A novel function of neuroglobin for neuroregeneration in mice after optic nerve injury

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Highlights

・A novel function of Ngb for neuroregeneration.

・Mammalian Ngb has neuroprotective and neuroregenerative properties.

・Ngb injection into mouse eye promotes cell survival and axonal regeneration *in vivo*.

A novel function of neuroglobin for neuroregeneration in mice after optic nerve injury

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Abbreviations : Ngb, Neuroglobin; c-Ngb, chimeric Ngb; h-Ngb, human Ngb; RAGs, regeneration-associated genes; RGC, retinal ganglion cell; CNS, central nervous system; GCL, ganglion cell layer; GDI, guanine nucleotide dissociation inhibitor; NO, nitrite oxide; GAP43, growth associated protein 43

Running title: A neuroregenerative role for neuroglobin in the mouse optic nerve

Abstract

Neuroglobin (Ngb) is a recently discovered heme protein in the vertebrate brain that can bind to oxygen molecules. Mammalian Ngb plays a crucial role in neuroprotection under conditions of oxidative stress. To investigate other potential functions of Ngb, we investigated the mouse retinal Ngb system following optic nerve injury. In the retina of control mice, Ngb immunoreactivity was limited to the retinal ganglion cell (RGC) layer, and this immunoreactivity rapidly decreased to less than 50% of the control level 5 days after optic nerve injury. On the basis of this decrease, we designed *in vivo* experiments with enhanced expression of Ngb using adult mouse retina. The enhanced expression of Ngb was achieved by injecting chimeric human Ngb protein, which included the cell membrane-penetrating module of fish Ngb. One-day pretreatment with chimeric Ngb increased immunoreactivity levels of Ngb two-fold in mouse RGCs and increased the number of surviving RGCs three-fold by 14 days after optic nerve injury compared with vehicle controls. Furthermore, in the mouse retinas showing enhanced Ngb expression, several regenerating central optic axons exhibited outgrowth and were found to pass through the nerve crush site 14 days after nerve injury. No such regenerating optic axons were observed in the control mouse optic nerve during the same time frame. The data obtained from *in vivo* experiments strongly indicate that mammalian Ngb has neuroprotective and neuroregenerative properties.

Keywords

Neuroglobin; neuroprotection; neuroregeneration; mouse; retina; optic nerve injury

1. Introduction

Neuroglobin (Ngb) is the third type of heme protein to be discovered after hemoglobin and myoglobin. It was originally identified in the mammalian brain and has the ability to bind to oxygen (O_2) molecules [1]. Because of the low concentration of Ngb in brain tissue, Ngb likely does not serve as an O_2 carrier; rather, it has neuroprotective properties under conditions of oxidative stress, such as those caused by ischemia, reperfusion *in vitro* [2, 3], and reperfusion *in vivo* [4]. Although Ngb was initially identified in mammalian species, it is also present in non-mammalian vertebrate species such as the zebrafish [5, 6]. Human and zebrafish Ngb proteins share about 50% of their amino acid sequence identity, and their associated genes comprise four exons interrupted by three introns [5−7].

Mammalian Ngb displays guanine nucleotide dissociation inhibitor (GDI) activity that is tightly correlated with its neuroprotective properties [8−12]. In contrast, zebrafish Ngb does not show similar GDI activity but instead presents a cell membrane-penetrating module [13−16]. Given these distinct properties, we constructed chimeric human Ngb by replacing a human module with a zebrafish cell membrane-penetrating module to produce a recombinant cell membrane-penetrating protein of human Ngb [14, 16].

Over the past two decades, our laboratory has identified many nerve regeneration-associated genes (RAGs) in the fish visual system. Generally, fish central nervous system (CNS) neurons can regenerate even after nerve transection, whereas mammalian CNS neurons cannot [17−19]. We have previously cloned RAGs from the axotomized zebrafish visual system, including the retina, and found that they are largely classified into two groups: one that displays anti-oxidant or anti-apoptotic activity and another that displays neurite outgrowth activity [19−26]. The former type can be induced at an early stage of nerve regeneration immediately after optic nerve injury, whereas the latter can typically be induced at a later stage of nerve regeneration [19]. Moreover, we have compared

