

SCIENTIFIC REPORTS

OPEN

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

Yuriko Azuchi^{1,2}, Kazuhiko Namekata^{1,2}, Tadayuki Shimada³, Xiaoli Guo¹, Atsuko Kimura¹, Chikako Harada¹, Atsuko Saito², Kanato Yamagata³ & Takayuki Harada¹

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.

Traumatic optic neuropathy is a common clinical problem that occurs in 0.5–5% of patients with closed head injury¹. A damage to the optic nerve induces secondary swelling within the optic canal, accompanied by subsequent retinal ganglion cell (RGC) loss and optic nerve atrophy². Although effective treatments are not established, previous studies have shown that neurotrophins, such as brain-derived neurotrophic factor (BDNF), protect RGCs in animal models of optic nerve injury (ONI)^{3–5}. In addition, suppression of glutamate neurotoxicity, neuroinflammation, oxidative stress and histone deacetylases (HDACs) may be effective for RGC protection^{6–11}. Since the ONI model mimics some aspects of glaucoma, it is also a useful animal model for glaucoma¹¹.

Neuritin, also known as candidate plasticity gene 15 (CPG15), was first identified as one of the activity-dependent gene products in the brain¹². Neuritin is an extracellular, glycosylphosphoinositide-linked protein, which can be secreted as a soluble form by various cells including neural and glial cells^{13–15}. Neuritin induces neuritogenesis, neurite arborization, neurite outgrowth and synapse formation, which are involved in the development and functions of the central nervous system^{15–18}. Loss of neuritin delayed development of the neuropil, including RGC axons and lateral geniculate nucleus, but these deficits were overcome in adult mice¹⁵. In addition, neuritin is recently thought to be a kind of neurotrophin that regulates neural survival¹⁹. Exposure of rat cerebellar granule neurons to neuritin markedly induced phosphorylation of Akt, ERK and mammalian target of rapamycin, in part by activating the insulin receptor signaling pathway¹⁹. 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^{20,21}. Since the insulin receptor is expressed in the retina including RGCs²², in the present study, we examined the effects of ONI on retinal degeneration in neuritin knockout (KO) mice.

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 ($106.7 \pm 1.1\%$, $n = 8$; $p = 0.307$) and 5 days ($108.5 \pm 0.7\%$, $n = 7$; $p = 0.157$) after ONI, but significantly increased at 10 days ($123.6 \pm 5.0\%$, $n = 6$; $p < 0.0001$) and 15 days ($122.6 \pm 1.2\%$, $n = 6$; $p < 0.0001$) after ONI compared with normal mice (Fig. 1B). These results suggest that neuritin plays an important role in the retina following ONI.

¹Visual Research Project, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan. ²Department of Environmental Science, Graduate School of Science, Toho University, Chiba, Japan. ³Synaptic Plasticity Project, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan. Correspondence and requests for materials should be addressed to K.N. (email: namekata-kz@igakuken.or.jp)

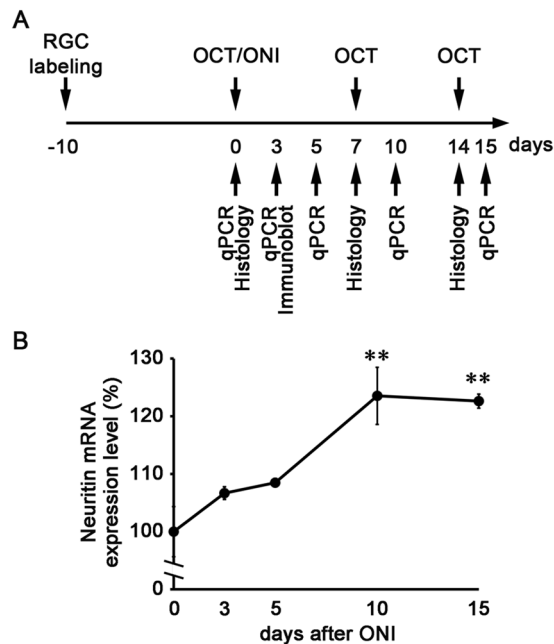


Figure 1. Expression levels of *neurtin* mRNA after ONI in WT mice. **(A)** Experimental timeline. **(B)** mRNA expression levels of *neurtin* in whole retinas at 0, 3, 5, 10 and 15 days after ONI was determined using quantitative real-time PCR analysis. The result is expressed as a percentage of the normal WT mice. Data are presented as means \pm 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. ** $p < 0.01$.

