A proteolytic C-terminal fragment of Nogo-A (reticulon-4A) is released in exosomes and potently inhibits axon regeneration

Yuichi Sekine#, Jane A. Lindborg, and Stephen M. Strittmatter*

From the Cellular Neuroscience, Neurodegeneration and Repair Program, Departments of Neurology and of Neuroscience, Yale University School of Medicine, New Haven, CT 06536, USA

Running title: Exosomal Nogo-66 inhibits axon regrowth

#Present Address: Wakayama Medical University, Department of Anatomy & Neurobiology, 811-1, Kimiidera, Wakayama, 641-8509, Japan

*To whom correspondence should be addressed: Stephen M. Strittmatter, CNNR Program, Yale University School of Medicine, 295 Congress Ave., BCMM 436, New Haven, CT 06536-0812 USA; stephen.strittmatter@yale.edu; Tel. 203-785-4878; Fax. 203-785-5098

Keywords: Nogo, Reticulon, Exosome, Nogo Receptor 1, NgR1, axon regeneration, BACE1, ß-secretase 1, ß-site amyloid precursor protein cleaving enzyme, oligodendrocyte

ABSTRACT

Glial signals are known to inhibit axonal regeneration and functional recovery after mammalian central nervous system (CNS) trauma, including spinal cord injury. Such signals include membrane-associated proteins of the oligodendrocyte plasma membrane and astrocyte-derived matrix-associated proteins. Here, using cell lines and primary cortical neuron cultures, recombinant protein expression, immunoprecipitation and immunoblotting assays, transmission EM of exosomes, and axon-regeneration assays, we explored the secretion and activity of the myelin-associated, neurite outgrowth inhibitor Nogo-A and observed exosomal release of a 24-kDa, C-terminal Nogo-A fragment from cultured cells. We found that the cleavage site in this 1192-amino-acid-long fragment is located between amino acids 961–971. We also detected a Nogo-66 receptor (NgR1)-interacting Nogo-66 domain on the exosome surface. Enzyme inhibitor treatment and siRNA knockdown revealed that ß-secretase 1 (BACE1) is the protease responsible for the Nogo-A cleavage. Functionally, exosomes with the Nogo-66 domain on their surface potently inhibited axonal regeneration of mechanically injured cerebral cortex neurons from mice. The production of this fragment was observed in the exosomal fraction from neuronal tissue lysates after spinal cord crush injury of mice. We also noted that relative to the exosomal marker Alix, a Nogo-immunoreactive 24-kDa protein is enriched in exosomes two-fold after injury. We conclude that membrane-associated Nogo-A produced in oligodendrocytes is processed proteolytically by BACE1, is released via exosomes, and represents a potent diffusible inhibitor of regenerative growth in NgR1-expressing axons.

After traumatic spinal cord injury (SCI), neurological deficits are frequently profound and persistent below the level of damage. Dysfunction persists despite the survival of nearly all neurons, due to axonal damage and disconnection. Regrowth of injured axons is strongly inhibited in the CNS environment. Membrane-associated Myelin associated inhibitors (MAIs) and matrix-associated Chondroitin sulfate proteoglycans (CSPGs) are identified as two major classes of inhibitors for axonal growth in the CNS.

There are multiple MAI proteins, most prominently Nogo-A (RT4A), Myelin Associated Glycoprotein (MAG) and Oligodendrocyte Myelin Glycoprotein (OMgp) (1-5). All three of these oligodendrocyte membrane-associated ligands bind to Nogo-66 Receptor 1 (NgR1 or RTN4R) (6-9) and to Paired Immunoglobulin-like Receptor B (PirB or LiLRB2) (10,11). Nogo-A, the best characterized MAI, is expressed by both oligodendrocytes and neurons, and localizes to the endoplasmic reticulum and a lesser degree to the oligodendrocyte plasma membrane at the innermost adaxonal and outermost
myelin membranes in spinal cord (12,13). Axonal growth is inhibited by two regions of Nogo-A. One aa stretch, termed amino-Nogo-A or Nogo-A-Δ20 acts via NgR1-independent mechanisms to prevent neural growth through integrins and S1PR2 (14,15). The other region, Nogo-66, binds to NgR1 to inhibit axonal regeneration (1,6). Further, Nogo-22, a 22 kDa fragment of Nogo-A that contains the Nogo-A-24 and Nogo-C39 regions in addition to Nogo-66 domain, is a more potent inhibitor of axon regeneration than Nogo-66 (11). The mRNA and protein expression levels of Nogo-A in spinal cord after injury are upregulated moderately (13,16). In contrast to Nogo-A, the extracellular matrix-associated CSPGs are secreted by reactive astrocytes of the glial scar and are strongly upregulated after injury (17,18).

After spinal cord trauma, damaged axons are frequently demyelinated, so axonal cell surface contact with membrane-associated MAIs may be limited. Another injury-induced transmembrane MAI, Semaphorin 4D, is proteolyzed and soluble protein fragments are released from cell membrane after SCI (19-21). There is no direct mechanistic data regarding the release of Nogo-A, MAG or OMgp the MAIs are secreted after injury.

While evidence for diffusible glia signals limiting axonal regrowth after traumatic SCI has been limited, there is accumulating evidence for oligodendrocyte-derived exosomes participating in axonal and neuronal regulation. This includes firing rate and biochemical signaling (22-24). There is evidence that peripheral nervous system Schwann cell-derived exosomes enhance axon regeneration (25).