the behavior of zebrafish and mammalian homolog RAGs after optic nerve injury. Interestingly, although RAG genes in mice are maximally expressed in the normal retina, expression rapidly decreases immediately after optic nerve injury, occasionally completely disappearing. In a previous study, we found that Ngb mRNA and Ngb proteins were transiently upregulated in the zebrafish retina 3 days after optic nerve injury, only to return to control levels 7 days later [16]. *In vitro* studies have shown that overexpression of mouse Ngb protein enhances cell viability under hypoxia / reoxygenation conditions [27]. Additionally, we have confirmed that chimeric human Ngb promotes neurite outgrowth activity in rat pheochromocytoma PC12 cells [28].

To investigate the neuroprotective and neuroregenerative properties of mammalian Ngb, we investigated the mouse retinal Ngb system both with and without chimeric cell membrane-penetrating human Ngb *in vivo*.

2. Materials and methods

2.1. Animals

All animals were maintained and handled in accordance with the Guiding Principles in the Care and Use of Animals and the Kanazawa University's guidelines for animal experiments. Male C57BL/6 mice (8–9 weeks old; Japan SLC, Inc., Shizuoka, Japan) were reared in clear plastic cages and kept under a 12 h light–dark cycle at 23°C. Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (30–40 mg/kg body weight). The optic nerve was crushed using forceps 1 mm posterior to the eyeball. The study was designed to minimize the number of experimental animals used and to minimize their pain and suffering.

2.2. Tissue preparation

At specific times following optic nerve injury, retinal and optic nerve samples were

prepared for histological analysis. Briefly, mouse eyes were enucleated under anesthesia, bisected and fixed in 4% paraformaldehyde solution containing 0.1 M phosphate buffer (pH 7.4) and 5% sucrose for 2 hours at 4°C. After infiltration with increasing concentrations of sucrose (5–20%), followed by an overnight incubation in 20% sucrose at 4°C, the tissues were embedded in an OCT compound (Sakura Fine technical, Tokyo, Japan), and sectioned at a thickness of 12 μm.

2.3. Immunohistochemistry

Following washing and blocking, retinal and optic nerve sections were incubated overnight at 4 °C with the following primary antibodies: rabbit anti-Ngb polyclonal antibody (1:250, Sigma-Aldrich, Saint Louis, MO, USA), mouse anti-Brn3a polyclonal antibody (1:400, Millipore Corporation, CA, USA), and sheep anti-GAP43 polyclonal antibody (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were then incubated with Alexa Fluor anti-IgG (1:1000, Molecular Probes, Eugene, OR, USA) at 23 °C. Positive fluorescent signals were detected using a fluorescence microscope (VB-7000, Keyence, Osaka, Japan).

2.4. Western blot analysis

Retinas were isolated and aliquots containing 30 μg of protein were subjected to polyacrylamide gel electrophoresis using a 15% gel as described previously [29]. The separated proteins were transferred to a nitrocellulose membrane and sequentially incubated with primary and secondary antibodies. By using primary antibodies, signals for the Ngb protein bands could be detected using a BCIP/NBT kit (KPL, Gaithersburg, MD, USA). Rabbit anti β-actin (1:500, GeneTex, San Antonio, TX, USA) was used as an internal standard. Protein bands isolated from retinal samples were densitometrically analyzed using Scion

Image Software (Scion Corporation). All experiments were repeated at least three times.

2.5. Preparation of chimeric Ngb proteins

Human and zebrafish Ngb genes comprise four exons and each exon encodes compact protein structural 'modules': M1, M2, M3, and M4 [16]. Plasmids expressing chimeric ZHHH Ngb, in which module M1 of human Ngb (HHHH) was replaced with zebrafish Ngb (ZZZZ), were prepared as described previously [9, 15]. Transduction of chimeric Ngb protein was induced in *E. coli* strain BL 21 (DE 3) after treatment with isopropyl-β-D-thiogalactopyranoside, and each Ngb protein was purified as described previously [8, 9, 30, 31] and briefly described in supplemental methods .

2.6. Intraocular injection of chimeric Ngb into mouse eye

To assess the neuroprotection properties of Ngb, an eye injection was performed as previously described [32]. We intraocularly injected 2 μL of recombinant Ngb protein with a final concentration of 5 μM using a 30-gauge needle coupled to a Hamilton microsyringe. One day after the injection of Ngb, the optic nerve was crushed as described above. As a control for each experiment, we also injected PBS alone (vehicle solution).

