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*Original Article*

# Antibodies neutralizing Nogo-A increase pan-cadherin expression and motor recovery following spinal cord injury in rats

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**Study design:** A rat model of spinal cord injury was used to test the hypothesis that Nogo-A monoclonal antibody (NEP1-40) promotes morphologic and functional recoveries of injured spinal cord.
**Objective:** Nogo-A is a myelin-associated neurite outgrowth inhibitory protein, which blocks elongation nerve fibers and limits neuronal regeneration after central nervous system injury.
**Methods:** Forty-four rats were utilized and allocated into control (vehicle) and NEP1-40-treated groups. In all animals, the spinal cord was hemi-transected at Th-10 and phosphate-buffered saline solution was immediately applied on the injured area in the control group. NEP1-40 solution was immediately applied on the hemi-transected area in the treatment group. Each group was subdivided into three subgroups according to the postsurgical day of killing (3, 8 and 21 days). The spinal cords were removed for analysis.
**Results:** Motor scores in the NEP1-40-treated groups were significantly higher than those in the vehicle groups both at 8 and 21 days post injury. Immunohistochemical staining for pan-cadherin, a marker of neuronal cell adhesion and axonal sprouting, revealed a significant increase in staining in the NEP1-40 treatment group at 8 and 21 days post injury. Transmission electron microscopical evaluation revealed degeneration of the myelin and loss of cytoarchitectural organization in the axons of controls. Better preservation and normal histologic features were observed in the NEP1-40-treated groups.
**Conclusion:** We have demonstrated improved preservation of injured axons and significant pan-cadherin expression after NEP1-40 treatment after the spinal cord injury. Inhibition of Nogo-A may improve the capacity for neuronal regeneration after spinal cord injury.
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**Keywords:** motor recovery; NCAM; neuroregeneration; Nogo-A; pan-cadherin

## Introduction

The adult mammalian central nervous system (CNS) has a limited capacity for nerve regeneration and structural plasticity.[1–4] One of many explanations for the decreased amount of anatomical plasticity that occurs after adult CNS injury in adults is the presence of myelin-associated inhibitory factors that block neurite outgrowth.[2–6] Currently, the best-characterized cells that mediate inhibitory signals in axonal growth are the oligodendrocytes. Nogo-A is a myelin-associated neurite-outgrowth inhibitory protein that limits neuronal regeneration and plasticity after CNS injury. It is a protein product of the *Nogo* gene,[3–6] which is expressed on the cell surface of oligodendrocytes.[4] The presence of glia-derived inhibitory factor Nogo-A may provide a nonpermissive environment for the elongation of nerve fibers.[5] Both regeneration and axonal sprouting are very limited in the fully matured CNS of higher vertebrates, but can be enhanced by neutralizing the neurite outgrowth inhibitory protein Nogo-A.[6]

Adhesion molecules have a very important role in the pathogenesis of the inflammation that develops after spinal trauma.[7] Neural cell adhesion molecules (NCAM), which promote adhesion between the surfaces of neural cells, are found in the gray and white matter and are expressed on glial cells, especially on reactive astrocytes and neurons that have completed their differentiation.[8] *N*-cadherin, which is one of those adhesion molecules found outside the cellular plasma membrane, promotes hemophilic adhesions.[9] Neurons lose their connections and transform into single cellular structures when antibodies to *N*-cadherin are applied.[10]

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Cadherin activity is essential for axon-dendritic spine contacts, synaptic plasticity and rearrangement.[9,10]

In this experimental study, we demonstrated the increased expression of adhesion molecules (cadherins) from the nerve cells of injured spinal cords that occurred after we applied a treatment that neutralized the myelin-associated growth inhibitor Nogo-A. Electron microscopic evaluation confirmed axonal preservation.

## Materials and methods

Forty-four male Sprague–Dawley rats (weighing 150–180 g) were used. All protocols were approved by the University of Baskent Animal Care and Use Committee (no. DA0214) and we certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research. The rats were randomly allocated into two groups: the vehicle group (the control group) and the group treated with Nogo-A monoclonal antibody (the NEP1-40-treated group). Those two groups were divided into three subgroups according to the date of killing (days 3, 8 and 21 after the surgery) (Table 1).