Here, we report that cells proteolyze Nogo-A at carboxy-terminal sites to produce a 24 kDa fragment. The C-terminal Nogo-A-fragment is secreted into culture medium as an exosome component. Biochemical studies reveal that the inhibitory Nogo-66 region is exposed on the exosome surface. Alkalization decreases the level of C-terminal fragment, suggesting a role for lysosomal endopeptidases. Pharmacological and genetic studies indicate that BACE1 participates in Nogo-A C-terminal cleavage. We explored a role for the exosomal C-terminal fragment in axonal regeneration. Cortical neurons lacking NgR1 do not respond to the exosomal Nogo-A fragment, while WT neurons exhibit decreased axonal regeneration by low concentrations of exosomal Nogo-A. Moreover, the exosomal Nogo-A fragment level is increased after SCI in vivo. Thus, Nogo-A is proteolyzed and secreted as an exosome protein after injury to provide a potent diffusible inhibitor of axonal regeneration.

**RESULTS**

**Nogo-A C-terminal region is secreted into culture supernatant as an exosome component**

In C-terminal Myc-tagged Nogo-A overexpressing HEK293T cells, both full length Nogo-A and a number of proteolytic fragments were detected in the cell lysate fraction. Similar protein bands were detected when HEK293T cells overexpressed either Myc-tagged or untagged Nogo-A, excluding the possibility that the addition of an epitope tag result causes the proteolysis (Supplementary Fig. 1). In contrast, immunoprecipitation with anti-Myc antibody from culture supernatant detected predominantly a cleaved C-terminal fragment of approximately 24 kDa and little or no full length protein (Fig. 1A). When an N-terminal FLAG-tagged Nogo-A was expressed in HEK293T cells, the lysate again showed full length protein and several fragments. However, FLAG immunoprecipitates from culture supernatant showed no specific bands (data not shown). Thus, the culture medium contains exclusively the C-terminal 24 kDa fragment of Nogo-A.

To examine whether the culture medium Nogo-A 24 kDa fragment exists as a free protein or an extracellular vesicle component of the culture medium, we fractionated the media (Fig. 1B). The C-terminal fragment was detected predominantly in the exosome fraction, and not in the non-exosome fraction (Fig. 1C). Specifically, exosome enrichment of the 24 kDa fragment is such that the ratio for 24kDa:FL Nogo-A species shifts 100-fold, from 45:1 to 1:20, in exosome versus cell lysates (Fig. 1D).

Transmission electron microscopy analysis of fractionated HEK293T culture media confirmed the presence of exosomes in these preparations (Fig. 2A). Exosomes were identified by their particle size distribution of 30-100 nm in diameter (26). The minor percentage of particles with a size distribution >100 nm is likely due to microvesicles (26). We performed sucrose density gradient analysis to compare the distribution of Nogo-A fractions to exosome marker protein (Fig. 2B). The C-terminal 24 kDa fragment is detected in the same fractions as the exosome marker, Alix. Furthermore, the levels of the C-terminal Nogo-A fragment in culture media and exosome fraction were significantly decreased after culture with the
Exosomal Nogo-66 inhibits axonal regrowth

Exo1 (Fig. 2C and D).

These data indicate that the C-terminal 24 kDa Nogo-A fragment is secreted as an exosomal protein.

**Proteolytic cleavage site in Nogo-A**

To localize the cleavage site for the Nogo-A 24 kDa fragment, we expressed truncated protein and compared the size with the fragment derived from the full length construct (Fig. 3A). The exosomal fragment of Nogo-A migrated as a doublet by SDS-PAGE, with the Nogo-A 961-1192 and 971-1192 constructs showing similar migration in the same gel (Fig. 3B). This result suggests the proteolysis occurs around 961 and 971 aa. We also created a series of 10 amino acid deletion mutant constructs from 891 to 1050 aa and assessed the secretion of a Nogo-A fragment into exosomes (data not shown). None of these 10 aa deletion mutant constructs strongly reduced the level of Nogo-A fragment in exosome. In addition, we made a series of single aa substitution mutation constructs from 951 to 980 aa and examined the Nogo-A fragment in exosome (data not shown). As for the 10 aa deletion constructs, the point mutation analysis failed to identify a single aa essential for exosomal secretion of the 24 kDa Nogo-A fragment. These data suggest that Nogo-A is cleaved for exosomal secretion in the vicinity of aa 961-971 immediately amino terminal to the first hydrophobic segment, but that the topographic restriction may be more critical than a specific aa sequence.

**Determination of the topology of Nogo-66 loop region in exosome**

There is evidence that Nogo-A assumes several different topologies within lipid bilayers in different subcellular compartments (27). Because we could immunoprecipitate the vast majority of the 24 kDa Nogo-A fragment with anti-Myc antibody in the absence of detergent, the C-terminus is most likely exposed on the surface of exosome (Fig. 3C, two models on the right). Based on prior evidence that the C-terminal hydrophobic segment assumes a hairpin topology (28), the Nogo-66 domain is also expected to be exposed on the exosome surface (Fig. 3C, two models on the right). However, due to controversy regarding hairpin versus transmembrane topology, we investigated the Nogo-66 loop topology within the exosome fraction, using a non-permeable maleimide-PEG11-biotin reagent. Because there is one cysteine amino acid at 1101 aa in the Nogo-66 sequence and maleimide reacts efficiently and specifically with free sulfhydryls. The C1101A point mutated Nogo-A was generated and used as a negative control for this experiment. HEK293T cells were transfected with Nogo-A WT or the C1101A mutant and exosome fractions were prepared from those culture media. The exosomes were resuspended in PBS and incubated with maleimide-PEG11-biotin, and then the reaction stopped with PBS prior to lysis in RIPA buffer. Lysed exosomes were immunoprecipitated with anti-Myc antibody and blotted with anti-Myc antibody or streptavidin (Fig. 3C). Equal amount of C-terminal fragments (in anti-Myc blot) was detected in both Nogo-A WT and CA mutant either incubated with or without maleimide-PEG11-biotin (Fig. 3D). However, streptavidin signal was detected only in a Nogo-A WT with maleimide-PEG11-biotin condition, suggesting that the Nogo-66 loop is exposed on exosome surface. To calculate how much Nogo-66 loop is exposed on the exosome surface, we compared maleimide-PEG11-biotin binding ratio between exosome suspended in PBS and RIPA. Streptavidin intensity in PBS suspended exosome was 96% of that detected in RIPA suspended exosome indicating that nearly all of the Nogo-66 loop is exposed on the surface of the exosome (Fig. 3E and F).