Accelerated retinal degeneration after ONI in neurtin KO mice. To examine the functions of neurtin, we next investigated the severity of ONI-induced retinal degeneration in WT and neurtin 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^{9,23–25}. The SD-OCT images revealed that the mean thickness of the ganglion cell complex (GCC) in neurtin KO mice was significantly decreased compared with WT mice at 7 days after ONI ($68.6 \pm 0.9 \mu\text{m}$ vs $74.9 \pm 0.3 \mu\text{m}$, $n = 6$; $p < 0.001$). A similar difference between neurtin KO and WT mice was found at 14 days after ONI ($65.6 \pm 0.9 \mu\text{m}$ vs $69.0 \pm 0.8 \mu\text{m}$, $n = 6$; $p = 0.022$) (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 neurtin KO mice following ONI, but the cell loss was more severe in neurtin KO mice compared with WT mice at 7 days (329 ± 6 cells/section vs 377 ± 13 cells/section, $n = 6$; $p = 0.008$) 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 neurtin KO mice was also decreased compared with WT mice at 7 days after ONI ($91.5 \pm 1.0 \mu\text{m}$ vs $96.4 \pm 1.6 \mu\text{m}$, $n = 6$; $p = 0.025$). A similar difference between neurtin KO and WT mice was also found at 14 days after ONI ($84.1 \pm 1.2 \mu\text{m}$ vs $89.6 \pm 1.3 \mu\text{m}$, $n = 6$; $p = 0.010$) (Fig. 3A,C).

Because GCL contains cell types other than RGCs including displaced amacrine cells²⁶, we next performed retrograde labeling of RGCs with Fluoro-Gold (FG) and determined the effect of neurtin on RGC survival. Consistent with the results of cell counting in the GCL (Fig. 3B), the RGC number was decreased in neurtin KO mice compared with WT mice at both 7 days (1046 ± 74 cells/ mm^2 vs 1441 ± 142 cells/ mm^2 , $n = 6$; $p = 0.033$) and 14 days (790 ± 64 cells/ mm^2 vs 1018 ± 71 cells/ mm^2 , $n = 6$; $p = 0.037$) after ONI in the central retina (Fig. 4A,B). In addition, the RGC number was significantly decreased in neurtin KO mice compared with WT mice at 7 days (900 ± 62 cells/ mm^2 vs 1301 ± 104 cells/ mm^2 , $n = 6$; $p = 0.008$) and 14 days (724 ± 72 cells/ mm^2 vs 949 ± 58 cells/ mm^2 , $n = 6$; $p = 0.034$) after ONI in the middle retina (Fig. 4C). In the peripheral retina, a similar difference between neurtin KO and WT mice was observed at 7 days (843 ± 64 cells/ mm^2 vs 1276 ± 72 cells/ mm^2 , $n = 6$; $p = 0.001$) and 14 days (624 ± 47 cells/ mm^2 vs 786 ± 29 cells/ mm^2 , $n = 6$; $p = 0.015$) after ONI (Fig. 4D). Taken together, these results suggest that neurtin slows the process of RGC loss all across the retina and retinal degeneration following ONI.

