Role of neuritin in retinal ganglion cell death in adult mice following optic nerve injury

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Neuritin is a small extracellular protein that plays important roles in the process of neural development, synaptic plasticity, and neural cell survival. Here we investigated the function of neuritin in a mouse model of optic nerve injury (ONI). ONI induced upregulation of neuritin mRNA in the retina of WT mice. The retinal structure and the number of retinal ganglion cells (RGCs) were normal in adult neuritin knockout (KO) mice. In vivo retinal imaging and histopathological analyses demonstrated that RGC death and inner retinal degeneration following ONI were more severe in neuritin KO mice. Immunoblot analyses revealed that ONI-induced phosphorylation of Akt and ERK were suppressed in neuritin KO mice. Our findings suggest that neuritin has neuroprotective effects following ONI and may be useful for treatment of posttraumatic complication.

Results

Upregulation of neuritin in the retina following ONI. We first examined neuritin mRNA expression levels in the mouse retina before and after ONI. Quantitative real-time PCR analyses were carried out at 0, 3, 5, 10 and 15 days after ONI (Fig. 1A). Neuritin expression was normal at 3 days (106.7 ± 1.1%, n = 8; p = 0.307) and 5 days (108.5 ± 0.7%, n = 7; p = 0.157) after ONI, but significantly increased at 10 days (123.6 ± 5.0%, n = 6; p < 0.0001) and 15 days (122.6 ± 1.2%, n = 6; p < 0.0001) after ONI compared with normal mice (Fig. 1B). These results suggest that neuritin plays an important role in the retina following ONI.

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Accelerated retinal degeneration after ONI in neuritin KO mice. To examine the functions of neuritin, we next investigated the severity of ONI-induced retinal degeneration in WT and neuritin KO mice. We visualized retinal layers using spectral-domain optical coherence tomography (SD-OCT), a noninvasive imaging technique that is useful for monitoring the changes in retinal structures after injury in living animals. The SD-OCT images revealed that the mean thickness of the ganglion cell complex (GCC) in neuritin KO mice was significantly decreased compared with WT mice at 7 days after ONI (68.6 ± 0.9 μm vs 74.9 ± 0.3 μm, n = 6; p < 0.001). A similar difference between neuritin KO and WT mice was found at 14 days after ONI (65.6 ± 0.9 μm vs 69.0 ± 0.8 μm, n = 6; p = 0.022) (Fig. 2).

We then examined histopathology of the retina at 7 and 14 days after ONI. The cell number in the ganglion cell layer (GCL) was decreased in both WT and neuritin KO mice following ONI, but the cell loss was more severe in neuritin KO mice compared with WT mice at 7 days (1046 ± 104 cells/mm² vs 71 ± 71 cells/mm², n = 6; p = 0.008) and 14 days (221 ± 5 cells/section vs 248 ± 3 cells/section, n = 6; p = 0.002) after ONI (Fig. 3A,B). In addition, the thickness of the inner retinal layer (IRL; between the internal limiting membrane and the interface of the outer plexiform layer and the outer nuclear layer) in neuritin KO mice was also decreased compared with WT mice at 7 days after ONI (91.5 ± 1.0 μm vs 96.4 ± 1.6 μm, n = 6; p = 0.025). A similar difference between neuritin KO and WT mice was also found at 14 days after ONI (84.1 ± 1.2 μm vs 89.6 ± 1.3 μm, n = 6; p = 0.010) (Fig. 3A,C).

Because GCL contains cell types other than RGCs including displaced amacrine cells, we next performed retrograde labeling of RGCs with Fluoro-Gold (FG) and determined the effect of neuritin on RGC survival. Consistent with the results of cell counting in the GCL (Fig. 3B), the RGC number was decreased in neuritin KO mice compared with WT mice at both 7 days (1046 ± 74 cells/mm² vs 1441 ± 142 cells/mm², n = 6; p = 0.033) and 14 days (790 ± 64 cells/mm² vs 1018 ± 71 cells/mm², n = 6; p = 0.037) after ONI in the central retina (Fig. 4A,B). In addition, the RGC number was significantly decreased in neuritin KO mice compared with WT mice at 7 days (900 ± 62 cells/mm² vs 1301 ± 104 cells/mm², n = 6; p = 0.008) and 14 days (724 ± 72 cells/mm² vs 949 ± 58 cells/mm², n = 6; p = 0.034) after ONI in the middle retina (Fig. 4C). In the peripheral retina, a similar difference between neuritin KO and WT mice was observed at 7 days (843 ± 64 cells/mm² vs 1276 ± 72 cells/mm², n = 6; p = 0.001) and 14 days (624 ± 47 cells/mm² vs 786 ± 29 cells/mm², n = 6; p = 0.015) after ONI (Fig. 4D). Taken together, these results suggest that neuritin slows the process of RGC loss all across the retina and retinal degeneration following ONI.