**Identification of enzyme for Nogo-A proteolysis**

To identify proteolytic enzymes responsible for the production of the 24 kDa fragment, we used several endopeptidase inhibitors. Nogo-A-Myc overexpressed HEK293T cells were treated with inhibitors and exosomes were collected and immunoblotted with anti-Myc and anti-CD9 (exosome marker) antibodies (Fig. 4A and B). Caspase inhibitor, ZVAD-fmk, and Cathepsin inhibitor, E64d, had no effect on the Nogo-A C-terminal fragment levels in exosomes compared to DMSO control. The Calpain inhibitor, MG101, increased fragment level, while lysosomal proteinase inhibitor NH₄Cl decreased the Nogo-A fragment level in exosomes. NH₄Cl is known to reduce the acidification of lysosomes, which contributes to optimal lysosomal enzyme activity.

Amongst endosomal/lysosomal proteases we considered β-site amyloid precursor protein cleaving enzyme 1 (BACE1, β-secretase 1) as a candidate protease with a known acidic pH optimum. It has been reported that BACE1 interacts with Nogo-A related Reticulon family proteins (29). Treatment of cells with a BACE1 inhibitor decreased the level of Nogo-A C-terminal fragment in the exosome fraction dose dependently and as
completely as NH$_4$Cl (Fig. 4C and D). BACE inhibition acted selectively on the Nogo-A 24 kDa fragment in exosomes, while expression of full-length Nogo-A in cell lysates remained relatively constant (Supplementary Fig. 2A). The very low to undetectable levels of the 24 kDa fragment in cell lysate preparations prevented detection of any effects of BACE inhibition in that fraction. Since the pharmacological study suggested that BACE1 might mediate Nogo-A proteolysis, we used silencing RNAs to suppress BACE1 level in HEK293T cells. BACE1 knock down with two unrelated siRNA species significantly reduced the level of Nogo-A fragment in exosomes compared to control siRNA, but did not change the level of the exosome marker CD9 (Fig. 4E and F). BACE1 knock down was confirmed by qPCR (Fig. 4G).

Function of Nogo-A C-terminal fragment

Since the Nogo-A C-terminal fragment contains axon inhibitory region, Nogo-66, we sought to examine whether the exosome fraction is inhibitory for axonal regeneration. For functional studies, we conducted in vitro axon regeneration assessment with cultured cortical neurons. Neurons were cultured for 8 days and scraped with a metal pin tool for axotomy, and then incubated with exosome preparations for 3 days to allow regeneration. Axotomized WT neurons treated with exosome secreted from Nogo-A overexpressing HEK293T cells showed decreased axonal regeneration compared to vector control, consistent with the exosomal 24 kDa Nogo-A fragment being an active inhibitor of regeneration (Fig. 5A and B). Because Nogo-A signaling is transduced primarily through NgR1, we assessed regeneration with NgR1-/- neurons. In contrast to the case with WT neurons, the regeneration of NgR1-/- axons was not suppressed by exosomes from Nogo-A expressing HEK293T cells (Fig. 5C). Thus, the C-terminal fragment Nogo-A in exosomes limits axonal regeneration in an NgR1-dependent manner.

A purified recombinant 22 kDa protein, Nogo22, containing the three known NgR1 binding domains of Nogo-A (Nogo-A-24, Nogo-66, and Nogo-C39) is a substantially more potent inhibitor of axonal regeneration than Nogo-66 alone (11). As shown above, C-terminal fragment of Nogo-A includes all components of this Nogo-22 domain associated with the exosome lipid bilayer, constituting a physiologically relevant version of Nogo22. We sought to compare the relative potency of Nogo22 versus the exosomal 24 kDa Nogo-A fragment. To measure the amount of Nogo-A fragments in the exosome, fractions were blotted with anti-Nogo22 antibody and titrated with purified Nogo-22 (Supplementary Fig. 3A and B). While high concentrations of Nogo in the exosomal preparation could not be tested, it is clear that at lower concentrations the exosomal Nogo-A fragment is more potent than purified Nogo22 (Fig. 5D). The Nogo protein concentration required for inhibition of regeneration to 80% (IC-80) for Nogo22 and for Nogo in exosomes from each separate experiment was calculated and compared. The exosomal Nogo fragment exhibits a significantly lower IC-80 (45 nM) than Nogo22 (97 nM) (Fig 5E).

Increased Nogo-A fragment levels after spinal cord trauma in vivo

The evidence that a 24 kDa Nogo-A fragment is secreted as an exosomal protein from HEK293T cells and inhibits axon regeneration after axotomy prompted us to assess the production of the Nogo-A fragment in vivo. To evaluate injured spinal cord tissue, we chose a mid-thoracic spinal cord crush model because the injury is severe but the dura matter remains intact, preventing a CSF leak from the lesion which might release local exosome accumulation. Spinal cords from sham and injured (SCI) animals were homogenized in TBS and fractions separated as in Figure 1B. A Nogo-immunoreactive 24 kDa protein was detected in the exosome fraction from injured animals but not from the sham surgery group (Fig. 6A). Quantification of the Nogo-immunoreactive 24 kDa protein level relative to the exosomal marker, Alix, showed a significant increase in the injured spinal cord (Fig. 6B).