2.7. Surviving RGCs counts in whole-mounted retina

RGC survival was evaluated in flat-mounted retinas with immunohistochemistry using a mouse antibody against TUJ1 (1:500, R&D Systems, Inc., Minneapolis, USA), followed by a fluorescent secondary antibody 14 days after the injury. Images of eight pre-specified retinal areas 3 mm from the optic disc were captured by fluorescent microscopy (under ×200 magnification; E600, Nikon), and positive cells were counted using Image J software (Wayne Rasband, NIH, Bethesda, MD). Cell densities were calculated as mean values of surviving

RGCs per 0.14 mm^2 in the eight specified areas.

2.8. Quantitation of optic nerve regeneration in vivo

Optic nerves samples embedded in OCT compound were cut into longitudinal sections of 14 μm thickness. Regenerating optic axons were visualized by staining with mouse anti-GAP43 antibody (1∶250, Santa Cruz Biotechnology, Santa Cruz, CA) followed by a fluorescently labeled secondary antibody and captured by fluorescent microscopy (BZ-9000, Keyence, Osaka, Japan). Axons were counted manually in at least eight sections per conditions (six mice in each treatment group) at 200 μm and 400 μm away from the injury site. The numbers of regenerating axons were counted as described by Leon et al [33].

2.9. Statistics

All results are reported as the mean ± SEM, and each result included data from 3–5 experiments. Differences between groups were analyzed using one-way ANOVAs, followed by Dunnett's multiple comparison test with PASW Software (SPSS Inc., Chicago, IL, USA). P values < 0.05 were considered statistically significant.

3. Results

3.1. Reduction of Ngb in mouse retina after optic nerve injury

←Fig. 1

Ngb is specifically expressed in the vertebrate CNS and peripheral nervous systems, with the retina having a 100-fold higher concentration than other CNS tissues [34].

To characterize how Ngb concentration is altered in mouse retina following optic nerve injury, we performed Ngb immunohistochemical staining at various times after nerve injury (Fig. 1). Ngb was localized in all nuclear layers, especially in the ganglion cell layer (GCL) in control mice retina (Fig. 1A, 0 d). These Ngb signals in the GCL were additionally stained with anti-Brn3a, which is a protein marker of RGCs (Fig. 1A). Ngb immunoreactivity in RGCs rapidly decreased 1 day after nerve injury, and was less than 50% of control values (0 d) by 5 days after injury (Fig. 1A, B). Although western blot analysis showed that Ngb protein levels slightly increased 1 day after optic nerve injury, they were then found to decrease 2 days later, followed by a further decrease by 5 days after optic nerve injury (Fig. 1C, D).

3.2. Injection of recombinant Ngb protein into mouse eye promotes RGC survival after optic nerve injury

←Fig. 2

Next, we investigated the effects of Ngb introduction and the survival rate of RGCs with chimeric human Ngb after optic nerve injury *in vivo*. Recombinant chimeric Ngb (c-Ngb) was used to enhance the expression of Ngb via intraocular injections into mouse eyes 24 hours before optic nerve injury. Based on data from *in vitro* studies [16, 28], the concentration of c-Ngb was 5 μM in these *in vivo* studies. c-Ngb has a cell membrane-penetrating module obtained from the zebrafish, as opposed to the standard mouse Ngb corresponding module. Twenty-four hours after c-Ngb injection, almost all RGCs showed Ngb signals (Fig. 2A), and

eventually produced Ngb signal levels approximately twice the levels observed in controls (Fig. 2B). Thus, the intraocular injection of c-Ngb unequivocally increased Ngb expression in RGCs 1 day after injection (Figs. 2A, B)

We then compared the cell survival rate of RGCs with or without enhanced Ngb expression (Fig. 2C, D). Fig. 2C shows immunofluorescent staining of flat-mounted retinas using TUJ1 antibody, a marker of surviving RGCs [35]. Optic nerve injury drastically reduced the number of surviving RGCs compared with the control (vehicle only), as measured 2 weeks after injury (Fig. 2C, D). The enhanced expression of Ngb rescued many RGCs from cell death after optic nerve injury (Fig. 2C, D). Fourteen days following injury, the number of surviving RGCs with enhanced expression of Ngb was three times larger than RGCs without enhanced Ngb expression (Fig. 2D).