Effects of neurtin on cell survival signaling in the retina after ONI. We also investigated if neurtin 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^{20,21}. We therefore examined the effects of neurtin on ONI-induced activation of the Akt and ERK signaling. Immunoblot analysis revealed that ONI induces expression of phosphorylated (activated) Akt in WT mice ($200.1 \pm 26.4\%$, $n = 8$; $p = 0.004$), but not in neurtin KO mice ($119.4 \pm$

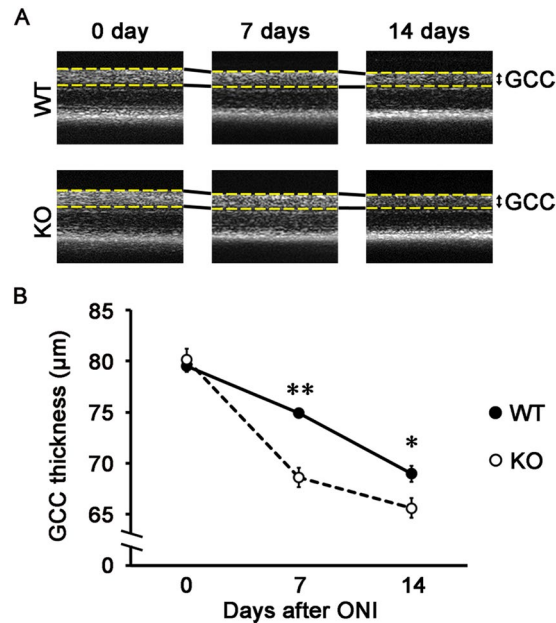


Figure 2. *In vivo* imaging of the retina in WT and neuritin KO mice. **(A)** Representative OCT cross-sectional images of retinas at 0, 7, 14 days after ONI in WT and neuritin KO mice. The dotted yellow lines indicate the ganglion cell complex (GCC). **(B)** Corresponding longitudinal evaluation of the GCC thickness. Data are presented as means \pm S.E.M. $n = 6$ per group. * $p < 0.05$, ** $p < 0.01$.

20.1%, $n = 8$; $p = 0.453$) (Fig. 5A,B). ONI also activated the ERK signaling in WT mice ($191.3 \pm 25.6\%$, $n = 8$; $p = 0.008$), but not in neuritin KO mice ($99.6 \pm 17.9\%$, $n = 8$; $p = 0.370$) (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^{20,21}, were inhibited in neuritin KO mice. Previous studies have reported that neuritin exerts neuroprotective effects by activating the insulin receptor signaling pathway¹⁹. The insulin receptor is expressed in various cell types in the retina^{22,27,28}, 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^{19,22,29,30}. In addition, insulin-like growth factor-1 (IGF-1), an important factor in retinal development, prevents ONI-induced RGC death^{31,32}. 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^{3-5,23,24,33-38}. Interestingly, neuritin expression is induced by neuronal activity and by the activity-regulated neurotrophins BDNF and NT-3¹³. 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 neurons¹⁴. In addition, transcorneal electrical stimulation rescues the axotomized RGCs by increasing IGF-1 production in Müller glial cells^{39,40}. Since neuritin and IGF-1 are soluble secreted proteins that can bind to the insulin receptor¹⁹, 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 ONI⁴¹. Similar protective effects of neuritin was reported in a rat model of sciatic nerve injury⁴².

Our present study demonstrated that ONI induces upregulation of *neuritin* mRNA in the retina of C57BL/6J 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 ONI⁴³. 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 injury⁴⁴. 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^{24,45}. VPA is also effective for RGC protection after ONI¹⁰. Interestingly, VPA stimulates productions of nerve growth factor and BDNF in cultured Müller glial cells²⁴. These results suggest that VPA may induce neuritin expression by stimulating productions of neurotrophins. Although further *in vivo* studies are