DISCUSSION

The major finding of the current study is that exosomes contain a proteolytic fragment of the MAI Nogo-A. A role for exosomes as diffusible inhibitors of axon regeneration after SCI is supported by several observations. A specific fragment of Nogo-A containing the three NgR1
Exosomal Nogo-66 inhibits axonal regrowth

Interacting domains is present in exosomes and the Nogo-66 domain is exposed to the medium. Functional tests show that exosomes containing the 24 kDa Nogo-A fragment are as active as the most previously described NgR1 ligand, Nogo22, in blocking axon regeneration in vitro. Furthermore, the levels of exosomal Nogo-immunoreactive 24 kDa protein are increased in spinal cord after SCI in mice.

The exosome fraction is highly enriched in the 24 kDa Nogo-A fragment but not full length Nogo-A. Thus, proteolysis of Nogo-A is invariably associated with secretion in exosomes. This may imply that production of Nogo-A-containing exosomes requires passage of the protein through a subcellular compartment in which this cleavage is robust, such as the endo-lysosome pathway. It is also possible that this proteolytic processing plays an instructive role in targeting Nogo-A to the exosomal pathway.

The BACE1 enzyme is required for Nogo-A cleavage to the exosomal 24 kDa fragment. There are data demonstrating a direct interaction of BACE1 with reticulon family members, most prominently Reticulon3 (RTN3), but to a lesser extent Nogo-A (RTN4) (29-31). In this context, the focus has been on reticulon inhibition of BACE1 activity with respect to amyloid precursor protein (APP), rather than reticulons serving as BACE1 substrates or participating in exosomal processing. The current data show that BACE1 inhibitors reduce the release of potent Nogo-A fragments into exosomes. Administration of BACE1 inhibitors is therefore predicted to shift the balance of Nogo-A inhibition from membrane-associated to secreted exosomes. Therefore, BACE1 inhibition might alter the regional distribution of Nogo-A action through NgR1 on neurons.

The exosome fraction contains all of the NgR1-interacting domains of Nogo-A, that have previously been linked in Nogo22 (11). However, localization to the surface of exosomes appears to further enhance potency for NgR1-mediated axon growth inhibition. In this context, the increase of the Nogo-immunoreactive 24 kDa protein after SCI implies greater Nogo-A-related inhibitory activity post-trauma. Further studies will be required to define both the mechanism of this increase and the regional distribution of exosomal Nogo-A in the injured nervous system.

Overall, existence of the proteolysed exosomal Nogo-A fragment broadens the action of the MAI Nogo-A from cellular membrane-associated to secreted exosomes. Further, BACE1 activity may titrate axonal growth inhibition after CNS trauma independent of APP.

EXPERIMENTAL PROCEDURES

Cell lines and primary cortical neuron culture

Human embryonic kidney 293T (HEK293T) cells were maintained in DMEM containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Mouse E17 cortical neurons were dissected in ice-cold Hibernate E medium (catalog #HE-Ca; BrainBits) and incubated in digestion HBSS medium containing 30 U/ml Papain (catalog #LS003127; Worthington Biochemical), 1.5 mM CaCl2, 2.5mM EDTA, and 2 mg/ml DNaseI (catalog #DN25; SIGMA) at 37°C for 20 min. Digested tissues were triturated and suspended in Neurobasal-A media supplemented with B-27, GlutaMAX, and penicillin-streptomycin (all from Invitrogen). Neurons were plated on tissue culture plates coated with poly-D-lysine.

Expression plasmids, antibodies and reagents

C-terminal Myc-tagged wild-type human Nogo-A plasmid has been previously described (1) and used for generating mutant constructs by PCR-methods using KOD Hot start DNA polymerase (TOYOBO) and sequenced. Anti-Myc (#M4439 or #C3956) and anti-beta-actin (#A1978) antibodies (SIGMA), anti-ßIII-Tubulin antibody (#G7121, Promega), anti-Alix (#2171), CD9 (#13174) and Cathepsin D (#2284) antibodies (Cell Signaling Technology), anti-Nogo-A-H300 antibody (#sc-25660) (Santa Cruz Biotechnology), and anti-Nogo-A-M (#AF3515) and anti-Nogo-A-N (#AF3098) antibodies (R&D) were used for the following experiments. Rabbit polyclonal antibody against Nogo22 was generated using purified Nogo22 as an antigen (Covance). Nogo22 protein has been described previously (11). Maleimide-PEG11-Biotin was purchased from Thermo Scientific. MG101 (R&D), Z-VAD-FMK (Promega), E64d (Santa Cruz Biotechnology) and β-Secretase Inhibitor IV (Merck) were used for inhibitor experiments.

Transfection and transduction

HEK293T cells were transfected with Myc-tagged or untagged Nogo-A plasmids using Lipofectamine 2000 (Invitrogen) and siRNA NC (sense: rCrGrUrUrArUrCrGrGrUrArUrArUrCrGrG rCrUrUAT, antisense: rArUrArCrGrCrGrUrUrArUrArCrGrCrGrArUr rUrArUrCrGrArC) or targeting human BACE1 (#1
Exosomal Nogo-66 inhibits axonal regrowth

rCrUrArCrArUrArCrUrGrCrCrUrCrUrGrArGrUr
rArUrCAA

antisense:
rGrArGrArUrGrArUrArGrCrGrGrArArGr
UrCrArUrGrArUrCrArGrGrUrAr
UrCrGrACC,

#2 sense:
rCrCrUrArCrUrGrArGrArUrArCrArUrGr
rGrCrGrArArGrArGrArArGrGrGrArGrGrUrAr
GrCrArUrArGrGrG), human Cathepsin D

#1 sense:
rArGrUrArUrUrArCrArArGrGrGrUrUrCrUrCrUrG
rUrCrCTA

antisense:
rUrArGrArCrArGrArGrArCrCrUrGrUrA
rArUrCrUrUrG,

#2 sense:
rGrUrUrGrArCrCrGrArGrArGrArArCrCrA
rGrGrGTG,

antisense:
rCrArCrCrCrUrGrUrUrGrUrUrGrUrCrArCrGrGrU
rCrArArCrArC,

#3 sense:
rArUrCrArCrGrGrGrArUrArCrArUrGrAr
UrCrCrCCT,

antisense:
rArGrGrGrArUrArCrUrUrGrCrCrCrC
rUrGrArArCrA), using Lipofectamine RNAiMAX (Invitrogen).