Effects of neuritin on cell survival signaling in the retina after ONI. We also investigated if neuritin has any effects on stimulation of cell survival signaling pathways in the retina following ONI. Previous studies have reported that Akt activation promotes RGC survival after ONI and activation of the ERK signaling pathway leads to RGC protection in glaucomatous eyes. We therefore examined the effects of neuritin on ONI-induced activation of the Akt and ERK signaling. Immunoblot analysis revealed that ONI induces expression of phosphorylated (activated) Akt in WT mice (200.1 ± 26.4%, n = 8; p = 0.004), but not in neuritin KO mice (119.4 ± 26.0% vs 104.2 ± 12.0%, n = 6; p = 0.015) (Fig. 5A,B). In contrast, neuritin significantly increased the phosphorylation of ERK2 in both WT and neuritin KO mice following ONI (Fig. 5C,D). These results indicate that neuritin protects RGCs by promoting Akt activation and ERK2 phosphorylation in the retina after ONI.

Figure 1. Expression levels of neuritin mRNA after ONI in WT mice. (A) Experimental timeline. (B) mRNA expression levels of neuritin in whole retinas at 0, 3, 5, 10 and 15 days after ONI was determined using quantitative real-time PCR analysis. The result is expressed as a percentage of the normal WT mice. Data are presented as means ± S.E.M. n = 8 at 0, 3 days after ONI, n = 7 at 5 days after ONI, n = 6 at 10, 15 days after ONI. **p < 0.01.
20.1%, $n = 8; p = 0.453$) (Fig. 5A, B). ONI also activated the ERK signaling in WT mice (191.3 ± 25.6%, $n = 8; p = 0.008$), but not in neuritin KO mice (99.6 ± 17.9%, $n = 8; p = 0.370$) (Fig. 5A, C). These results suggest that neuritin is associated with activation of Akt- and ERK-mediated cell survival signaling.

**Discussion**

In this study, we reported that loss of neuritin accelerates RGC loss and retinal degeneration in adult mice following ONI. We also demonstrated that ONI-induced activation of Akt and ERK, which mediate pro-survival signaling in RGCs, were inhibited in neuritin KO mice. Previous studies have reported that neuritin exerts neuroprotective effects by activating the insulin receptor signaling pathway. The insulin receptor is expressed in various cell types in the retina, and insulin and its receptor system may play a critical role in maintaining neuronal function and survival in the retina by activating Akt and ERK. In addition, insulin-like growth factor-1 (IGF-1), an important factor in retinal development, prevents ONI-induced RGC death. Thus, neuritin-insulin receptor-Akt and -ERK pathways in RGCs may be valid therapeutic targets for treatment of post-traumatic complication and glaucoma.

Previous studies have shown that neurotrophins, such as BDNF and neurotrophin-3 (NT-3), and their receptors are involved in the development of visual systems and protection of retinal neurons from various types of injury. Interestingly, neuritin expression is induced by neuronal activity and by the activity-regulated neurotrophins BDNF and NT-3. A recent study reported that the soluble form of neuritin was secreted from astrocytes in response to an ischemic insult and this could ameliorate the recovery of the ischemia-injured hippocampal neurons. In addition, transcorneal electrical stimulation rescues the axotomized RGCs by increasing IGF-1 production in Müller glial cells. Since neuritin and IGF-1 are soluble secreted proteins that can bind to the insulin receptor, in response to neural activity and neurotrophins, neuritin and other trophic factors may be secreted from various cell types and stimulate RGC survival via autocrine and paracrine manners. A recent study supported neuroprotective effects of neuritin by reporting that adeno-associated virus (AAV)-mediated overexpression of neuritin delayed RGC apoptosis, regenerated injured axons, and maintained RGC function following ONI. Similar protective effects of neuritin was reported in a rat model of sciatic nerve injury.