*Nogo monoclonal antibody preparation*

Hundred micrograms of Nogo-A monoclonal antibody (Human Nogo 1-40 peptide (NEP1-40); Alpha Diagnostic International Inc., San Antonio, TX, USA) was diluted with 2.5 ml of phosphate-buffered saline (PBS) (pH 7.5; 0.1–0.2% bovine serum albumin (BSA); containing 0.05% sodium azide or merthiolate) and was instilled via micropipettes into 25 tubes, each of which contained 0.1 ml NEP1-40. Then, 0.1 ml of NEP1-40 was applied to each spinal cord hemi-transection area.

**Table 1** All rats were randomly allocated into two groups as control-vehicle group and Nogo monoclonal antibody-treated (NEP1-40) group

| | *Vehicle group* | *NEP1-40 group* |
|---|---|---|
| Killed on day 3 | 6 | 7 |
| Killed on day 8 | 8 | 8 |
| Killed on day 21 | 7 | 8 |

Each group divided into three subgroups and killed on days 3, 8 and 21 after the surgical procedure

*Surgical procedure*

Anesthesia was provided by an intraperitoneal (i.p.) ketamine 60 mg/kg (Ketalar, Pfizer, Turkey) and 2% xylazine 10 mg/kg (Rompun, Bayer, Turkey). During surgery, the body temperature was maintained at 37°C. The femoral artery was catheterized, and blood gas values were analyzed (Table 2). A preoperative dose of enrofloxacin (Baytril K, Bayer, Turkey) 10 mg/kg was administered subcutaneously to all subjects. The thoracolumbar area was shaved and prepared with povidone-iodine (Betadine, Pharma, Turkey). A midline incision was made from the Th-9 level to the Th-11. Surgery was performed with the aid of a surgical microscope (OPML – 9 FC Zeiss, Germany). The fascia and paraspinous muscles were sharply incised and total laminectomy was performed at Th-10. The spinal cord was hemi-transected with a microscissor in all rats. The amount of spinal cord transected was the same in all groups, and that hemi-transection was made lateral to the anatomic midline of the cord at the Th-10 level. PBS (0.1 ml) solution was applied to the hemi-transected area in the controls. NEP1-40 (0.1 ml) solution was applied to the hemi-transected area in the NEP1-40 treatment group immediately after injury. The surgical site was closed, and the antibacterial spray Pyedif (Sanofi-Dif, Turkey) was applied to the skin incision. Immediately after surgery, all rats received 5 ml of subcutaneously administered lactated Ringer's solution and were maintained in an incubator until thermoregulation had been re-established. Postoperative care was provided, and each rat received one additional subcutaneous injection of enrofloxacin (10 mg/kg). Lactated Ringer's solution

**Table 2** Physiological variables in rats subjected to spinal cord hemi-transection

| *Parameter* | *Experimental group* | *Time before and after spinal cord insult* | | | |
|---|---|---|---|---|---|
| | | *−30 min* | *−15 min* | *15 min* | *30 min* |
| MABP (mm Hg) | Vehicle | 97±6.3 | 89±7.1 | 85±7.4 | 94±4.2 |
| | NEP1-40 | 95±4.4 | 91±5.3 | 88±5.1 | 91±4.9 |
| PH | Vehicle | 7.46±0.02 | 7.42±0.01 | 7.43±0.03 | 7.41±0.01 |
| | NEP1-40 | 7.43±0.01 | 7.41±0.02 | 7.43±0.02 | 7.43±0.01 |
| $Po_2$ (mm Hg) | Vehicle | 87±4 | 83±3 | 86±4 | 88±5 |
| | NEP1-40 | 85±4 | 88±6 | 91±7 | 84±5 |
| $P_{aCO2}$ (mm Hg) | Vehicle | 43±2 | 41±4 | 40±4 | 39±5 |
| | NEP1-40 | 41±4 | 39±4 | 42±5 | 40±4 |

Abbreviation: MABP, mean arterial blood pressure
Blood gas values analyzed at intervals are shown. Note all these values are within the normal physiological range. All values are expressed as means±s.e.

was administered to prevent dehydration. Rats were visually inspected to identify skin irritation or decubitus ulcers. Although many scales have been developed since Tarlov (BBB, AOB, and so on.), we have evaluated each rat's functional status with the modified Tarlov's motor grading scale.[11–13] The motor scale scores were noted on the days 3, 8 and 21 after surgery.[12,13]

Rats in both the control and NEP1-40 treatment groups were killed on days 3, 8 and 21 after surgery by a lethal dose of i.p. ketamine 150 mg/kg (Ketalar, Pfizer, Turkey). After transcardiac perfusion with 10% formalin had been performed, the spinal cord at the Th-9 to Th-11 level was removed *en bloc* and was immersed overnight in 10% formalin. The hemi-transected medulla spinalis was prepared for immunohistochemical and electron microscopic analysis.