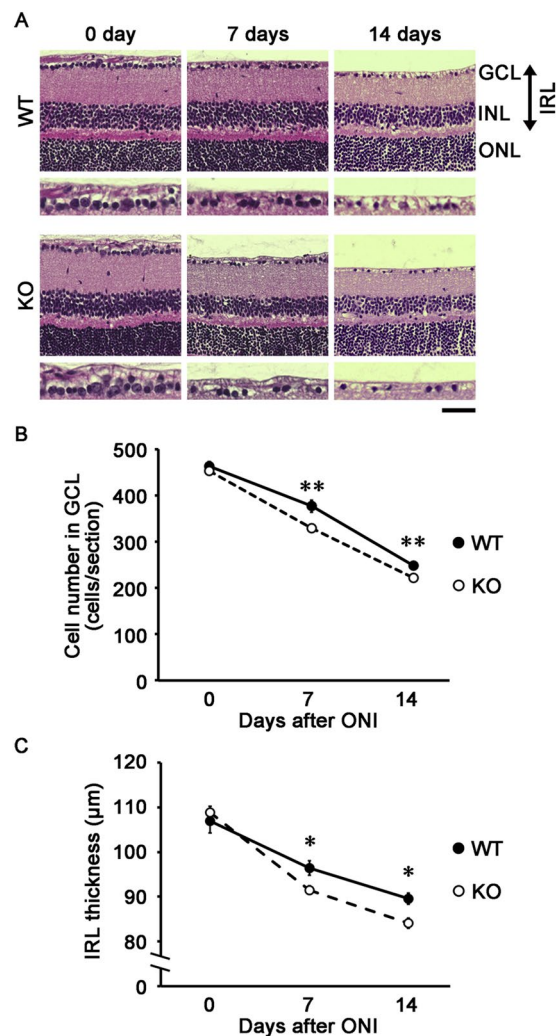


Figure 3. Accelerated retinal degeneration after ONI in neuritin KO mice. **(A)** Representative retinal sections stained with hematoxylin and eosin at 0, 7, 14 days after ONI in WT and neuritin KO mice. Scale bar: 50 and 25 μm in the upper and immediately lower panels, respectively. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IRL, inner retinal layer. **(B,C)** Quantitative analyses of the cell number in the GCL per section **(B)** and IRL thickness **(C)**. Data are presented as means ± S.E.M. $n = 6$ per group. * $p < 0.05$, ** $p < 0.01$.

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 (*Nrn1*^{tm1.2Ndiv}; stock # 018402, Jackson Laboratory, Bar Harbor, ME, USA)¹⁸, 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 report¹⁶. A WT forward primer (5'-GTGCGAGCCCAATCTGCATTCC-3'), a neuritin KO forward primer (5'-GCCGTTGTGGTCTTCCAAAGACC-3'), and a common reverse primer (5'-CGGGTTTCCAAAATAATGAGCGAC-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'-GGTCAGTAGTGGGGCAGAGTGGCGGTGATG-3') and a reverse primer (5'-AAGGGAACCCAGGGTCAGAGAGGACACTT-3') were used. For glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) control amplification, a forward primer (5'-TGCACCACCAACTGCTTAG-3') and a reverse primer (5'-GGATGCAGGGATGATGTTTC-3') were used (Supplementary Figure 2).

