Axonal regeneration of optic nerve after crush in Nogo66 receptor knockout mice

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Mature retinal ganglion cells (RGCs) cannot regenerate injured axons because some neurite growth inhibitors, including the C-terminal of Nogo-A (Nogo66), myelin-associated glycoprotein (MAG) and Omgp, exert their effects on neuron regeneration through the Nogo receptor (NgR). In this study, the axonal regeneration of retinal ganglion cells (RGCs) after optic nerve (ON) crush was investigated both in vivo and in vitro in NgR knockout mice. We used NgR knockout mice as the experimental group, and C57BL/6 mice as the control group. Partial ON injury was induced by using a specially designed ON clip to pinch the ON 1 mm behind the mouse eyeball with 40 g pressure for 9 s. NgR mRNA was studied by in situ hybridization (ISH). NgR protein was studied by Western blot. Growth Associated Protein 43 (GAP-43), a plasticity and growth protein expressed highly during axon regeneration, was studied by immunofluorescence staining on the frozen sections. RGCs were cultured and purified. The axonal growth of RGCs was calculated by a computerized image analyzer. We found that compared with the control group, the GAP-43 expression was significantly higher and the axonal growth was significantly more active at every observation time point in the experimental group. These results indicate that NgR genes play an important role in the axonal regeneration after ON injury, while knockout of NgR is effective for eliminating this inhibition and enhancing axonal regeneration.

\section*{Introduction}

Failing of CNS neurons to regenerate after injury is a popular phenomenon that has attracted intense investigation at both the preclinical and clinical levels. Regeneration failure has been attributed in part to the proteins associated with CNS myelin and the scar at the injury site.

Some neurite growth inhibitors include the C-terminal of Nogo-A (Nogo66), myelin-associated glycoprotein (MAG) and Omgp, which share similar domains [4,8,18]. The NgR belongs to a family of three CNS-enriched glycosyl phosphatidylinositol-linked proteins [3,11,17]. NgR has been proposed to function as the ligand binding component of a tripartite receptor system consisting of Lingo-1 and the tumor necrosis factor (TNF) receptor family members p75NTR or TROY [13]. Perturbation of NgR with function-blocking antibodies [6,12] or short hairpin RNA interference (shRNAi)-mediated knockdown [1] have been reported to support that NgR is essential for Nogo66, MAG and Omgp inhibition.

Growth Associated Protein 43 (GAP-43), a plasticity and growth protein expressed highly in neuronal growth cones during development and axonal regeneration, is considered a crucial component of the axon and presynaptic terminal, its null mutation leading to death within days after birth due to axon pathfinding defects.

Optic nerve trauma and glaucoma can lead to optic nerve injury. The role of NgR in axonal regeneration after optic nerve injury has not been investigated. Expression of NgR is mainly in the retinal ganglion cells (RGCs) of retina [5]. In this study, knockout of NgR was used to clarify the possible contribution of NgR to inhibit the axonal regeneration of RGCs. Here we provide the evidence that NgR is important for the inhibition of neurite outgrowth.

An NgR-targeted embryonic stem cell clone was identified from the Omnibank sequence Tag database. Omnibank mutations were created. Heterozygotic mice harboring the disrupted allele were bred to C57Bl/6 mice for expansion in our animal housing facility and intercrossed to maintain the NgR mutation on a hybrid 129SvEvBrd_C57Bl/6 background. The mice were raised in the laminar air flow cage rack. The present investigation adhered to the principles regarding the care and use of animals according to the...
American Physiologic Society, the Society for Neuroscience, and the ARVO Statement for the use of animals in ophthalmic and vision research.

The left eyes of 30 female NgR knockout mice (8- or 12-week-old) were used as the experimental group, and 30 left eyes of female C 57BL/6 mice (8- or 12-week-old) in the control group. Both groups were further divided into three subgroups representing the first day (n = 10), third day (n = 10), and seventh day (n = 10), outcomes to depict the axonal growth at successive time points.

