesion kinase (FAK) through fibronectin, another key protein in axonal pathfinding. Thus, by inhibiting activation of integrin, amino-nogo prevents axonal sprouting not just by inactivating integrin, but also FAK (30).

This helps shine some light on the mystery of the Nogo-A/B deficient mice having different recovery results. The answer partially lies in the characteristics of the extracellular matrix. This experiment not just helped connect amino-nogo to the integrin-inhibition pathway of axonal inhibition, but also connecting the unsatisfactory results found when NgR was inhibited. Integrin antagonism is a minor, but highly important factor to consider in regards to inhibition.

Nogo-A role in Disease

Multiple sclerosis

Multiple sclerosis is a disease that involves the demyelination and degeneration of axons in the CNS. The mechanism of this demyelination remains largely unknown, but we do know that CRMP-2 is involved. CRMP-2 is a tubulin-associated protein that helps regulate axonal growth; tubulin is inhibited when phosphorylated. In chronic multiple sclerosis, tubulin is found phosphorylated in the CNS. Nogo-66 receptor activation may be upstream of CRMP-2 phosphorylation. To study this, we used an autoimmune encephalomyelitis model, a disease also characterized by CRMP-2 phosphorylation. We injected mice with an anti-NgR antibody that inhibited NgR function and measured phosphorylated CRMP-2 levels. Phosphorylated CRMP-2 levels were lower, associated with an improved clinical outcome (31). This meant that NgR activation was upstream of CRMP-2 phosphorylation and that blockade of the nogo-nogo receptor pathway in multiple sclerosis is a viable therapy.

Alzheimer’s disease

Alzheimer’s disease (AD) is characterized by insoluble plaques composed of Aβ, tau dysfunction, and the presence of toxic Aβ oligomers. Aβ is derived from amyloid precursor protein APP. Knowing the pathology of AD consists of aberrant axonal sprouting, we wanted to see if the nogo-nogo receptor pathway played a significant role. Subcellular localization of Nogo-A and NgR is misplaced in AD models in the CNS. Increasing NgR expression in Alzheimer’s models in vivo decreased Aβ production, while disrupting NgR expression in vitro raised Aβ levels, amyloid plaque deposits, and increased dystrophic neurites. With such a direct link visible, we wanted to see if the precursor of Aβ, APP, had any association with NgR. We found that APP directly associates with NgR, with higher NgR concentration resulting in lower APP levels. The next step was seeing if it was possible to pharmacologically lower NgR. We injected NgR ecto into Alzheimer’s models in vitro and the results were similar to our genetically overexpressed NgR mice. Our results indicate that NgR may play a role in blocking secretase processing of APP (32). Such an inverse correlation indicates that NgR ecto may be a novel therapeutic in halting the progression of AD. To test whether increased NgR regulates Aβ production or increases Aβ clearance, we used an AD mouse model injected with NgR ecto. Subcutaneous NgR ecto injections resulted in lower Aβ brain levels and higher Aβ serum levels. This indicates a clearance of Aβ from the brain. This clearance of Aβ was correlated with higher test scores on the radial arm water maze (33). This supports our theory that NgR ecto may represent a novel therapeutic agent, with peripheral administration being effective even after the onset of the disease.

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal genetically inherited motor neuron disease. Previous research has linked ER stress and ER chaperone protein dysfunction to ALS disease progression. Genetic studies have shown that the reticulon family is highly expressed in the ER, along with an isoform of a reticulin 4 (Nogo-A) in human and mice ALS. These high levels of expression of Nogo lead to the hypothesis that nogo may influence the progression of ALS pathology. Using cell culture and a mSOD1 murine model for ALS we found that reticulin family alters the intracellular distribution of protein disulfide bonds, PDI, an ER chaperone protein. We show that reticulin family is a regulator of PDI. Increased expression of reticulin proteins such as nogo-A can help alter PDI distribution throughout the neuron in a way that can slow disease progression. Survival and genetic studies show on ALS mice model show that single deletion of Nogo-A/B gene accelerates disease progression and onset. Deletion of both alleles significantly altered disease progression and mortality rate (34). These results give insight into the nature of nogo and how, while having a major role in axonal inhibition and plasticity, it may also have other secondary roles.

Conclusions

Damage to the CNS by traumatic injury, stroke, and neurode-
generative disorders can result in permanent loss of sensory, motor, and cognitive functions. Many research labs studying neurodegeneration have focused on the prevention of tissue damage by disease pathology, but despite much effort there has been limited success finding preventative treatment. In the past fifteen years my lab and others in the field have made major contributions to identify the molecular pathways hindering neurite inhibition after CNS injury. We have revolutionized the field by the identification of the Nogo-A and its receptor, NgR, as they have led to the developed peptide and protein inhibitor of the Nogo-receptor signaling pathway. We identified two mechanisms that promote Nogo-based CNS inhibition: the Rho-ROCK kinase pathway that is selectively activated by Nogo-66, and the integrin-actin pathway that is activated by second inhibitory domain of Nogo-A known as Amino-nogo. (figure 2). In addition, we have found that these antagonists can selectively overcome the inhibitory effects of myelin-associated proteins (figure 2). The α-Nogo-A, NEP1-40 and NgR(310)-ecto-Fc antagonists have been extensively studied by my lab and by others in SCI and stroke mice models and have shown tremendous promise as they promote axonal growth in the SCI and recovery of motor function without side effects. The success in promoting axonal regeneration has prompted other labs to study the role Nogo-NgR binding in a range of neurological diseases. Genetic disruption of Nogo and/or NgR in disease models has provided a novel target for modifying the course of pathology in AD, MS, ALS, and schizophrenia.

Future research will focus on further understanding the interaction of Nogo/NgR in the neurodegenerative disease pathways when it has already been implicated and also test possible nogo-interactions in other unexplored neurological disorders. Genetic deletion of Nogo-A/B has shown promising therapy to promote axonal outgrowth and slow disease progression in many disease models as described above (30, 31, 32, 33). The next step is to test the therapeutic value of NgR antagonists to disrupt Nogo-A/NgR interaction in vivo and in vitro in schizophrenia, MS, and AD.

Finally, it is important to note that blocking Nogo/NgR interactions is not the only promising treatment for CNS regeneration. Other studies that have indicated other potential targets that promoted neuronal regeneration are: control of reactive astrocyte secretions, introduction of neurophins, and transplantation of special peripheral nerve and stem cells to site of injury. Leaders in stem cell research have shown that stem cell implantation after SCI hold tremendous therapeutic value in SCI injury. Studies have indicated that neural progenitor cells derived from embryonic stem cells can promote axonal outgrowth over long distances when implanted into sites of SCI (34). Other findings indicate that grafts of neural progenitor cells applied to the site of injury also remyelinate injured axons and may have neuroprotective properties (35, 36). Recently, Lu et al., showed that human induced pluripotent stem cells (iPSCs) that differentiated into neural stem cells and were grafted into adult immunodeficient rats after SCI resulted in the extension of tens of thousands of axons from the lesion site over the entire length of the rat CNS (37). Although, stem cell research is promising and may someday serve as therapy to replace injury in a range of neurological diseases. Genetic disruption of Nogo and/or NgR in disease models has provided a novel target for modifying the course of pathology in AD, MS, ALS, and schizophrenia.

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