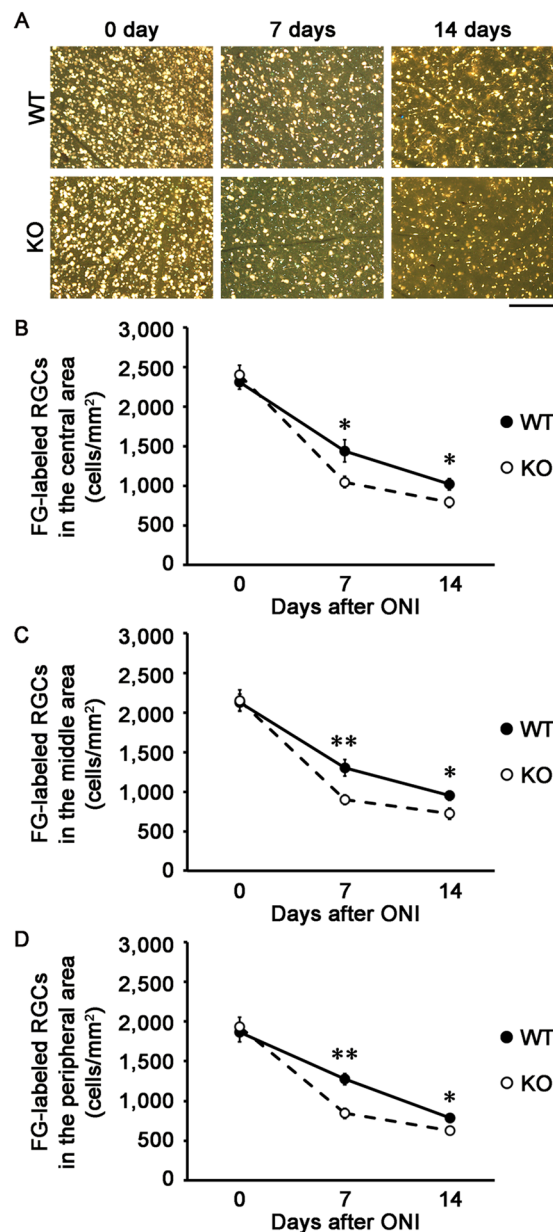


Figure 4. Accelerated RGC loss after ONI in neuritin KO mice. **(A)** Representative images of retrograde-labeled RGCs from the middle areas of the retinas in WT and neuritin KO mice at 0, 7, 14 days after ONI. Scale bar: 100 μm . **(B–D)** Quantification of FG-labeled RGCs in the central (B), middle (C) and peripheral (D) area. Data are presented as means \pm S.E.M. $n = 6$ per group. * $p < 0.05$, ** $p < 0.01$.

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^{23,24}. 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 s^{23,25,46}. 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 mm^2) 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)⁴⁷. FG-labeled cells were counted, and the mean number of RGCs per square millimeter was calculated.

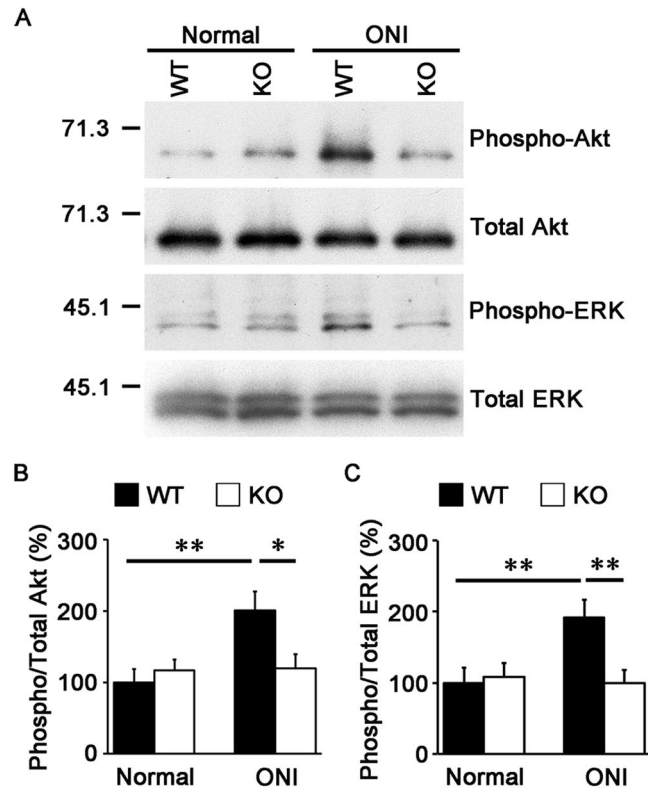


Figure 5. Effects of neuritin on ONI-induced activation of Akt and ERK in the retina. **(A)** Immunoblot analysis of phosphorylated (Phospho-) and total Akt and ERK before and 3 days after ONI in the retina of WT and neuritin KO mice. Full length blot images are presented in Supplementary Figure 3. **(B,C)** Relative expression levels of phosphorylated proteins are quantified. The results are expressed as percentage of the normal WT mice and are presented as means \pm S.E.M. $n = 8$ 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 2 h. Paraffin embedded retinal sections of 7 μ m thickness were cut through the optic nerve and stained with hematoxylin and eosin. The extent of retinal degeneration was quantified in two ways^{48,49}. 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 (Advantax, 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 (ImageJ 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 \pm 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.