3.3. Injection of recombinant Ngb protein into mouse eye promotes optic nerve regeneration after optic nerve injury

←Fig. 3

Finally, we tested the effects of Ngb on neuroregenerative activity after optic nerve injury. Intraocular injections of c-Ngb induced significant optic nerve regeneration *in vivo* (+c-Ngb, Fig. 3B) compared with the vehicle control condition (Fig. 3A), as revealed by GAP43 staining [36, 37]. Indeed, the +c-Ngb group showed many regenerating optic fibers passing through the crush site (asterisk, Fig. 3B). Fig. 3C illustrates the quantitative optic nerve regeneration data *in vivo* at 200 μm and 400 μm away from the optic nerve crush site. +c-Ngb groups show more than double the number of regenerating axons compared to the control at the same distances from the crush site.

4. Discussion

4.1. Comparative studies of the Ngb system in the mouse and zebrafish retina

Previously, we found that in the zebrafish retina, Ngb mRNA is expressed in amacrine cells, whereas Ngb proteins are expressed in both amacrine cells and the inner plexiform layer [16]. In the present study, we confirmed that Ngb proteins are only expressed in the RGCs of the mouse retina [27, 38]. The localization of Ngb proteins in the inner plexiform layer of the zebrafish is easily imaged due to the secretion of Ngb from the amacrine cells and the cell membrane-penetrating module of zebrafish Ngb. Moreover, optic nerve injury induces a transient increase in Ngb protein levels within amacrine cells and the inner plexiform layer of the zebrafish retina 3–5 days after axotomy [16, 27]. This period is considered to be the early stage of the zebrafish optic nerve regeneration process (the preparation period, [19]). However, in the present study we show that optic nerve injuries induce a rapid decrease in Ngb protein levels in mouse RGCs 3–5 days following nerve lesion (Fig. 1), and that immunoreactive RGCs gradually disappear during the following days. This opposing pattern of Ngb expression in the mouse and zebrafish retina following optic nerve injury suggests that Ngb is important for the survival of mouse RGCs. The secretion of Ngb from presynaptic amacrine cells in the zebrafish might help RGCs survive. This inter-species difference suggests that Ngb introduced into mouse RGCs can serve as a viable candidate for regeneration-associated genes (RAGs). Indeed, we have previously shown that the supplementation of RAGs derived from fish retina with transglutaminase [23] and retinoic acid metabolites [19, 39] successfully rescued RGCs and regenerated the optic axons of mammals. In the present study, the introduction of Ngb into mouse RGCs was performed using chimeric human Ngb possessing cell membrane-penetrating activity.

4.2. Neuroregenerative activity of mammalian Ngb in the mouse retina after optic nerve

injury

The injection of chimeric human Ngb into mouse eyes increased the levels of Ngb protein two-fold compared with vehicle controls, 1–2 days after injection (Fig. 2). This increase was accompanied by a comparable three-fold increase in the number of surviving RGCs in the mouse retina compared with vehicle controls, 14 days after optic nerve injury (Fig. 2C, D). Furthermore, several central regenerating optic axons displayed significant outgrowth beyond the crush site by more than 400 μm (Fig. 3B, C), whereas almost no regenerating fibers penetrated beyond the lesion site in the vehicle control group (Fig. 3A, C). Similar neuroprotective (cell survival) and neurite outgrowth activities have been observed in the mouse RGC-5 retinal cell line [27] pretreated with mammalian Ngb, and almost all PC12 cells treated with c-Ngb showed significant long neurites compared to controls [28]. Optic nerve injuries *in vivo* and hypoxia/reoxygenation insults *in vitro* are very different experimental procedures that can both affect RGCs. However, optic nerve injuries resulting from oxidative stress to RGCs can be achieved by glutamate toxicity via the NMDA receptor, nitrite oxide (NO) toxicity, and reactive oxygen species [40─43]. The molecular mechanism of Ngb in RGC protection and optic nerve regeneration remains unclear. However, Ngb may regulate the NO signaling pathway that contributes to cell survival during hypoxia [44─47] and optic nerve regeneration [19, 47]. Therefore, the fact that mouse RGCs overexpressed Ngb after it was introduced into the eye and those RGCs were protected for 14 days after optic nerve injury strongly suggests that Ngb plays a neuroprotective role in both hypoxia/reoxygenation and nerve injury. Furthermore, the present findings provide the first data to indicate that Ngb plays neuroregenerative roles as well as neuroprotective roles in injured mouse RGCs during the early stage of optic nerve regeneration.