Retina were prepared in extraction buffer containing 50 mM Tris–HCl (pH7.4), 150 mM NaCl, 1% Triton-100, 0.1% SDS, 1 mM EDTA, 1 mM AEBSF, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. Equal amounts of total protein (10 μg) were separated by 10% SDS-PAGE and transferred to Hybond-P polyvinylidene difluoride (PVDF) membrane (Amerham Pharmacia Biotech, Piscataway, NJ, USA). After blocking with 5% nonfat dry milk in PBS with 0.1% Tween-20, membranes were probed with rabbit anti-NgR antibody(1:500, Abcam company, Cambridge, USA), followed by subsequent incubation with goat anti-rabbit secondary antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Visualization of the protein bands was performed by the enhanced chemiluminescence kit (Santa Cruz Biotechnology). Parallel Western blot was probed with anti-GAP-43 monoclonal antibody (Abcam company, Cambridge, USA) as a loading control. Band intensity was quantified using Quantity One 4.4.1 software (Bio-Rad).

Prior to ON crush, general anesthesia was induced in each animal with an intraperitoneal injection of 1% sodium pentobarbital solution (50 mg/kg bodyweight). After the meninges was opened longitudinally, the ON was crushed at 1 mm distal to the eyeball by an ON forceps with 40 g pressure for 9 s. Injury to the ophthalmic artery was carefully avoided during the procedure. The left ON was crushed in both groups, but the right ON was intact in the control group.

Seven days after ON crush, thoracotomy was performed. The animals were perfused as described previously for in situ hybridization [21]. Frozen sections (15 μm) of eyes with ON segments up to the optic chiasm were cut longitudinally on a cryostat, thaw mounted onto coated glass slides, and stored at −80 °C.

The serial number (NM-022982) and cDNA array of NgR gene was achieved from GenBank. The common probe, recognizing all NgR, contains transcript A sequence between nucleotides 2326 and 4515.

Hybridization was performed as described before [21] and signals were detected with alkaline phosphatase-coupled anti-digoxigenin antibodies using spreptavidin–biotin–peroxidase complex (SABC) as color reaction substrates.

Frozen sections were stained in GAP-43 monoclonal antibody (1:500 dilution), followed by fluorescent secondary antibody (1:500).

RGCs from the retina were isolated and purified as previously described [2,16]. The cell suspension was incubated successively in a polypropylene tube coated with a monoclonal anti-rat macrophage IgG to remove macrophages, and then in another one coated with a monoclonal anti-Thy 1.1 IgG. RGCs were cultured at a concentration of 6 × 106 cells/ml in DMEM that B-27 and incubated at 37 °C with 5% CO2 ventilation. Cells were examined daily under phase-contrast microscope.

RGCs were fixed in 4% paraformaldehyde for 15 min and blocked with 5% goat serum. The cells were incubated successively with primary antibody anti-GAP-43 (1:1000) at 4 °C for 24 h, secondary antibody goat-anti-rabbit IgG at 37 °C for 1 h, and SABC at 37 °C for 30 min. After coloration with DAB, dehydration, and dimethyl benzene treatment, slides were mounted and examined with phase-contrast microscopy.

Regeneration was quantified as follows. In four longitudinal sections per case, under 400 magnification, we counted the number of GAP-43-positive axons extend ≥1 mm from the injury site and divided these numbers by the cross-sectional width of the nerve at the point where axons were counted. The number of axons per unit width of optic nerve was averaged across the four sections and used to calculate the total number of axons regenerating in the nerve.

The data were analyzed by one-way variance to determine the statistical significant, using software Origin (version 6.0, OriginLab Corp., Northampton, MA). P value of less than 0.05 was considered statistically significant and less than 0.01 statistically borderline significant.

NgR expressed in the retinal ganglion cells. Stain of brown indicates positive expression of NgR. Expression of NgR was calculated using Image-Pro Plus (version 4.0, Media Cybernetics, Silver Spring, MD). Positive area of stain of NgR expression of experimental group was 1.25 ± 0.03 × 102 μm2, whereas that of negative control group was 23.26 ± 1.36 × 102 μm2. There was significant difference between the experimental and control group (P= 0.008). There is little expression of NgR mRNA in the ON of NgR knockout mice.