References

- Steinsapir, K. D. & Goldberg, R. A. Traumatic optic neuropathy. *Surv. Ophthalmol.* **38**, 487–518 (1994).
- Sarkies, N. Traumatic optic neuropathy. *Eye (Lond)*. **18**, 1122–1125 (2004).
- Kimura, A., Namekata, K., Guo, X., Harada, C. & Harada, T. Neuroprotection, growth factors and BDNF-TrkB signalling in retinal degeneration. *Int. J. Mol. Sci.* **17**, 1584, <https://doi.org/10.3390/ijms17091584> (2016).
- Parrilla-Reverter, G. *et al.* Effects of different neurotrophic factors on the survival of retinal ganglion cells after a complete intraorbital nerve crush injury: a quantitative *in vivo* study. *Exp. Eye Res.* **89**, 32–41 (2009).
- Park, K., Luo, J. M., Hishah, S., Harvey, A. R. & Cui, Q. Cellular mechanisms associated with spontaneous and ciliary neurotrophic factor-cAMP-induced survival and axonal regeneration of adult retinal ganglion cells. *J. Neurosci.* **24**, 10806–10815 (2004).
- Kimura, A., Namekata, K., Guo, X., Harada, C. & Harada, T. Dock3-NMDA receptor interaction as a target for glaucoma therapy. *Histol. Histopathol.* **32**, 215–221 (2017).
- Namekata, K. *et al.* Dock3 attenuates neural cell death due to NMDA neurotoxicity and oxidative stress in a mouse model of normal tension glaucoma. *Cell Death Differ.* **20**, 1250–1256 (2013).
- Tonari, M. *et al.* Blocking endothelin-B receptors rescues retinal ganglion cells from optic nerve injury through suppression of neuroinflammation. *Invest. Ophthalmol. Vis. Sci.* **53**, 3490–3500 (2012).
- Noro, T. *et al.* Spermidine promotes retinal ganglion cell survival and optic nerve regeneration in adult mice following optic nerve injury. *Cell Death Dis.* **6**, e1720, <https://doi.org/10.1038/cddis.2015.93> (2015).
- Biermann, J. *et al.* Valproic acid-mediated neuroprotection and regeneration in injured retinal ganglion cells. *Invest. Ophthalmol. Vis. Sci.* **51**, 526–534 (2010).
- Kimura, A. *et al.* Targeting oxidative stress for treatment of glaucoma and optic neuritis. *Oxid. Med. Cell. Longev.* **2017**, 2817252, <https://doi.org/10.1155/2017/2817252> (2017).
- Nedivi, E., Hevroni, D., Naot, D., Israeli, D. & Citri, Y. Numerous candidate plasticity-related genes revealed by differential cDNA cloning. *Nature*. **363**, 718–722 (1993).
- Naeve, G. S. *et al.* Neuritin: a gene induced by neural activity and neurotrophins that promotes neuritogenesis. *Proc. Natl. Acad. Sci. USA* **94**, 2648–2653 (1997).
- Zhao, J. J. *et al.* Soluble cpg15 from astrocytes ameliorates neurite outgrowth recovery of hippocampal neurons after mouse cerebral ischemia. *J. Neurosci.* **37**, 1628–1647 (2017).
- Putz, U., Harwell, C. & Nedivi, E. Soluble CPG15 expressed during early development rescues cortical progenitors from apoptosis. *Nat. Neurosci.* **8**, 322–331 (2005).
- Fujino, T. *et al.* CPG15 regulates synapse stability in the developing and adult brain. *Genes Dev.* **25**, 2674–2685 (2011).
- Shimada, T., Sugiura, H. & Yamagata, K. Neuritin: a therapeutic candidate for promoting axonal regeneration. *World J. Neurol.* **3**, 138–143 (2013).
- Shimada, T., Yoshida, T. & Yamagata, K. Neuritin mediates activity-dependent axonal branch formation in part via FGF signaling. *J. Neurosci.* **36**, 4534–4548 (2016).
- Yao, J. J. *et al.* Neuritin activates insulin receptor pathway to up-regulate Kv4.2-mediated transient outward K^+ current in rat cerebellar granule neurons. *J. Biol. Chem.* **287**, 41534–41545 (2012).