*Immunohistochemical analysis*

The formaldehyde-fixed and paraffin-embedded spinal cords were sectioned. After deparaffinization and rehydration, each section was immunostained with antibodies to cadherin (Pan Ab-4, rabbit polyclonal, Neomarkers Thermo Scientific (Fremont, CA, USA)). Immunohistochemical analyses were conducted via the standard avidin–biotin complex method according to the following procedure. For both antigens, antigen retrieval was performed in a microwave oven in 10 mM citrate buffer + EDTA (pH 6.0) at 700 W for 15 min. Then, endogenous peroxidase activity was blocked with 0.3% $H_2O_2$ for 30 min. After incubation with 5% BSA in Tris-buffered solution (TBS) (50 mM Tris–HCl, 150 mM NaCl, pH 7.4) to block nonspecific binding for 10 min, the sections were incubated overnight with primary antibodies for pan-cadherin in a humidified chamber at room temperature. The sections were then incubated with peroxidase-labeled polymer for 30 min, after which a diaminobenzidine plus substrate-chromogen solution was applied. The sections were counterstained with hematoxylin and were mounted. Between steps, the slides were washed twice in TBS. For negative controls, the primary antibodies were omitted, and nonimmune serum was used instead. For positive controls, we stained sections of tissues that were considered suitable according to the manufacturer's protocol.

All histological sections were evaluated by a neuropathologist who was blinded to whether the rat studied was a control or had received treatment. The extent and intensity of pan-cadherin expression were evaluated semiquantitatively. Scoring was classified into the following three groups: 0, no expression; 1, low expression; and 2, high expression.

*Electron microscopic technique*

Before electron microscopic examination was performed, all tissues were fixed in PBS containing 2.5% glutaraldehyde for 2–3 h and were then post-fixed in 1% osmium tetroxide ($OsO_4$) and were dehydrated in a series of graded alcohol baths (25, 50, 75, 95% and absolute alcohol). After having been passed through propylene oxide, the specimens were embedded in 2-dodecenyl succinic anhydride (Araldyte CY 212, DDSA), benzyldimethyl amine and dibutylphthalate. Semithin sections were cut, stained with toluidine blue and examined with a light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined with a LEO 906E EM transmission electron microscope (TEM).

*Statistical evaluation*

Statistical Package for the Social Sciences (SPSS), version 11.0, was used for statistical analysis. Data were evaluated using the Wilcoxon-signed rank test to compare changes among the individual groups. A *P*-value of less than 0.05 was considered statistically significant.

## Results

*Motor grading results*

According to the modified Tarlov's scale scores (Figure 1), during the early period after spinal cord injury (day 3), there was no significant difference between the mean scores of NEP1-40-treated group ($0.17 \pm 0.17$) and the control group ($0.43 \pm 0.3$) ($P > 0.05$). However, on day 8 post injury, the motor scores in the NEP1-40 group were significantly higher ($2.75 \pm 0.41$) than those in the control group ($1 \pm 0.33$) ($P = 0.019$). On day 21 after injury, the motor scores of the NEP1-40 group were significantly