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Figure Legends

Fig. 1

Reduction of Neuroglobin in mouse retina after optic nerve injury

(A) Immunohistochemical results of the mouse retina 0 (control, no injury), 1, 3, and 5 days after optic nerve injury. Decreased Ngb intensity in RGCs was observed as early as 1 day after the injury and became substantially evident 3–5 days after the nerve was lesioned. (B) Graphical representation of Ngb positive staining using immunofluorescent intensity. (C) Western blot analysis of Ngb in the retina for 0 (control), 1, 3 and 5 days after optic nerve injury. A small increase in Ngb levels was observed 1 day after optic nerve injury, which was followed by a 60 % decrease in Ngb protein levels three to 5 days after the optic nerve lesion, compared with controls. Each experiment was repeated three times. RGC, retinal ganglion cell. Scale bar = 50 μm. *$p<0.05$, **$p<0.01$ ANOVA Dunnett's test

Fig. 2

Recombinant Ngb protein promotes mouse RGC cell survival after *in vivo* optic nerve injury

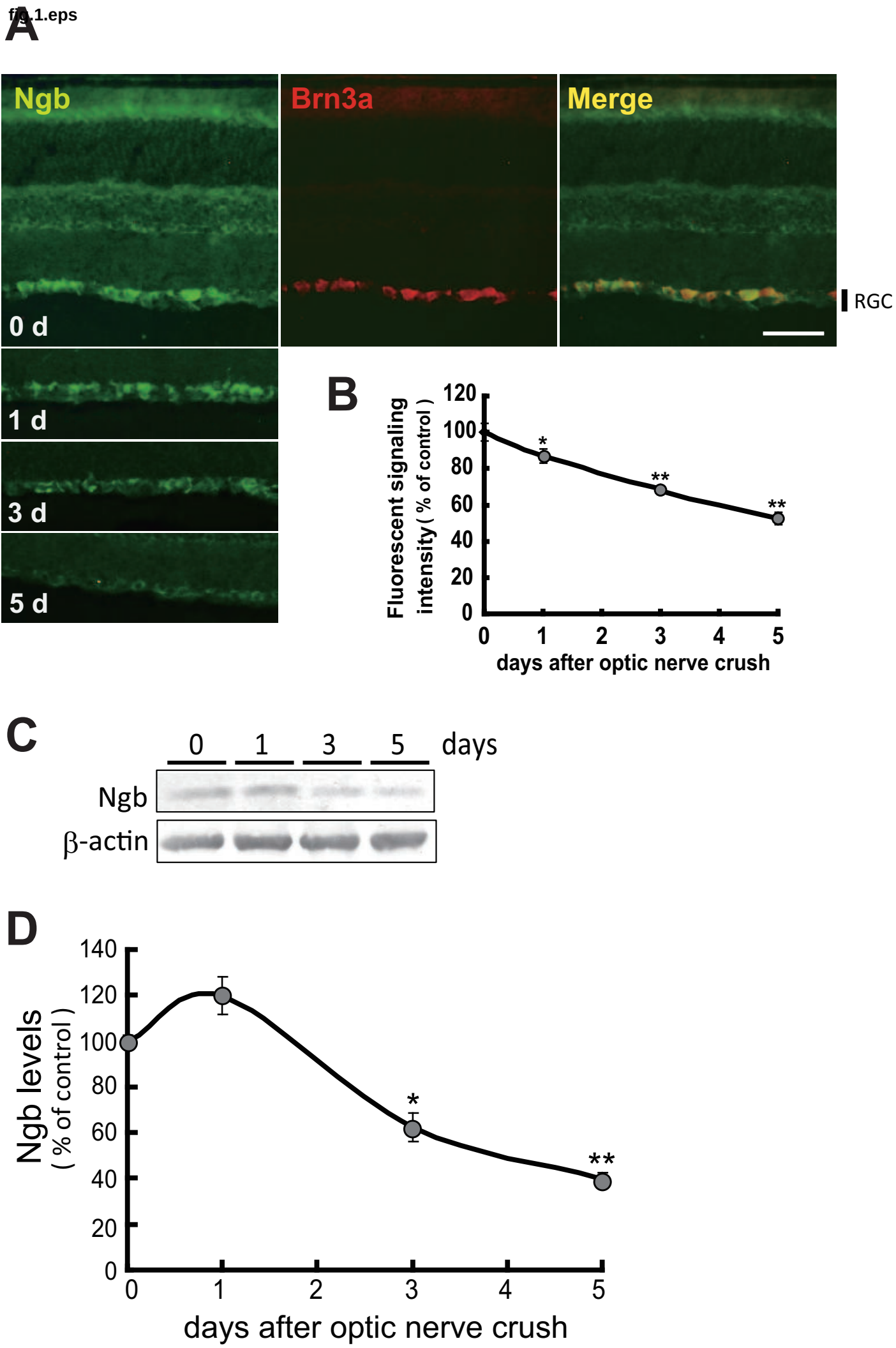
(A) Intraocular injection of chimeric Ngb (c-Ngb) in mouse retina (5 μM/eye) results in a high accumulation of Ngb proteins in RGCs as revealed by immunofluorescent intensity. (B) Ngb protein levels were approximately twice that observed in controls 24 hours after intraocular injection. (C) Surviving RGCs stained by TUJ1 in a whole-mounted retina. Control (no injury), injury (14 days after optic nerve injury) and +c-Ngb (injury plus c-Ngb). Scale bar = 50 μm. (D) Quantification of surviving RGCs 14 days after nerve injury with and without c-Ngb. *$P<0.01$ vs control, +$P<0.01$ vs injury.