- Guo, X., Snider, W. D. & Chen, B. GSK3 β regulates AKT-induced central nervous system axon regeneration via an eIF2B-dependent, mTORC1-independent pathway. *Elife*. **5**, e11903, <https://doi.org/10.7554/eLife.11903> (2016).
- Zhou, Y., Pernet, V., Hauswirth, W. W. & Di Polo, A. Activation of the extracellular signal-regulated kinase 1/2 pathway by AAV gene transfer protects retinal ganglion cells in glaucoma. *Mol. Ther.* **12**, 402–412 (2005).
- Kanamori, A. *et al.* Akt is activated via insulin/IGF-1 receptor in rat retina with episcleral vein cauterization. *Brain Res.* **1022**, 195–204 (2004).
- Harada, C. *et al.* TrkB signaling in retinal glia stimulates neuroprotection after optic nerve injury. *Am. J. Pathol.* **185**, 3238–3247 (2015).
- Kimura, A. *et al.* Valproic acid prevents NMDA-induced retinal ganglion cell death via stimulation of neuronal TrkB receptor signaling. *Am. J. Pathol.* **185**, 756–764 (2015).
- Katome, T. *et al.* Inhibition of ASK1-p38 pathway prevents neural cell death following optic nerve injury. *Cell Death Differ.* **20**, 270–280 (2013).
- Perry, V. H. Evidence for an amacrine cell system in the ganglion cell layer of the rat retina. *Neuroscience*. **6**, 931–944 (1981).
- Rajala, A., Tanito, M., Le, Y. Z., Kahn, C. R. & Rajala, R. V. Loss of neuroprotective survival signal in mice lacking insulin receptor gene in rod photoreceptor cells. *J. Biol. Chem.* **283**, 19781–19792 (2008).
- Lofqvist, C. *et al.* Quantification and localization of the IGF/insulin system expression in retinal blood vessels and neurons during oxygen-induced retinopathy in mice. *Invest. Ophthalmol. Vis. Sci.* **50**, 1831–1837 (2009).
- Barber, A. J. *et al.* Insulin rescues retinal neurons from apoptosis by a phosphatidylinositol 3-kinase/Akt-mediated mechanism that reduces the activation of caspase-3. *J. Biol. Chem.* **276**, 32814–32821 (2001).
- Diaz, B., Serna, J., De Pablo, F. & De la Rosa, E. J. *In vivo* regulation of cell death by embryonic (pro)insulin and the insulin receptor during early retinal neurogenesis. *Development*. **127**, 1641–1649 (2000).
- Kermer, P., Klöcker, N., Labes, M. & Bähr, M. Insulin-like growth factor-I protects axotomized rat retinal ganglion cells from secondary death via PI3-K-dependent Akt phosphorylation and inhibition of caspase-3 *in vivo*. *J. Neurosci.* **20**, 722–728 (2000).
- Gutiérrez-Ospina, G., D la Barrera, A. G., Larriva, J. & Giordano, M. Insulin-like growth factor I partly prevents axon elimination in the neonate rat optic nerve. *Neurosci. Lett.* **325**, 207–210 (2002).
- Harada, C. *et al.* Glia- and neuron-specific functions of TrkB signalling during retinal degeneration and regeneration. *Nat. Commun.* **2**, 189, <https://doi.org/10.1038/ncomms1190> (2011).
- Harada, T. *et al.* Modification of glial-neuronal cell interactions prevents photoreceptor apoptosis during light-induced retinal degeneration. *Neuron*. **26**, 533–541 (2000).
- Harada, T. *et al.* Microglia-Müller glia cell interactions control neurotrophic factor production during light-induced retinal degeneration. *J. Neurosci.* **22**, 9228–9236 (2002).
- Ma, L. *et al.* Neurotrophin-3 is required for appropriate establishment of thalamocortical connections. *Neuron*. **36**, 623–634 (2002).