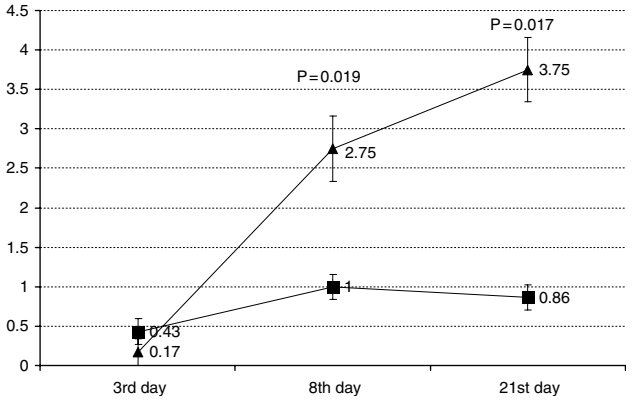


**Figure 1** Modified Tarlov's scale scores: during the early period after spinal cord injury (day 3), there was no significant difference between the mean scores of NEP1-40-treated group ($0.17 \pm 0.17$) and the control group ($0.43 \pm 0.3$) ($P > 0.05$). However, on day 8 post injury, the motor scores in the NEP1-40 group were significantly higher ($2.75 \pm 0.41$) than those in the control group ($1 \pm 0.33$) ($P = 0.019$). On day 21 after injury, the motor scores of the NEP1-40 group were significantly higher ($3.75 \pm 0.41$) than those in the control group ($0.86 \pm 0.46$) ($P = 0.017$). (Numeric values represent the means and the bars are $\pm$s.e.m.: triangles (▲), NEP1-40-treated groups; squares (■), vehicle-treated control groups.)

higher ($3.75 \pm 0.41$) than those in the control group ($0.86 \pm 0.46$) ($P = 0.017$).

*Pan-cadherin expression*

Membranous immunoreactivity was considered a positive result for the presence of pan-cadherin. On day 3 after injury in the control group, a faint cytoplasmic pan-cadherin expression was observed in ependymal cells. On day 8 (Figure 2a) and day 21 after injury (Figure 3a), there was no significant staining, although weak cytoplasmic staining was noted in the neuronal and ependymal cells in the control group. On day 3 after injury, we observed no significant difference in the results of staining for pan-cadherin in the control and NEP1-40-treated groups; however, on day 8 (Figure 2b) and day 21 (Figure 3b) after injury in the NEP1-40-treated rats, neuronal cells (particularly those located in the ventral regions of spinal cord) exhibited marked membranous positivity for pan-cadherin. The immunostaining pattern was more striking on day 21 after injury in the NEP1-40-treated group (Figure 3b). The ependymal cell layer also demonstrated strong membranous immunostaining with that antibody.

The semiquantitative pan-cadherin expression results also revealed that membranous positivity for pan-cadherin on day 21 after injury in the NEP1-40 group ($1.62 \pm 0.26$) was significantly higher than that in the control group ($P = 0.020$). Pan-cadherin expression was significantly greater on day 8 after injury in the NEP1-40 group ($0.75 \pm 0.16$) than in the control group ($P = 0.014$).

*Electron microscopy*

On day 3 after injury, diffuse degeneration of the myelin sheath was observed via electron microscopy in all controls and in the rats treated with NEP1-40. In those groups, the lamellar pattern of myelin was disrupted. The myelin sheaths had segregated, and tears, breaks and vacuolization had developed between the lamellae of the myelin. Cytostructural organization in the

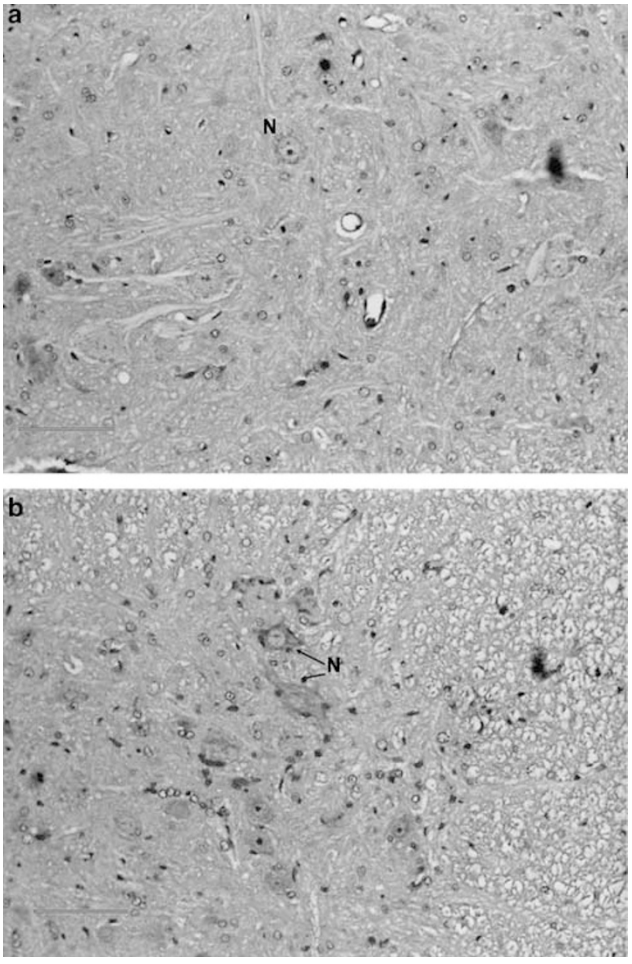


**Figure 2** Day 8 vehicle group (**a**) without marked expression of Cadherin immunohistochemistry ($\times 20$). Day 8 NEP1-40 group (**b**) showing moderate degree ($1+$) expression of cadherin immunohistochemistry ($\times 20$). N, neuron; bars, 100 $\mu$m