Fig. 3

Recombinant Ngb induces axonal regeneration in mouse optic nerve after injury

Longitudinal sections of adult mouse optic nerve with GAP-43 immunoreactive positive staining. The regenerated optic axons extended centrally passing through lesion site (*) 2 weeks after optic nerve injury. (A) Vehicle control, (B) plus c-Ngb injection (5 μM/eye). Scale = 200 μm. (C) Quantification of axonal regrowth at two indicated proximal points from the injury site. *$P < 0.01$ vs injury plus c-Ngb injection (200 μm). +$P < 0.01$ vs injury plus c-Ngb injection (400 μm).

fig.1.eps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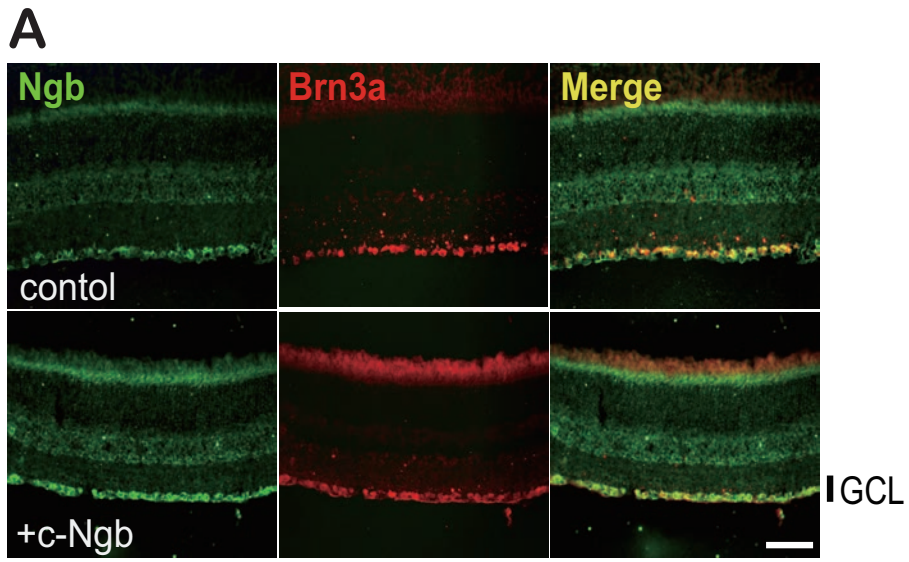
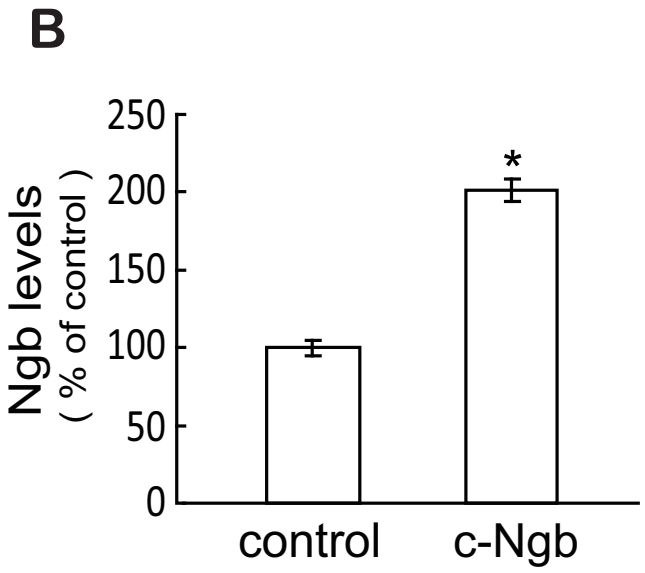
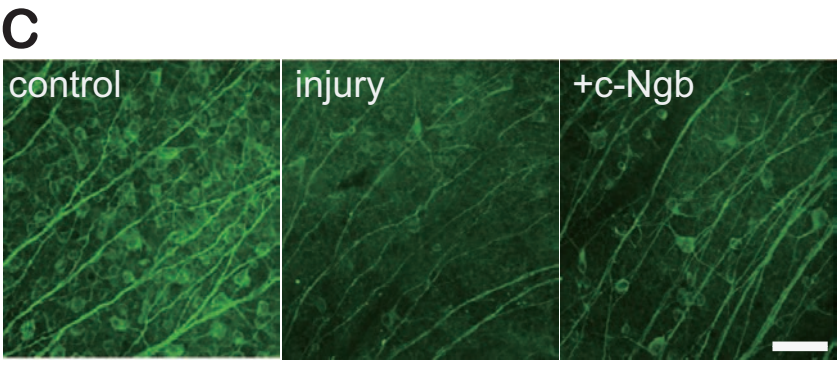
1 d
3 d
5 d
B
Fluorescent signaling intensity (% of control)
120
100
80
60
40
20
0
*
**
**
0
1
2
3
4
5
days after optic nerve crush
C
0
1
3
5
days
Ngb
β-actin
D
Ngb levels
(% of control)
140
120
100
80
60
40
20
0
*
**
0
1
2
3
4
5
days after optic nerve crush