Our present study demonstrated that ONI induces upregulation of *neuritin* mRNA in the retina of C57BL/6 J mice at 10 and 15 days after ONI. On the other hand, in BALB/cj mice, *neuritin* mRNA displayed a biphasic level of expression with significantly decreased expression from basal levels at 3 and 21 days after ONI and modestly decreased expression at 14 days after ONI. In a rat model of spinal cord injury, *neuritin* mRNA showed significantly reduced expression at 1 day, with subsequent expression recovery between 7 and 14 days after spinal cord injury. The discrepancy may be due to differences in experimental animals, injuries and time points.

We recently reported that some existing drugs are useful for RGC protection. For example, valproic acid (VPA), one of the HDAC inhibitors, protects RGCs from glutamate neurotoxicity and in a mouse model of normal tension glaucoma. VPA is also effective for RGC protection after ONI. Interestingly, VPA stimulates productions of nerve growth factor and BDNF in cultured Müller glial cells. These results suggest that VPA may induce neuritin expression by stimulating productions of neurotrophins. Although further in vivo studies are...
required, our findings raise intriguing possibilities for the management of ONI and RGC degeneration by existing drugs such as oral VPA in combination with local application of exogenous neurotrophins and neuritin.

**Methods**

**Mice.** Experiments were performed using C57BL/6J mice (CLEA Japan, Tokyo, Japan) or neuritin KO mice (Nrn1tm1.2Ndiv: stock # 018402, Jackson Laboratory, Bar Harbor, ME, USA) in accordance with the Tokyo Metropolitan Institute of Medical Science Guidelines for the Care and Use of Animals. Light intensity inside the cages ranged from 100 to 200 lux and a 12 hours light/12 hours dark cycle was maintained. All experiments were approved by the Tokyo Metropolitan Institute of Medical Science. Neuritin gene KO was confirmed by PCR genotyping of mouse tail DNA according to the protocol provided by Jackson Laboratories, which was based on a previous report. A WT forward primer (5′-GTGCCAGCCTCAATCTGGCAGCTGATCC-3′), a neuritin KO forward primer (5′-GCCGTTGTGGTCTTCCAAAGACC-3′), and a common reverse primer (5′-CGGGTTTTCAAAAATAATGAGGAC-3′) were used (Supplementary Figure 1). To further confirm the deletion, another PCR of tail genomic DNA was performed. For the neuritin exon 2 amplification, a forward primer (5′-GTGCAGTAGTGCGACAGTGGCAGGTTGATG-3′) and a reverse primer (5′-AAGGGAAAACCAGGTCAGAGAGGAGCCTTT-3′) were used. For glyceraldehyde-3-phosphate dehydrogenase (gpdh) control amplification, a forward primer (5′-TGCACCACCACTGCTTAG-3′) and a reverse primer (5′-GGATGCAGGATGATTTCT-3′) were used (Supplementary Figure 2).

![Figure 3. Accelerated retinal degeneration after ONI in neuritin KO mice.](image-url)
Retrograde RGC labeling and optic nerve injury. Mice were deeply anesthetized with isoflurane (Intervet, Tokyo, Japan), placed on stereotaxic frame, and received an injection of 2 µL FG (1% in phosphate-buffered saline; Fluorochrome LLC, Denver, CO, USA) into the superior colliculus. At 10 days after FG application, mice were anesthetized by intraperitoneal injection of sodium pentobarbital (87.5 mg/kg) before subjected to an ONI procedure. Optic nerves were exposed intraorbitally and crushed at about 0.5 to 1.0 mm from the posterior pole of the eyeball with fine surgical forceps for 5-10 s. On 7 and 14 days after ONI, mice were killed by cervical dislocation, eyes were enucleated, and retinas were isolated for whole mount preparation. Retinas were fixed in Zamboni’s fixative (2% paraformaldehyde and 15% picric acid in 0.1 M phosphate buffer) for 20 min, mounted on a slide glass with a mounting medium (Vectashield; Vector Laboratories Inc., Burlingame, CA, USA), and the RGC density was examined with a fluorescent microscope. Six standard areas (0.09 mm²) were selected from each retina as follows: one was from the central area (0.1 mm from the optic disc), two were from the middle area (0.8 mm from the optic disc), three were from the peripheral area (1.5 mm from the optic disc). FG-labeled cells were counted, and the mean number of RGCs per square millimeter was calculated.