There is no expression of NgR protein after NgR knockout (group A, Fig. 1, lane 2), while expression of NgR protein can be seen in the control group (group B, Fig. 1, lane 1).

Axon of positive GAP-43 expression stained with green fluorescence distributes along the longitudinal axis of optic nerve. GAP-43

Fig. 1. No expression of NgR protein after knockout of NgR gene (lane 2). Expression of NgR protein can be seen in C 57BL/6 control mice (lane 1).
expression of optic nerve increased significantly with the survival time.

The number of axonal length ≥ 1 mm from the crush position in experimental group is $816.12 \pm 1.23$ after optic nerve crush for seven days. The number of axonal length ≥ 1 mm from the crush position in control group is $12.26 \pm 1.35$. There was a significant difference in axonal length ≥ 1 mm from the crush position that stained positively for GAP-43 on the ON sections between experimental and control group ($P = 0.006$). The crush position of ON of mice can be investigated under normal light microscopy.

Adherence of RGCs began at 12 h after seeded into poly-lysine-coated 24-well plates then incubated at 37°C 5% CO$_2$ ventilation, and finished in 24 h. After this, a single layer of round and oval RGCs was seen on the plates.

For neurite length determinations, the neurites were traced and the morphometry analysis was performed using Image-Pro Plus. The length of the axon was defined as the distance from the soma to the tip of the process. An average of 50 neurons from each group was selected from a number of chamber slides. Axonal length of the experimental group was longer in the third day ($P = 0.006$) than that of the control group ($P = 0.08$) on the first postoperative day. While significant difference was observed in axonal length by the third and seventh days between the experimental and control groups ($P = 0.005$).

Significant corticospinal tract fiber sprouting in young Nogo-A/B−/− mice has been observed by Kim et al. [9], Simonen et al. [19] investigated corticospinal tract fiber regeneration in Nogo-A−/− mice. However, Zheng et al. [24] found no significant regeneration in either Nogo-A/B−/− line or Nogo-A/B(C−/−) line mice. Our results showed that knockdown of Nogo-A or Nogo-A/B/C can effectively improve axonal regeneration of ON after crush [20].

Kim et al. [10] reported that NgR-deficiency improves the raphe spinal and rubrospinal axon regeneration but not that of corticospinal neurons. Zheng et al. [23] also failed to observe any improvement of NgR knockout mice in terms of corticospinal regeneration compared with wild-type controls. In several ways, the regenerative phenotypes in NgR knockout appear to be inferior to that promoted by NEP1-40 treatment.

NgR expresses in RGC in retina. We detected the expression level of NgR by ISH and Western blot, and found that there is no expression of NgR protein after knockout of NgR gene, while the expression
of NgR was detected in the control group. There is significant difference between the experimental and control group. Our results indicate that it is possible to knockout NgR in retina. We confirmed that NgR can be successfully knockeddown. Knockdown of NgR makes it possible to study the effect of NgR on axonal regeneration of RGCs in vivo and in vitro.

GAP-43 expression in growing cone is a symbol of regenerated axon. We did detect the expression of GAP-43 in RGC after culturing for seven days in both experimental and control group. Our data indicate that knockout of NgR can effectively enhance axonal regeneration of RGCs in vivo. So RGCs have instinctive competence of axonal regeneration in vivo. Axonal length of experimental group increased from the first day to the seventh day, but the axonal length of the RGCs in control group grows slowly. The difference in axonal lengths of RGCs between the experimental and control group was significant either at the third day or the seventh day. Extinguishing of NgR enhances axonal regeneration of RGCs in vitro.

To sum up, our data suggest that both NgR and Nogo play important roles in inhibiting axonal regeneration of RGCs. Finally, in view of the growing interest in NgR as a target for drug development in optic nerve regeneration, it is possible that our observation will help to promote going efforts to further elucidate the role of molecular mediators of optic nerve outgrowth inhibition.

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