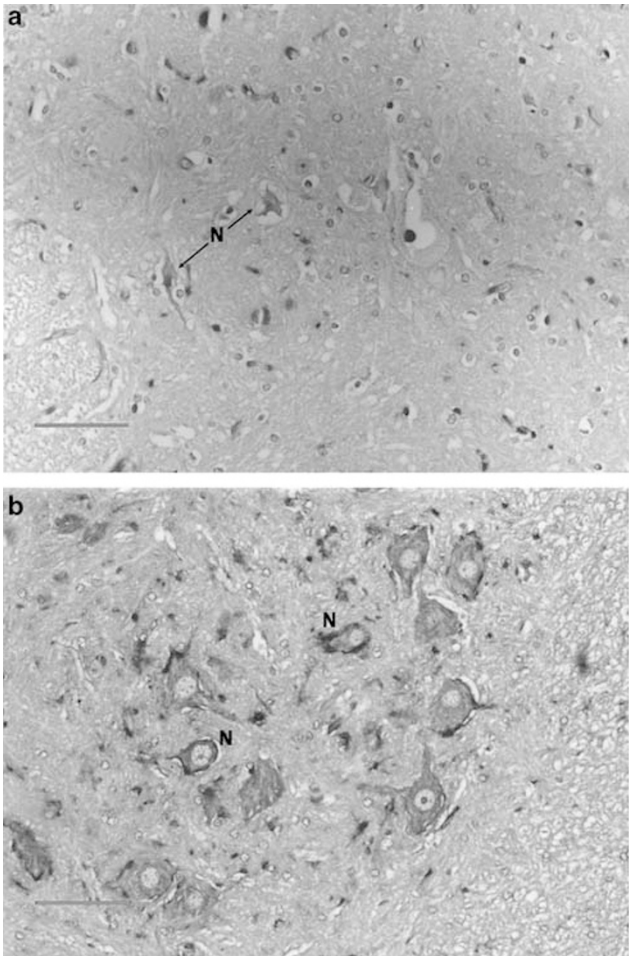


**Figure 3** Day 21 vehicle group (**a**) without marked expression of cadherin immunohistochemistry ($\times 20$). Day 21 NEP1-40 group (**b**) showing marked degree ($2+$) expression of cadherin immunohistochemistry ($\times 20$). N, neuron; bars, 100 $\mu$m

neuronal axons had been destroyed. The density of the axoplasms had increased and the volume had decreased. Unmyelinated nerve fibers in a widely degenerated form were noted (Figures 4a and 5a). The myelin sheath, axoplasm and unmyelinated nerve fibers were found to have degenerated on day 8 after injury in the NEP1-40-treated group, but those structures were preserved better (Figure 4b) than their corresponding structures in the control group. On day 21 after injury, better preservation of the cytostructure was noted in the NEP1-40 group than in the control group. The lamellar pattern and the density of the axoplasms were normal in appearance, and the volume of the axoplasms had increased in the NEP1-40-treated group (Figure 5b).

## Discussion

In this study, post-injury motor scores in the NEP1-40-treated groups were significantly higher than the control groups. We confirmed our motor score results with the