Immunoprecipitation and immunoblotting
HEK293T cells were lysed with a RIPA buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate and 1% Triton X-100) and centrifuged at 20,000 x g for 20 min at 4°C. Antibody and protein G-sepharose mixture were added to supernatants or lysates and incubated for 2 h at 4°C with gentle rotation. The beads were washed three times and the immune complexes were then resolved by SDS-PAGE. After transfer, nitrocellulose membranes were incubated in blocking buffer (Blocking Buffer for Fluorescent Western Blotting, Rockland MB-070-010) for 1 h at RT and immunoblotted with the appropriate primary antibodies. Following primary antibody incubation, secondary antibodies (Odyssey IRDye 680 or 800) were applied for 1 h at RT. Membranes were then washed and visualized using a Licor Odyssey Infrared imaging system.

Exosome purification and sucrose density gradient experiment
Exosomes from culture supernatant of HEK293T were prepared by differential centrifugation. HEK293T cells were transfected with vector or Nogo-A-Myc (3 µg) for 24 h, then medium was changed with Neurobasal A and cultured for another 24 h. Culture medium was centrifuged at 2,000 g for 10 min to remove the large cell debris and dead cells and at 20,000 g for 20 min to eliminate small debris. Then, exosomes were pelleted by ultracentrifugation at 120,000 g for 70 min. All procedures were performed at 4 °C. Exosome pellets were resuspended in 100 µl PBS and measured the protein concentration with Bradford assay for cortical axon regeneration assay, and resuspended in 10 µl laemmli buffer for immunoblot.

Transmission electron microscopy of exosomes
Exosomes from HEK293T culture supernatant were isolated by ultracentrifugation as described above. The exosome pellet was resuspended in 10 µl PBS and submitted to Yale University’s Center for Cellular and Molecular Imaging Electron Microscopy Facility. Five µl of exosome solution was placed on freshly glow-discharged copper grids (carbon-coated, 300 mesh, Electron Microscopy Services). Following a 2 min incubation the grids were rinsed twice on deionized water droplets and then stained for 2 min with 2% aqueous uranyl acetate. The excess staining solution was blotted off by Whatman #1 filter paper and the grids were allowed to air-dry for 20 min. Samples were examined using a FEI Tecnai TF20 transmission electron microscope operated at 200 kV of accelerating voltage. Images were acquired using an AMT NanoSprint 1200 CMOS camera. Particle diameter was measured using ImageJ, and size distribution was represented as a percent of the total number of particles analyzed.

Maleimide-PEG11-Biotin labeling
Exosomes prepared from HEK293T cells were resuspended in PBS or RIPA and incubated with membrane non-permeable Maleimide-PEG11-Biotin for 2 h. Then DTT was added to terminate the reaction, and exosomes were lysed with RIPA buffer for immunoprecitiation with anti-Myc antibody. The immune complexes were washed 3 times and resolved by SDS-PAGE and blotted with anti-Myc antibody and Alexa Fluor 488 streptavidin (Thermo Fisher Scientific).

RT-PCR and Quantitative PCR
Total RNA was prepared using TRIZol (SIGMA) and subjected to reverse transcriptase (RT)-PCR using the M-MuLV Reverse
Transcriptase (New England BioLabs). cDNAs were used for real-time qPCR with iQ supermix (BioRad) and TaqMan Gene Expression Assay (#Hs06633371 for huBACE1 and #Hs02758991 for hGapdh from Applied Biosystem) on a Bio-Rad CFX Connect Real-Time PCR Detection System using standard cycles. Each sample was analyzed in triplicate.

**Cortical Axon Regeneration Assay**

The Ngr1<sup>-/-</sup> mouse line has been described (32,33) and backcrossed for more than ten generations to C57BL/6 wild-type (Ngr1<sup>+/+</sup>) mice. Primary neuron cultures were obtained from these mice. Regeneration assay was performed as described previously (34). Primary cortical cultures were established from E17 C57BL/6 mice. Digested cells were plated on 96-well poly-d-lysine coated plates at a density of 25,000 cells per well in 200 µL of plating medium. On 8 div, 96-well cultures were scraped using a custom-fabricated 96-pin array and the indicated amount of Nogo22 or exosomes were added. Neurons were allowed to regenerate for another 72 h before fixing with 4% paraformaldehyde. Regenerating axons in the scrape zone were visualized using an antibody against β III tubulin (1:2,000). Growth cones were visualized by staining for F-actin using rhodamine conjugated phalloidin (1:2,000, #R415, Life Technologies). Images were taken on a 10x objective in an automated high-throughput imager (ImageXpress Micro XLS, Molecular Devices) under identical conditions. Regeneration zone identification, image thresholding, skeletonizing, and quantitation were performed in ImageJ to score axon regeneration extent. Measurements from different wells for the same condition in any one experiment were averaged together for one n value, and statistical differences were calculated between cultures from N embryos.