37. Harada, C. *et al.* Role of neurotrophin-4/5 in neural cell death during retinal development and ischemic retinal injury *in vivo*. *Invest. Ophthalmol. Vis. Sci.* **46**, 669–673 (2005).
38. Harada, T., Harada, C. & Parada, L. F. Molecular regulation of visual system development: more than meets the eye. *Genes Dev.* **21**, 367–378 (2007).
39. Morimoto, T. *et al.* Transcorneal electrical stimulation rescues axotomized retinal ganglion cells by activating endogenous retinal IGF-1 system. *Invest. Ophthalmol. Vis. Sci.* **46**, 2147–2155 (2005).
40. Sato, T. *et al.* Effect of electrical stimulation on IGF-1 transcription by L-type calcium channels in cultured retinal Müller cells. *Jpn. J. Ophthalmol.* **52**, 217–223 (2008).
41. Sharma, T. P., Liu, Y., Wordinger, R. J., Pang, I. H. & Clark, A. F. Neuritin 1 promotes retinal ganglion cell survival and axonal regeneration following optic nerve crush. *Cell Death Dis.* **6**, e1661, <https://doi.org/10.1038/cddis.2015.22> (2015).
42. Wang, H. *et al.* Recombinant hNeuritin promotes structural and functional recovery of sciatic nerve injury in rats. *Front. Neurosci.* **10**, 589, <https://doi.org/10.3389/fnins.2016.00589> (2016).
43. Sharma, T. P. *et al.* Optic nerve crush induces spatial and temporal gene expression patterns in retina and optic nerve of BALB/c mice. *Mol. Neurodegener.* **9**, 14, <https://doi.org/10.1186/1750-1326-9-14> (2014).
44. Di Giovanni, S. *et al.* Neuronal plasticity after spinal cord injury: identification of a gene cluster driving neurite outgrowth. *FASEB J.* **19**, 153–154 (2005).
45. Kimura, A. *et al.* Valproic acid prevents retinal degeneration in a murine model of normal tension glaucoma. *Neurosci. Lett.* **588**, 108–113 (2015).
46. Namekata, K. *et al.* Dock3 induces axonal outgrowth by stimulating membrane recruitment of the WAVE complex. *Proc. Natl. Acad. Sci. USA* **107**, 7586–7591 (2010).
47. Chen, H. *et al.* Optic neuropathy due to microbead-induced elevated intraocular pressure in the mouse. *Invest. Ophthalmol. Vis. Sci.* **52**, 36–44 (2011).
48. Harada, C. *et al.* Role of apoptosis signal-regulating kinase 1 in stress-induced neural cell apoptosis *in vivo*. *Am. J. Pathol.* **168**, 261–269 (2006).
49. Harada, C. *et al.* ASK1 deficiency attenuates neural cell death in GLAST-deficient mice, a model of normal tension glaucoma. *Cell Death Differ.* **17**, 1751–1759 (2010).
50. Noro, T. *et al.* Spermidine ameliorates neurodegeneration in a mouse model of normal tension glaucoma. *Invest. Ophthalmol. Vis. Sci.* **56**, 5012–5019 (2015).

Acknowledgements

We would like to thank Mayumi Kunitomo, Keiko Okabe and Sayaka Ihara for their technical assistance. This work was supported in part by JSPS KAKENHI Grants-in-Aid for Scientific Research (K.N. JP16K08635, T.S. JP17K07086, X.G. JP16K07076, A.K. JP17K07123, C.H. JP16K11308, K.Y. JP25293239, T.H. JP15H04999), AMED (K.Y. JP18ek0109311), the Japan Epilepsy Research Foundation (K.Y.), and the Takeda Science Foundation (T.H.).

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.

Additional Information

Supplementary information accompanies this paper at <https://doi.org/10.1038/s41598-018-28425-7>.

Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2018