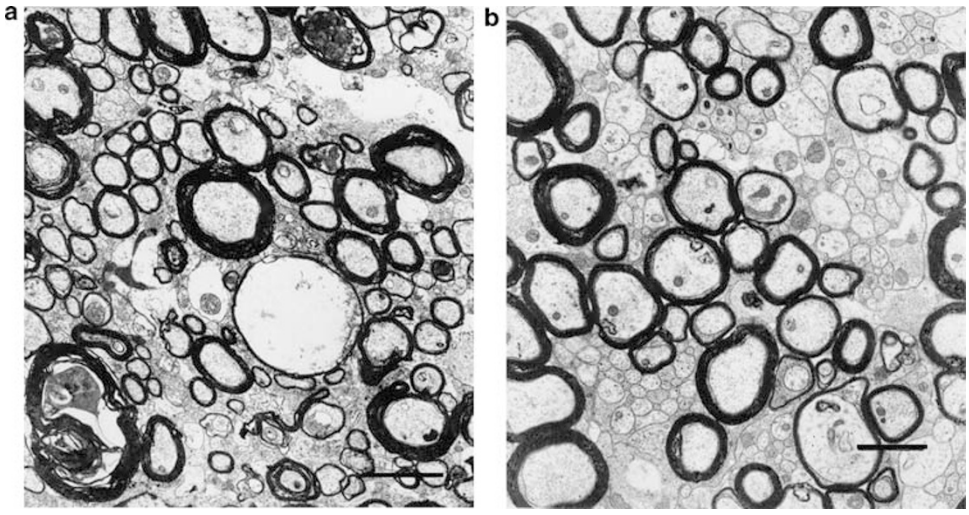


**Figure 4** Transmission electron microscopic findings on day 8: diffuse degeneration of the myelin sheath can be seen in the vehicle group on day 8. Myelin sheath have been spreaded, tears and breaks together with vacuolizations between the lamels of the myelin have occurred. Cytostructural organization have been lost in the axons. Density of the axoplasms have increased and the volume of the axoplasms have decreased. (lead citrate–uranyl acetate $\times 4646$, bar, 2.5 $\mu$m). (**a**) Degeneration of the myelin sheath, axoplasm and unmyelinated nerve fibers can also be seen in the NEP1-40-treated group on day 8, but these structures were preserved better than the vehicle group (lead citrate–uranyl acetate $\times$ 7750, bar, 1.25 $\mu$m) (**b**)

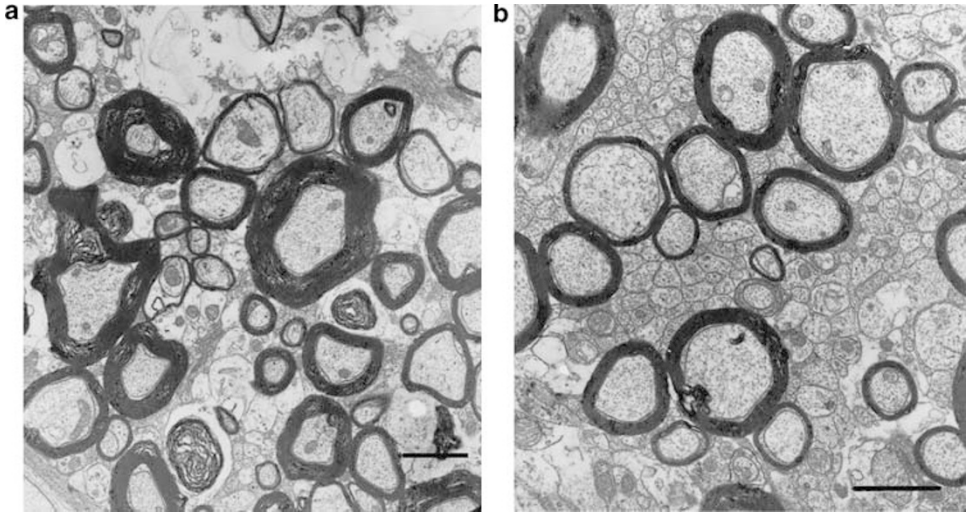


**Figure 5** Transmission electron microscopic findings on day 21: in the day 21 vehicle group, diffuse degeneration of the myelin sheath was observed and the lamellar pattern of myelin was disrupted. Vacuolizations between the lamels of the myelin have occurred. Axons have lost their cytostructural organization. Density of the axoplasms have increased and the volume of the axoplasms have decreased (lead citrate–uranyl acetate $\times$ 7750, bar, 1.25 $\mu$m) (**a**). On day 21, better preservation of the cytostructure and near-normal histological features were observed in NEP1-40-treated group. Preserved myelin sheath, axoplasms and the unmyelinated nerve fibers can be seen as a good indication for neuronal regeneration (lead citrate–uranyl acetate $\times$ 10 000, bar, 1.25 $\mu$m) (**b**)

contribution of immunohistochemistry and TEM. Following NEP1-40 treatment on day 8 and day 21 post injury, immunohistochemical pan-cadherin stainings demonstrated increased activity. TEM observations supported the findings of both motor score of the rats and pan-cadherin stainings, and we observed preservation of the nerves on days 8 and 21 in NEP1-40 treatment groups. By inhibiting the Nogo peptide we promoted axonal recovery and regeneration and increased the amount of adhesion molecules in the trauma site.