**Spinal cord injury**

Wild-type female mice were subjected to spinal cord crush surgery as described previously (35,36). Animals received subcutaneous injection of buprenex (0.01 mg/kg) 30 minutes before surgery and were deeply anesthetized with ketamine (100 mg/kg) and xylazine (15 mg/kg). To expose the dorsal spinal cord at T7 and T8 levels, laminectomy was performed. Then spinal cord was fully crushed with forceps for 3 s. Forceps (Dumont No. 5) had been filed to a width of 0.2 mm tips. The tips were inserted to include whole spinal cord across the ventral bone to avoid any spare tissue ventrally and laterally. Sham animals were laminectomized but not crushed. Muscle and skin overlying the lesion were sutured. Animals received subcutaneous injections of 100 mg/kg ampicillin and 0.1 mg/kg buprenex twice a day for the first two days after surgery. Procedures and postoperative care were performed in accordance with the guidelines of the Institutional Animal Use and Care Committee at Yale University. Three days after injury, animals were sacrificed, spinal cords were homogenized with TBS, and exosomes were fractionated.

**Statistics**

Statistical comparisons included one-way ANOVA and Student’s t test as specified in the Figure legends using Excel and Prism software. Statistical significance was set at P < 0.05. All data are mean ± standard error (SE). No statistical methods were used to calculate sample size estimates.

**ACKNOWLEDGMENTS**

This work was supported by grants from the N.I.H. and Falk Medical Research Trust to S.M.S. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. We thank Dr. Xinran Liu of Yale University’s Center for Cellular and Molecular Imaging Electron Microscopy Facility for sample preparation and image acquisition.

**CONFLICT OF INTEREST**

S.M.S. is a cofounder of ReNetX Bio, which seeks to develop NgR1-based therapeutics. The other author declares no competing interests.

**AUTHOR CONTRIBUTIONS**

Y.S. and J.A.L. conducted the experiments. Y.S. and S.M.S. conceived the study, analyzed data and wrote the manuscript.

**REFERENCES**
Exosomal Nogo-66 inhibits axonal regrowth

1. 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

2. Chen, M. S., Huber, A. B., van der Haar, M. E., Frank, M., Schnell, L., Spillmann, A. A., Christ, F., and Schwab, M. E. (2000) Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. *Nature* **403**, 434-439

3. 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

4. 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

5. Kottis, V., Thibault, P., Mikol, D., Xiao, Z. C., Zhang, R., Dergham, P., and Braun, P. E. (2002) Oligodendrocyte-myelin glycoprotein (OMgp) is an inhibitor of neurite outgrowth. *Journal of Neurochemistry* **82**, 1566-1569

6. 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

7. 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

8. Wang, K. C., Koprivica, V., Kim, J. A., Sivasankaran, R., Guo, Y., Neve, R. L., and He, Z. G. (2002) Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. *Nature* **417**, 941-944

9. Lauren, J., Hu, F., Chin, J., Liao, J., Airaksinen, M. S., and Strittmatter, S. M. (2007) Characterization of myelin ligand complexes with neuronal Nogo-66 receptor family members. *The Journal of biological chemistry* **282**, 5715-5725

10. Atwal, J. K., Pinkston-Gosse, J., Syken, J., Stawicki, S., Wu, Y., Shatz, C., and Tessier-Lavigne, M. (2008) Pirb is a functional receptor for myelin inhibitors of axonal regeneration. *Science* **322**, 967-970

11. Huebner, E. N., Kim, B. G., Duffy, P. J., Brown, R. H., and Strittmatter, S. M. (2011) A multi-domain fragment of Nogo-A protein is a potent inhibitor of cortical axon regeneration via Nogo receptor 1.. *J Biol Chem* **286**, 18026-18036

12. 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

13. 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

14. Hu, F., and Strittmatter, S. M. (2008) The N-terminal domain of Nogo-A inhibits cell adhesion and axonal outgrowth by an integrin-specific mechanism. *J Neurosci* **28**, 1262-1269

15. Kempf, A., Tews, B., Arzt, M. E., Weinmann, O., Obermair, F. J., Pernet, V., Zagrebelsky, M., Delekate, A., Iobbi, C., Zemann, A., Ristic, Z., Gullo, M., Spies, P., Dodd, D., Gygax, D., Korte, M., and Schwab, M. E. (2014) The sphingolipid receptor S1PR2 is a receptor for Nogo-a repressing synaptic plasticity. *PLoS Biol* **12**, e1001763

16. Huber, A. B., Weinmann, O., Brösamle, 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

17. Snow, D. M., Lemmon, V., Carrino, D. A., Caplan, A. I., and Silver, J. (1990) Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth in vitro. *Exp Neurol* **109**, 111-130

18. Shen, Y., Tenney, A. P., Busch, S. A., Horn, K. P., Cua, F. X., Liu, K., He, Z., Silver, J., and Flanagan, J. G. (2009) PTPsigma is a receptor for chondroitin sulfate proteoglycan, an inhibitor of neural regeneration. *Science* **326**, 592-596

19. Moreau-Fauvarque, C., Kumanogoh, A., Camand, E., Jaillard, C., Barbin, G., Boquet, I., Love, C., Jones, E. Y., Kikutani, H., Lubetzki, C., Dusart, I., and Chedotal, A. (2003) The transmembrane semaphorin Sema4D/CD100, an inhibitor of axonal growth, is expressed on oligodendrocytes and upregulated after CNS lesion. *J Neurosci* **23**, 9229-9239
Exosomal Nogo-66 inhibits axonal regeneration

20. Basile, J. R., Holmbeck, K., Bugge, T. H., and Gutkind, J. S. (2007) MT1-MMP controls tumor-induced angiogenesis through the release of semaphorin 4D. J Biol Chem 282, 6899-6905

21. Raissi, A. J., Staudenmaier, E. K., David, S., Hu, L., and Paradis, S. (2013) Sema4D localizes to synapses and regulates GABAergic synapse development as a membrane-bound molecule in the mammalian hippocampus. Mol Cell Neurosci 57, 23-32