fig.2.eps


A
Ngb
Brn3a
Merge
contol
+c-Ngb
GCL


B
Ngb levels
(% of control)
250
200
150
100
50
0
*
control
c-Ngb


C
control
injury
+c-Ngb

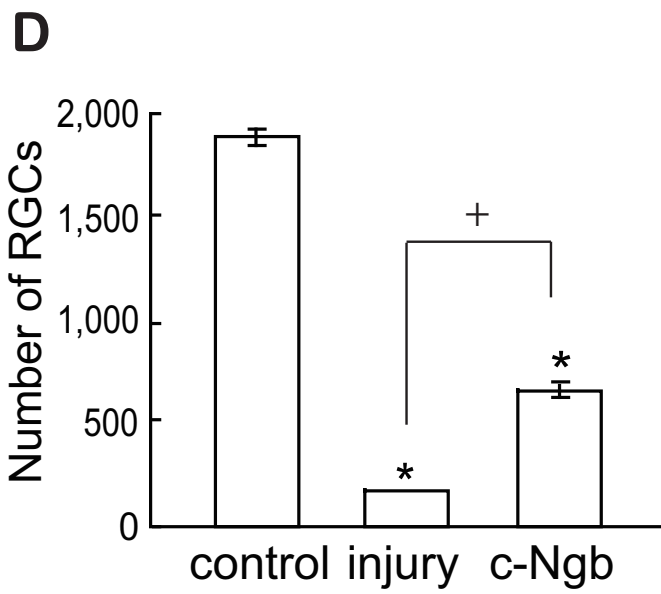
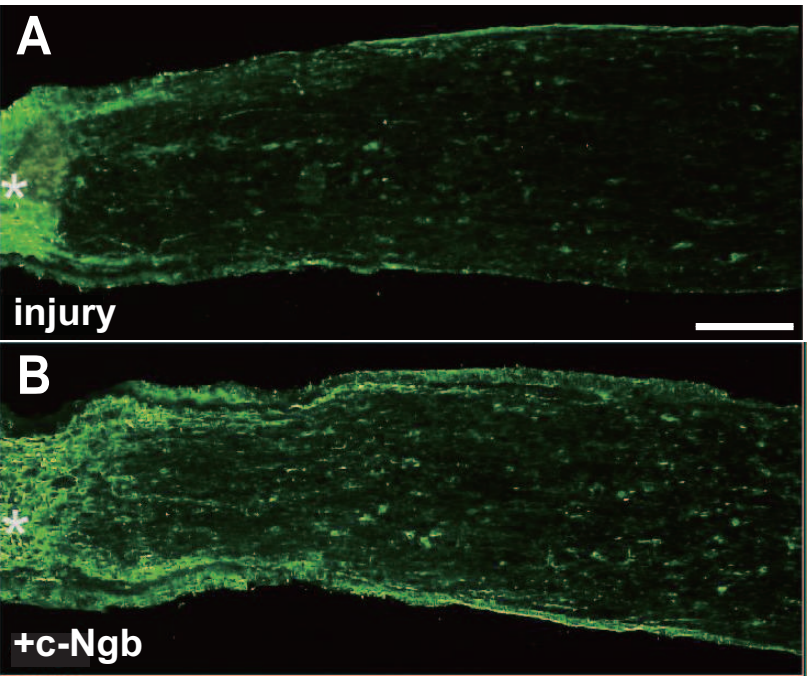
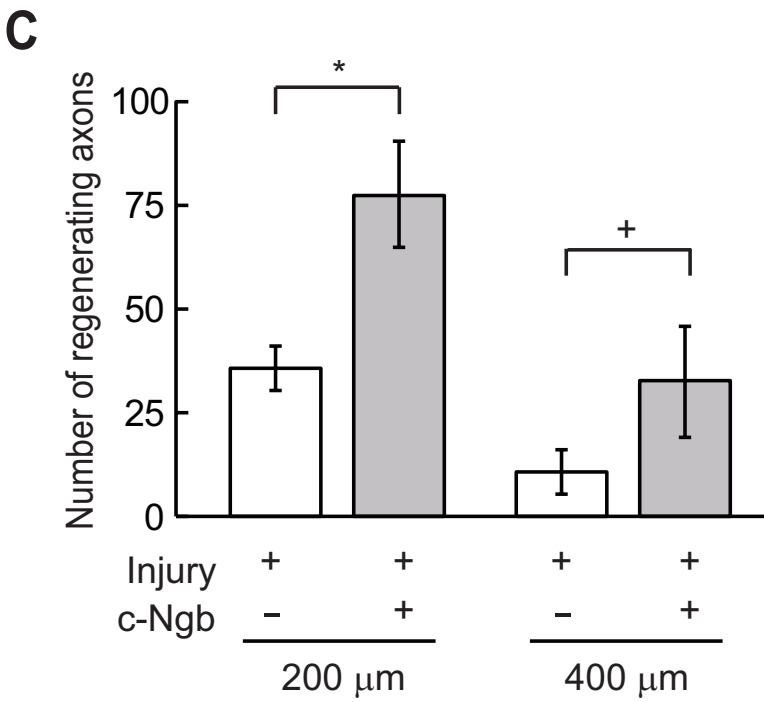

D
Number of RGCs
2,000
1,500
1,000
500
0
+
*
*
control
injury
c-Ngb

fig.3.eps


A
injury
B
+c-Ngb


C
Number of regenerating axons
100
75
50
25
0
*
+
Injury
c-Ngb
200 μm
400 μm

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