Spinal cord injury is a classic example of traumatic insult that leads to permanent disability, including the loss of sensory, motor and reflex activity.[14] Whether a mature neuron re-extends an axon depends on the following factors: the ability to re-express growth-related genes; the availability of neurotrophic factors and substrate molecules to which growing neurites may attach and extend; the presence of growth-inhibiting molecules; or the formation of a glial scar.[15]

Within the first 3 weeks after lesion formation, adult mammalian CNS axons demonstrate an initial growth response. However, sprouting axons extend only for very short distances before they cease growing and either degenerate or terminate locally at the lesion border. It is clear that the lesion area itself is of prime importance in the context of regeneration failure. The observation that cultured neurons are unable to extend neurites into optic nerve explants, even in the presence of optimal trophic factor conditions,[16] has led to the recent theory that neurite growth-inhibiting molecules inhibit the ability of adult mammalian CNS neurons to regenerate.[17] It has been hypothesized that myelin-associated molecules are exposed during the injury response and subsequently abort regeneration.[17] Various attempts to reduce or neutralize those proteins have been made. In one series of experiments, myelin-free spinal cord was produced in young rats by repeated irradiation or immunocytolysis.[18] Those treatments resulted in the substantial elongation of regrowing corticospinal and brainstem descending projections.

Bregman and colleagues[17] demonstrated that the neutralization of myelin-associated neurite growth inhibitors in adult rats with a spinal cord lesion can enhance regeneration and the anatomic plasticity of corticospinal and brainstem spinal pathways and can mediate noticeable improvements in important aspects of locomotor function. In Bregman's study, the continuous application of the antibodies via the implantation of a antibody-secreting hybridoma, either on top of a lesion or into the lateral ventricles restored the elongation of some corticospinal nerves, optic nerves and septo-hippocampal sprouts for considerable distances distal to the lesion site.[17] Brösamble and colleagues[19] subjected rats to dorsal spinal cord hemisection at level Th-8 and produced a recombinant humanized Fab fragment of the monoclonal antibody to Nogo-A (monoclonal antibody (mab) IN-1) in bacteria. They showed that mab IN-1 neutralized efficiently the neurite-growth inhibitory properties of a CNS myelin preparation. When infused subdurally at the site of a partial spinal cord transection in adult rats, mab IN-1 promoted long-distance axonal re-growth. In the mab IN-1-treated experimental group, regenerated fibers extended between 1.4 and 9.6 mm beyond the site of injury within 2 weeks after injury. No growth beyond typical lesion was observed in animals treated with a vehicle solution as controls.[19]

*N*-cadherin expression has been documented *in vivo* on normal unmyelinated fibers and regenerating fibers of the peripheral nervous system.[20] Sorting of axons into different pathways may depend on cadherin expression similar to that occurring during brain development and in cultured systems, NCAM and *N*-cadherin may promote neurite outgrowth and they are downregulated with the myelination.[8]

Thornton and colleagues,[20] who used quantitative immunohistochemical analysis to assess the expression of adhesion molecules in axotomized and crushed rat sciatic nerve, showed that the relative roles of those molecules proximal and distal to the site of injury are independent. They reported that 2 weeks after axotomy, regenerating axons were observed in the proximal stump. They concluded that NCAM has a role in the interactions between Schwann cells and axons and is expressed by Schwann cells after injury. Their results demonstrated the increased NCAM expression in proximal stumps after injury.

We suggest in our study that the key point in neuroregeneration or neuronal recovery was the inhibition of the Nogo peptide by NEP1-40, which stimulated the sprouting of new axons and inhibited myelin formation. Nogo mab may be a potential treatment in future, because Nogo mab could be applied immediately after spinal cord injury to an injured spine during surgery. Long-term treatment with that agent via an intradural infusion pump may be a therapeutic option for the patient with a spinal injury in future.

## Conclusion

Mabs to Nogo can permit injured CNS nerve fibers to regenerate by neutralizing myelin-associated growth-inhibitory proteins in the spinal cord.

## Acknowledgements

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