22. Frohlich, D., Kuo, W. P., Frühbeis, C., Sun, J. J., Zehndner, C. M., Luhmann, H. J., Pinto, S., Toedling, J., Trotter, J., and Kramer-Albers, E. M. (2014) Multifaceted effects of oligodendrogial exosomes on neurons: impact on neuronal firing rate, signal transduction and gene regulation. Philos Trans R Soc Lond B Biol Sci 369

23. Bakhti, M., Winter, C., and Simons, M. (2011) Inhibition of myelin membrane sheath formation by oligodendrocyte-derived exosome-like vesicles. J Biol Chem 286, 787-796

24. Frühbeis, C., Frohlich, D., Kuo, W. P., Amphornrat, J., Thilemann, S., Saab, A. S., Kirchhoff, F., Mobius, W., Goebbels, S., Nave, K. A., Schneider, A., Simons, M., Klugmann, M., Trotter, J., and Kramer-Albers, E. M. (2013) Neurotransmitter-triggered transfer of exosomes mediates oligodendrocyte-neuron communication. PLoS Biol 11, e1001604

25. Lopez-Verrilli, M. A., Picou, F., and Court, F. A. (2013) Schwann cell-derived exosomes enhance axonal regeneration in the peripheral nervous system. Glia 61, 1795-1806

26. Raposo, G., and Stoorvogel, W. (2013) Extracellular vesicles: exosomes, microvesicles, and friends. The Journal of cell biology 200, 373-383

27. Teng, F. Y., and Tang, B. L. (2008) Cell autonomous function of Nogo and reticulons: The emerging story at the endoplasmic reticulum. J Cell Physiol 216, 303-308

28. Voeltz, G. K., Prinz, W. A., Shibata, Y., Rist, J. M., and Rapoport, T. A. (2006) A class of membrane proteins shaping the tubular endoplasmic reticulum. Cell 124, 573-586

29. He, W., Lu, Y., Qahwash, I., Hu, X. Y., Chang, A., and Yan, R. (2004) Reticulon family members modulate BACE1 activity and amyloid-beta peptide generation. Nat Med 10, 959-965

30. He, W., Hu, X., Shi, Q., Zhou, X., Lu, Y., Fisher, C., and Yan, R. (2006) Mapping of interaction domains mediating binding between BACE1 and RTN/Nogo proteins. J Mol Biol 363, 625-634

31. Sharoar, M. G., and Yan, R. (2017) Effects of altered RTN3 expression on BACE1 activity and Alzheimer's neuritic plaques. Reviews in the neurosciences 28, 145-154

32. Kim, J. E., Liu, B. P., Park, J. H., and Strittmatter, S. M. (2004) Nogo-66 receptor prevents raphespinal and rubrospinal axon regeneration and limits functional recovery from spinal cord injury. Neuron 44, 439-451

33. Sekine, Y., Lin-Moore, A., Chenette, D. M., Wang, X., Jiang, Z., Cafferty, W. B., Hammarlund, M., and Strittmatter, S. M. (2018) Functional Genome-wide Screen Identifies Pathways Restricting Central Nervous System Axonal Regeneration. Cell Reports 23, 415-428

34. Sekine, Y., Algarate, P. T., Cafferty, W. B. J., and Strittmatter, S. M. (2019) Plexina2 and CRMP2 Signaling Complex Is Activated by Nogo-A-Ligated Ngr1 to Restrict Corticospinal Axon Sprouting after Trauma. J Neurosci 39, 3204-3216

35. Zukor, K., Belin, S., Wang, C., Keelan, N., Wang, X., and He, Z. (2013) Short hairpin RNA against PTEN enhances regenerative growth of corticospinal tract axons after spinal cord injury. J Neurosci 33, 15350-15361

36. Sekine, Y., Siegel, C. S., Sekine-Konno, T., Cafferty, W. B. J., and Strittmatter, S. M. (2018) The nociceptin receptor inhibits axonal regeneration and recovery from spinal cord injury. Science Signaling 11

ABBREVIATIONS

Abbreviations: Spinal cord injury, SCI; Central nervous system, CNS; Myelin associated inhibitor, MAI; Chondroitin sulfate proteoglycan, CSPG; Reticulon, RTN; Myelin Associated Glycoprotein, MAG; Oligodendrocyte Myelin Glycoprotein, OMgp; Nogo-66 Receptor 1, NgR1; Paired Immunoglobulin-like Receptor B, PirB; ß-site amyloid precursor protein cleaving enzyme 1, BACE1; amyloid precursor protein (APP); standard error, SE.
Figure 1. Nogo-A C-terminal region is secreted in culture supernatant as an exosome component. A, Culture supernatants collected from vector and Nogo-A-Myc transfected HEK293T cells were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-Myc antibody. Cell lysates were also immunoprecipitated and immunoblotted in the same membrane. B, Scheme of exosome fractionation by centrifugation. C, Each fraction from vector or Nogo-A-Myc transfected HEK293T cells was immunoblotted with anti-Myc antibody. D, Ratio of 24 kDa Nogo-A fragment to full-length Nogo-A in exosome and lysate fractions. Mean±SE, N=12 for each group. ***P<0.005, Student’s two-tailed t test.
Figure 2. Nogo-A C-terminal region is enriched in exosomes. A, Negative stain transmission electron microscopy images of the exosome fraction derived from HEK293T culture medium with particle diameter distribution represented in a histogram. N=546 particles. B, Culture medium from Nogo-A-Myc transfected HEK293T cells was separated by sucrose density centrifugation. Gradient fractions from the top were immunoblotted with anti-Alix and anti-Myc antibodies. C-D, HEK293T cells were transfected with vector (-) or Nogo-A-Myc. At 24 h after transfection, media were changed with DMSO or Exo1 containing medium and cultured further 12 h. Then, culture supernatants were immunoprecipitated with anti-Myc antibody or exosome fractionated. Mean±SE, N=3 independent experiments. *P<0.05, Student’s two-tailed t test.
Exosomal Nogo-66 inhibits axonal regrowth
Exosomal Nogo-66 inhibits axonal regrowth