Figure 4. Accelerated RGC loss after ONI in neuritin KO mice. (A) Representative images of retrograde-labeled RGCs from the middle areas of the retinas in WT and neuritin KO mice at 0, 7, 14 days after ONI. Scale bar: 100 µm. (B–D) Quantification of FG-labeled RGCs in the central (B), middle (C) and peripheral (D) area. Data are presented as means ± S.E.M. n = 6 per group. *p < 0.05, **p < 0.01.
Quantitative real-time PCR. Quantitative real-time PCR was performed using an MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with a THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) as described previously 24,33. Total RNA for PCR was prepared from whole retinas from six to eight different eyes at 0, 3, 5, 10 and 15 days after ONI. Complementary DNA reverse transcribed from total RNA was amplified by using primers specific for neuritin (sense: 5′-TCT TAC GGA TTG CCA GGA AG-3′, antisense: 5′-GCT AAA GCT GCC GAG AGA GA-3′) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; sense: 5′-TGC ACC ACC AAC TGC TTA G-3′, antisense: 5′-GGA TGC AGG GAT GAT GTT C-3′). Data were normalized to the level of GAPDH mRNA.

Imaging acquisition of SD-OCT. Mice were anesthetized by intraperitoneal injection of sodium pentobarbital and SD-OCT (RS-3000; Nidek, Aichi, Japan) examinations were performed at 0, 7, 14 days after ONI 9,23,25. To get fundus imaging, polymethyl methacrylate contact lenses optimal for mice (UNICON, Osaka, Japan) were placed on the corneas for prevention of anesthesia-induced cataract progression. A 60-D adaptor lens was placed on the objective lens of the Multiline OCT to focus on the mouse retina. All the images were location matched, scanning vertically through the center of the optic nerve head at 3-disk diameter lengths above the optic nerve head. The mean thickness of the GCC, between the internal limiting membrane and the interface of the inner plexiform layer and the inner nuclear layer, was measured. In this study, the maximum number of B-scans set by the manufacturer (50 for line scans) was used for averaging.

Histological and morphometric studies. At 0, 7, 14 days after ONI, mice were anesthetized with an intraperitoneal injection of sodium pentobarbital and perfused transcardially with saline, followed by Zamboni’s fixative. Eyes were removed and postfixed in 3% glutaraldehyde solution (3% glutaraldehyde, 9% formaldehyde, 37.5% ethanol, and 12.5% acetic acid in distilled water) for 2h. Paraffin embedded retinal sections of 7µm thickness were cut through the optic nerve and stained with hematoxylin and eosin. The extent of retinal degeneration was quantified in two ways48,49. First, the number of neurons in the GCL was counted from one ora serrata through the optic nerve to the other ora serrata. Second, in the same sections, the thickness of the IRL was measured.

Immunoblot analysis. Immunoblotting was performed for whole retina protein extracts from eight different eyes at 0 and 3 days after ONI, as described previously 23,50. Membranes were incubated with an antibody against Akt (1:1000; Cell Signaling Technology, Beverly, MA, USA), phospho-Akt (1:1000; Cell Signaling), ERK
(1:1000; Cell Signaling) or phospho-ERK (1:1000; BD Biosciences, Franklin Lakes, NJ, USA). Primary antibodies binding was detected using horseradish peroxidase-linked anti-mouse IgG or anti-rabbit IgG secondary antibodies (1:1000; Cell Signaling) and visualized by exposing to X-ray film (Advanta, Menlo Park, CA, USA) for 1 min. with Chemi-Lumi One Ultra (Nacalai Tesque, Kyoto, Japan). The band intensities were quantified using the NIH Image program (Image 1.50c4; NIH, Bethesda, MD, USA).

**Statistics.** For statistical analysis, we used a two-tailed Student’s t-test for comparison of two groups or one-way ANOVA followed by Dunnett’s post hoc test for multiple comparisons, as appropriate. Data are presented as means ± S.E.M. P < 0.05 was regarded as statistically significant. JMP version 13.1.0 (SAS Institute Inc., Cary, NC, USA) was used for the statistical analyses.

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Author Contributions
Y.A., K.N., K.Y. and T.H. designed the experiments and wrote the paper. Y.A., K.N., T.S., X.G., A.K., C.H. and A.S. organised or conducted the experiments and acquired data. All authors analysed data and reviewed the manuscript.

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