Figure 3. C-terminal cleavage site in Nogo-A. A, A series of Nogo-A-Myc C-terminal truncated constructs were generated. B, HEK293T cells were transfected with full length or a series of C-terminal constructs of Nogo-A-Myc. At 36 h after transfection, culture supernatants were collected and exosomes were fractionated. Cell lysates were also collected. Samples were then immunoblotted with anti-Myc antibody. C, Schematic images for Maleimide-PEG11-Biotin labeling. A cysteine residue of Nogo-A exposed on the surface of exosome can be labeled with non-permeable Maleimide-PEG11-Biotin reagent. Cysteine is expressed as a red circle. Alanine substituted from cysteine is shown as a yellow circle. D, Exosomes from vector, Nogo-A WT or Nogo-A C1101A transfected HEK293T cells were incubated with Maleimide-PEG11-Biotin. After the maleimide reaction, exosomes were lysed with RIPA and immunoprecipitated with anti-Myc antibody. Immunoprecipitates were washed 3 times and blotted with anti-Myc antibody and Alexa Fluor 488 streptavidin. E, Nogo-A-Myc-exosomes resuspended in PBS or RIPA buffer were incubated with Maleimide-PEG11-Biotin. After the maleimide reaction, exosomes were resuspended in RIPA and immunoprecipitated with anti-Myc antibody. Immunoprecipitates were washed 3 times and blotted with anti-Myc antibody and Alexa Fluor 488 streptavidin. F, Streptavidin intensity was divided by Myc intensity in both PBS and RIPA condition and expressed as normalized value as RIPA sample of 100 %. Mean±SE, N=9 independent experiments. P=0.70, Student’s two-tailed t test.
Figure 4. Nogo-66 loop region on the surface of exosomes. A and B, HEK293T cells were transfected with Nogo-A-Myc. At 24 h after transfection, media were changed and added DMSO, MG101 (20 µM), Z-VAD-FMK (20 µM), E64d (20 µM) and NH4Cl (20 mM), and cultured for further 12 h. Then, culture supernatants were collected and exosome fraction was immunoblotted with anti-Myc and anti-CD9 antibodies (A). The graph shows relative value to DMSO control of Myc intensity divided by CD9 intensity (B). Mean±SE, N=5-8 independent experiments. *P<0.05, ***P<0.005, one-way ANOVA followed by Dunnett's test. C and D, HEK293T cells were transfected with Nogo-A-Myc. At 24 h after transfection, media were changed and added DMSO, indicated amount of BACE inhibitors and NH4Cl (20 mM), and cultured for further 12 h. Then, culture supernatants were collected and exosome fraction was immunoblotted with anti-Myc and anti-CD9 antibodies (C). The graph shows relative value to DMSO control of Myc intensity divided by CD9 intensity (D). Mean±SE, N=4 independent experiments. **P<0.01, ***P<0.005, one-way ANOVA followed by Dunnett's test. E, HEK293T cells were transfected with Nogo-A-Myc and siNC, siBACE1 #1 or #2. Exosomes were purified at 36 h after transfection, and immunoblotted with anti-Myc and anti-CD9 antibodies. F, Quantification of Myc divided CD9 intensity normalized to DMSO of control. Mean±SE, N=6 independent experiments. **P<0.01, ***P<0.005, one-way ANOVA followed by Dunnett's test. G, Real time PCR for replicate of siBACE
Exosomal Nogo-66 inhibits axonal regrowth

transfected HEK293T cells. BACE1 mRNA expressions were normalized to its Gapdh mRNA expression. Mean±SE, N=4 independent experiments. ***P<0.005, one-way ANOVA followed by Dunnett's test.

Figure 5. Inhibitor function of Nogo-A C-terminal fragment. A and B, Cortical neurons were scraped and treated with indicated amount of exosomes at 8 DIV for 3 days (A). The microphotographs show βIII tubulin (in axons; green) and phalloidin (to stain F-actin; red). Scale bar represents 200 µm. (B) The graph shows
Exosomal Nogo-66 inhibits axonal regrowth

quantification of axonal regeneration. Mean±SE. N=3 biological replicates, *P<0.05, ***P<0.005, one-way ANOVA followed by Tukey's test. C, Cortical neurons from WT or NgR1-/- were scraped and treated with same amount of exosomes at 8 DIV for 3 days. The graph shows quantification of axon regeneration. *P<0.05, Student’s two-tailed t test. #, not significant, D, Cortical neurons were scraped and treated with exosomes (10, 20, 40 and 75 nM) or Nogo22 (0, 10, 30, 75, 100, 150, 300 and 600 nM) at 8 DIV for 3 days. The graph shows the quantification of axonal regeneration. Mean±SE. N=2-6 biological replicates. *P<0.05, Student’s two-tailed t test. E, IC-80 from each separate experiment was calculated. Mean±SE. N=15 (Nogo22), N=9 (Exosome) biological replicates. *P<0.05, Student’s two-tailed t test.

Figure 6. Increased Nogo-A fragment levels after spinal cord trauma in vivo. A, WT mice were spinal cord crushed and sacrificed 3 days after surgery. Spinal cord were then taken and homogenized in TBS. Exosome fractions were immunoblotted with anti-Nogo-A and anti-Alix antibodies. B, Quantification of Nogo-A divided by Alix intensity. Mean±SE, N=3 animals. *P<0.05, Student’s two-tailed t test.
A proteolytic C-terminal fragment of Nogo-A (reticulon-4A) is released in exosomes and potently inhibits axon regeneration
Yuichi Sekine, Jane A. Lindborg and Stephen M. Strittmatter

*J. Biol. Chem.* published online November 20, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA119.009896

Alerts:
  - When this article is cited
  - When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts