Ecel1 Knockdown With an AAV2-Mediated CRISPR/Cas9 System Promotes Optic Nerve Damage-Induced RGC Death in the Mouse Retina

Kota Sato,1,2 Yukihiro Shiga,1 Yurika Nakagawa,1 Kosuke Fujita,3 Koji M. Nishiguchi,4 Hiroshi Tawarayama,1,3 Namie Murayama,1 Shigeto Maekawa,1 Takeshi Yabana,1 Kazuko Omodaka,1 Shota Katayama,1 Qiwei Feng,1 Satoru Tsuda,1 and Toru Nakazawa1–4

1Department of Ophthalmology, Tohoku University Graduate School of Medicine, Miyagi, Japan
2Department of Ophthalmic Imaging and Information Analytics, Tohoku University Graduate School of Medicine, Miyagi, Japan
3Department of Retinal Disease Control, Tohoku University Graduate School of Medicine, Miyagi, Japan
4Department of Advanced Ophthalmic Medicine, Tohoku University Graduate School of Medicine, Miyagi, Japan

Correspondence: Toru Nakazawa, Department of Ophthalmology, Tohoku University Graduate School of Medicine, 1-1, Seiryo, Aoba, Sendai, Miyagi 980-8574, Japan; ntoru@oph.med.tohoku.ac.jp.
Submitted: January 4, 2018
Accepted: July 2, 2018

Purpose. To assess the therapeutic potential of endothelin-converting enzyme-like 1 (Ecel1) in a mouse model of optic nerve crush.

Methods. Ecel1 expression was evaluated with real time quantitative (qRT)-PCR, Western blotting, and immunohistochemistry in mouse retinas after optic nerve crush. Vinblastine administration to the optic nerve and the intravitreal injection of N-methyl-d-aspartate (NMDA) were used to assess Ecel1 gene expression. Ecel1 was deleted with an adeno-associated viral (AAV) clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas9 system, and retinal ganglion cell (RGC) survival was investigated with retrograde labeling, qRT-PCR, and visual evoked potential.

Results. Optic nerve crush induced Ecel1 expression specifically in the RGCs, peaking on day 4 after optic nerve crush. Ecel1 gene expression was induced by the vinblastine-induced inhibition of axonal flow, but not by NMDA-induced excitotoxicity, even though both are triggers of RGC death. Knockdown of Ecel1 promoted the loss of RGCs after optic nerve crush.

Conclusions. Our data suggest that Ecel1 induction is part of the retinal neuroprotective response to axonal injury in mice. These findings might provide insight into novel therapeutic targets for the attenuation of RGC damage, such as occurs in traumatic optic neuropathy.

Keywords: Ecel1, optic nerve injury, retinal ganglion cells, neuroprotection

Traumatic optic neuropathy after optic nerve damage is accompanied by the loss of retinal ganglion cells (RGCs) and optic nerve atrophy.1 Clinically, ocular diseases such as glaucoma are characterized by a similar loss of RGCs and result in visual defects. RGC loss is considered to have multifactorial disease mechanisms, and these have been proposed to include axonal injury caused by compression of the optic nerve at the level of the lamina cribrosa.2 Previously, we used an optic nerve injury animal model to investigate the molecular pathomechanisms of RGC death by analyzing the transcriptome with RNA sequencing technology.2 Confirming other research, our study showed that endothelin-converting enzyme-like 1 (Ecel1) was one of the most highly induced genes in mouse and rat retinas after optic nerve injury.3,4 Ecel1 was first identified with differential display PCR and was found to be markedly elevated during the response to nerve injury in rat brain tissue.5 Ecel1 is also called damage-induced neuronal endopeptidase and is induced by a variety of kinds of neuronal damage.5 Although Ecel1 is homologous with endothelin-converting enzyme as a putative zinc metalloprotease, its substrates are still unknown.5,7 Ecel1 protein is distributed in the plasma membrane and endoplasmic reticulum (ER).8 Transcriptional expression of Ecel1 occurs in the neurons of the central nervous system and peripheral nervous system from an early developmental stage.9 Furthermore, Ecel1-deficient mice undergo respiratory failure that results in neonatal lethality.10,11 Thus, past studies indicate that Ecel1 plays an important role in neural development, in particular in the formation of a normal neural network in the lungs.

Despite the incidence of neonatal lethality, studies have successfully used Ecel1-deficient mice to reveal the importance of this gene. Ecel1-deficient mice show atrophy of the diaphragm, as well as other skeletal muscles, because of a significant decrease in nerve terminal arborization in the diaphragm in the embryonic stage.11 This pathological phenotype does not occur in motor neuron-specific Ecel1-transgenic mice, but it does occur in protease domain–mutated Ecel1-transgenic mice.12 This suggests that Ecel1 has a crucial role in distal axonal arborization into the muscles, which depends on the proteolytic domain to establish neuromuscular junctions. Ecel1 is also known to be responsible for type 5 distal arthrogryposis (DA).13,14 Ecel1 knock-in mice with a pathogenic mutation causing type 5 DA have defects in the axonal arborization of the motor nerves in the limbs, suggesting that failure of the neuromuscular junctions could be the primary cause of DA in patients with Ecel1 mutation.15
Ecel1 gene expression is synergistically regulated by axonal regeneration-associated transcriptional factors, such as ATF3, c-Jun, and STAT3, through the activation of Sp1. This suggests that Ecel1 is associated with axonal regeneration. In the field of retinal research, a recent study of mature Ecel1-deficient mice (KO) showed that Ecel1 had a role in promoting axonal regeneration after optic nerve injury.

In addition, a protease-mediated mechanism might contribute to the activation of antioxidant enzymes and the prevention of neuronal death. However, the neuroprotective effect of Ecel1 in vivo is still unclear. In this study, we focused on the expression pattern of Ecel1 after optic nerve injury and attempted to determine whether its molecules have a therapeutic effect against underlying RGC death in a mouse model of traumatic optic neuropathy.

Materials and Methods

Animals

Eight- to 12-week-old male C57BL/6J mice were purchased from SLC (Shizuoka, Japan) and were maintained at Tohoku University Graduate School of Medicine under a 12-hour light/dark cycle. The animal experiments in this study were performed in accord with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the institutional animal care and use committee of Tohoku University, following the Guidelines for Animals in Research.

Induction of Axonal Injury in the Mice

Nerve crush was performed to induce RGC damage, as previously described. In brief, the mice underwent the surgical procedure under deep anesthesia, induced with the intraperitoneal administration of a mixture of ketamine (180 mg/kg) and xylazine (90 mg/kg). The optic nerve was exposed and crushed approximately 2 mm posterior to the eyeball with forceps for 5 seconds without damaging the blood vessels around the optic nerve.

Administration of N-Methyl-D-Aspartate (NMDA) and Vinblastine

The mice that underwent NMDA administration were anesthetized with sodium pentobarbital diluted with PBS (78 mg/kg), and the mice that underwent vinblastine administration were anesthetized with a mixture of ketamine (180 mg/kg) and xylazine (90 mg/kg) with saline. Ketamine is known as an NMDA receptor antagonist. To avoid the antagonistic effect of ketamine as a NMDA-receptor blocker, we used pentobarbital in the NMDA injection experiments. A 2-μL NMDA solution diluted with PBS (15 mM) was injected into the vitreous cavity, as previously described. In the controls that received NMDA injection, the same volume (2 μL) of PBS was injected. Vinblastine administration was also performed as described previously. In brief, the optic nerve was exposed under anesthesia, and a gelatin sponge (Spong, Yamanouchi, Japan) containing 3 mM vinblastine was placed on the nerve. In the controls that received vinblastine treatment, the sponge contained vehicle (saline).

Quantitative RT-PCR

Transcriptional gene expression analysis was performed as previously described. Briefly, retinal total RNA was isolated with a purification kit (miRNasy Mini Kit; Qiagen, Inc., Hilden, Germany), and cDNA was synthesized with reverse transcriptase (SuperScript III; Life Technologies, Inc., Carlsbad, CA, USA). Quantitative PCR (qPCR) was performed on a real-time PCR system (7500 Fast Real-Time PCR System; Life Technologies, Inc.) using a universal master mix (TaqMan Fast Universal PCR Master Mix; Life Technologies, Inc.). Pre-designed Taqman primers and probes were purchased (all from Life Technologies, Inc.), with the following catalog numbers: Brn3a (Mm02343791_m1), Brn3b (Mm00454754_s1), Brn3c (Mm04213795_s1), Tby-1.2 (Mm00493681_m1), Nefb (Mm01191456_m1), Rhbps (Mm00803908_m1), SOD1 (Mm01344233_g1), SOD2 (Mm01315000_m1), Gpx4 (Mm00315041_m1), and Gapdh (Mm99999915_g1). Relative transcriptional levels were determined with the comparative Ct method and normalized to Gapdh as an internal control.

Western Blotting

The mouse retinas were homogenized, and the extracted protein concentration was calculated with a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Retinal protein was separated with SDS-PAGE and transferred to PVDF membranes, as previously described. The membranes were blocked with 1% skim milk in Tw-PBS (PBS containing 0.04% Tween 20) for 1 hour at room temperature and then incubated with a goat anti-Ecel1 antibody (N-20, sc-11338, dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After washing the membranes with Tw-PBS, they were incubated with horseradish peroxidase (HRP)- conjugated donkey anti-goat IgG (dilution 1:5000; Sigma-Aldrich Corp., St. Louis, MO, USA) at room temperature for 1 hour. The immunoreactive signal was developed with detection reagent (ECL Prime; GE Healthcare, Piscataway, NJ, USA) and captured with chemiluminescence imager (ChemilDoc; Bio-Rad, Hercules, CA, USA). The membranes were reblotted with Restore Western blotting stripping buffer (Nacalai Tesque, Kyoto, Japan) and incubated with a mouse anti–beta-actin antibody (dilution 1:5000; Sigma-Aldrich Corp.) as an internal control. The density of the immunoreactive band was then determined with software (Image Lab; Bio-Rad). For antibody neutralization, incubation was performed with a goat anti-Ecel1 antibody and a five-fold (by weight) quantity of blocking peptide (sc-11338 p; Santa Cruz Biotechnology and was then used for immunoblotting.

Immunohistochemistry

For Ecel1 immunostaining, enucleated mouse eyes were immediately embedded and frozen in OCT compound with ethanol-dry ice. Fresh retinal cryosections (10–12 μm in thickness) were made with a cryostat (Leica CM3050S; Leica, Wetzlar, Germany), and these cryosections were fixed in cold acetone (at ~30°C) for 15 minutes. The acetone-fixed cryosections were washed with Tw-PBS, and 10% donkey serum in Tw-PBS was used as a blocking buffer. The blocked cryosections were then incubated with a goat anti-Ecel1 antibody (dilution 1:1000) and incubated with secondary antibodies (donkey anti-goat IgG, Alexa Fluor 488 or 568, dilution 1:500; Invitrogen Corp., Carlsbad, CA, USA), including Hoechst 33342. After washing with Tw-PBS, the cryosections were mounted with mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA). For TUNEL staining with Ecel1 immunohistochemistry, staining was first performed with an apoptosis detection kit (ApopTag Red In Situ Apoptosis Detection Kit; Merck-Millipore, Darmstadt, Germany) following the manufacturer’s procedure, and Ecel1 immunostaining was then performed as described above. For
Ecel1 Knockdown Promotes RGC Death

RBPMs or hemagglutinin (HA) immunostaining, mouse eyes were enucleated and fixed in 4% paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan). Skim milk (1%) in T-PBS, as a blocking buffer, and rabbit anti-RBPMs (dilution 1:200; Abcam, Cambridge, UK) or mouse anti-RBPMs (dilution 1:200; Novus Biologicals, Littleton, CO, USA) or rabbit anti-HA (sc-805, dilution 1:200; Santa Cruz Biotechnology) were used as primary antibodies. For HA staining, sections were treated with HRP-conjugated goat anti-rabbit IgG (dilution 1:2000; Sigma-Aldrich Corp.) overnight at 4°C and then incubated with signal enhancer (TSA Plus Fluorescein System; PerkinElmer, Inc., Waltham, MA, USA) following the manufacturer’s protocol. Immunofluorescence images were obtained with a fluorescence microscope (Axiophot 200; Carl Zeiss, Inc., Oberkochen, Germany). To count Ecel1-positive RGCs in the ganglion cell layer (GCL), whole retinal sections were observed through a microscope (BX9000 or BX800; Keyence Corporation, Osaka, Japan) in a blind fashion.

AAV2-Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR)/Cas9 Construction for Knockdown of Mouse Ecel1 Protein

Four different guide RNA sequences were designed to target exon 2 of the Ecel1 gene, which includes the translation start site, using an online CRISPR design tool (http://crispr.dbcls.jp/; provided in the public domain by CRISPRdirect). To construct adeno-associated virus (AAV) plasmids expressing *Staphylococcus aureus* Cas9 and single guide RNA sequences under control of the CMV and U6 promoters, respectively, double-strand DNA oligonucleotides corresponding to the designed guide RNA sequences were inserted into a Bsal recognition site, as follows: pX601-AAV-CMV::NLS-SaCas9-NLS-3xHA-bGHpA-U6::Bsal-sgRNA (pX601; a gift from Feng Zhang; Addgene plasmid no. 61591).27 Guide RNA sequences targeting Ecel1 and cyan fluorescent protein (CFP), as a negative control, were used in this study (see Fig. 4A). AAV2/2 vectors were generated and purified following a method described previously.27 Each AAV2/2 vector (1.0 × 10^12 gc/mL) was injected with 1.0 μL per injection into the vitreous of an anaesthetized mouse 4 weeks before optic nerve crush.

To construct a pAAV-CMV-mCherry expression vector, 716 bp of mCherry cDNA (pmCherry-c1; Clontech, Mountain View, CA, USA) was inserted into a pAAV-MCS expression vector (Cell Biolabs, Inc., San Diego, CA, USA).

For RGC purification, mCherry-positive cells were sorted using a microfluidic chip–based cell-sorting system (On-chip Sort; Staphylococcus aureus Cas9 and single guide RNA sequences under control of the CMV and U6 promoters, respectively, double-strand DNA oligonucleotides corresponding to the designed guide RNA sequences were inserted into a Bsal recognition site, as follows: pX601-AAV-CMV::NLS-SaCas9-NLS-3xHA-bGHpA-U6::Bsal-sgRNA (pX601; a gift from Feng Zhang; Addgene plasmid no. 61591).27 Guide RNA sequences targeting Ecel1 and cyan fluorescent protein (CFP), as a negative control, were used in this study (see Fig. 4A). AAV2/2 vectors were generated and purified following a method described previously.27 Each AAV2/2 vector (1.0 × 10^12 gc/mL) was injected with 1.0 μL per injection into the vitreous of an anaesthetized mouse 4 weeks before optic nerve crush.

To construct a pAAV-CMV-mCherry expression vector, 716 bp of mCherry cDNA (pmCherry-c1; Clontech, Mountain View, CA, USA) was inserted into a pAAV-MCS expression vector (Cell Biolabs, Inc., San Diego, CA, USA).

Four different guide RNA sequences were designed to target exon 2 of the Ecel1 gene, which includes the translation start site, using an online CRISPR design tool (http://crispr.dbcls.jp/; provided in the public domain by CRISPRdirect). To construct adeno-associated virus (AAV) plasmids expressing *Staphylococcus aureus* Cas9 and single guide RNA sequences under control of the CMV and U6 promoters, respectively, double-strand DNA oligonucleotides corresponding to the designed guide RNA sequences were inserted into a Bsal recognition site, as follows: pX601-AAV-CMV::NLS-SaCas9-NLS-3xHA-bGHpA-U6::Bsal-sgRNA (pX601; a gift from Feng Zhang; Addgene plasmid no. 61591).27 Guide RNA sequences targeting Ecel1 and cyan fluorescent protein (CFP), as a negative control, were used in this study (see Fig. 4A). AAV2/2 vectors were generated and purified following a method described previously.27 Each AAV2/2 vector (1.0 × 10^12 gc/mL) was injected with 1.0 μL per injection into the vitreous of an anaesthetized mouse 4 weeks before optic nerve crush.

To construct a pAAV-CMV-mCherry expression vector, 716 bp of mCherry cDNA (pmCherry-c1; Clontech, Mountain View, CA, USA) was inserted into a pAAV-MCS expression vector (Cell Biolabs, Inc., San Diego, CA, USA).

For RGC purification, mCherry-positive cells were sorted using a microfluidic chip–based cell-sorting system (On-chip Sort; On-chip Biotechnologies, Tokyo, Japan). The sorted samples were collected and resorted twice for further enrichment. For the T7El1 assay, genomic DNA was extracted with extraction kit (QIAamp DNA Mini Kit; Qiagen, Inc.) before PCR. The target site was amplified with PCR using the appropriate primer set (see Fig. 4A). The first reaction condition was 95°C for 5 minutes, 40 cycles at 95°C for 15 seconds, 68°C for 1 minute, and finally 12°C for cooling. The PCR amplicon was purified with a nucleospin kit (Marcherey-Nagel, Duren, Germany). The denaturing and annealing reactions were then conducted at 95°C for 5 minutes, 95°C to 85°C, 85°C to 25°C, and finally 12°C for cooling. This fragment was digested with an enzyme (T7 Endonuclease1; New England Biolabs, Ipswich, MA, USA). The denaturing and annealing reactions were then conducted at 95°C for 5 minutes, 95°C to 85°C, 85°C to 25°C, and finally 12°C for cooling. This fragment was digested with an enzyme (T7 Endonuclease1; New England Biolabs, Ipswich, MA, USA). The resulting products were analyzed with electrophoresis in a 3% agarose gel and were visualized with nucleic stain (Gel Red; Invitrogen). The intensity of the bands of the PCR amplicon and cleavage products were measured with Image J software (http://image.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). For the DNA sequencing, the PCR products were cloned into pcDNA-3 and transformed into DH5a (Toyobo, Osaka, Japan). DNA sequencing was performed using a genetic analyzer (ABI 3130xl; Life Technologies, Inc.) with a sequencing kit (BigDye Terminator v3.1 Cycle Sequencing Kit; Life Technologies).

Retrograde Labeling and Counting of RGCs

To determine the number of RGCs in the eyes of the mice, retrograde labeling was performed with 2% retrograde neuronal tracer (FluoroGold; Fluorochrome, Denver, CO, USA) 7 days before optic nerve injury, as previously described.28 Seven days after optic nerve injury, the eyeballs were enucleated and fixed in 4% paraformaldehyde for 1 hour. Retinal flat mounts were then prepared on glass slides, as previously described.29 The average number of RGCs was then counted, as previously described.28

Recording of Visual Evoked Potential in Mice

The electrophysiological response of the visual cortex was measured with the visual evoked potential (VEP), using a slight modification of a method we have previously described.25 Under anesthesia induced with the intraperitoneal administration of a mixture of ketamine (37.5 mg/kg) and medetomidine (0.625 mg/kg), overnight dark-adapted mice were used to measure the photopic VEP with a Ganzfeld dome, as well as an acquisition system and an LED stimulator (PuREC and LS-100, respectively; all from Mayo Corp., Inazawa, Japan).

Statistical Analysis

Statistical significance was calculated with unpaired Student’s *t*-test for comparisons of two groups and an analysis of variance (ANOVA) followed by Dunnett’s test for comparisons of the mean in three groups. *P* < 0.05 was considered significant.

RESULTS

RGC Injury Over Time After Optic Nerve Injury in Mice

To confirm whether the RGCs were damaged after optic nerve crush, we measured the gene expression levels of the following RGC markers: *Brn3a, Brn3b, Brn3c, Thy-1.2, Nefb*, and *Rbpms*. All these markers decreased over time after optic nerve crush (Fig. 1). In particular, the transcriptional levels of *Nefb* and *Rbpms* decreased significantly 2 days after optic nerve crush (Figs. 1E, 1F). In addition, the gene expression of *Brn3a, Brn3b, Brn3c*, and *Thy-1.2* decreased significantly 4 days after optic nerve crush (Figs. 1A–D), suggesting that the RGCs were severely injured and that the postoptic nerve crush loss of RGCs was highest on day 4.

Ecel1 Was Strongly Expressed in the Mouse Retinas After Optic Nerve Crush

A previous study showed that the transcriptional level of Ecel1 increased 2 days after optic nerve crush.3 To investigate the expression pattern of Ecel1 in the mouse retina, we performed real time quantitative PCR (qRT-PCR) and Western blotting on days 2 to 7 after optic nerve crush. Two days after optic nerve crush, the mRNA level of Ecel1 was significantly higher in the nerve crush group than in a sham operation group (21.4 ± 6.2-fold change). Four days and 7 days after optic nerve crush, there was a further increase in Ecel1 (129.6 ± 22.3- and 102.4
6.47-fold change, respectively) (Fig. 2A). The protein level of Ecel1 showed an increase similar to that of the transcriptional level, showing a very high increase in the retina 4 days and 7 days after optic nerve crush (Fig. 2B). These results were obtained by detecting the anti-Ecel1 antibody band, based on its predicted size. Therefore, we tested whether this band truly represented Ecel1 protein by performing antibody neutralization. The anti-Ecel1 immunoreactive band completely disappeared after Ecel1 peptide absorption (Fig. 2C), suggesting that the anti-E cel1 antibody specifically recognized Ecel1 protein in the mouse retina. Furthermore, Ecel1 was clearly detected in the GCL, where the RGCs are located, 4 days after optic nerve crush (Fig. 2D), but not in other cell layers, such as the inner nuclear layer or outer nuclear layer (Fig. 2E). Ecel1-positive cells in the GCL 4 days after optic nerve crush significantly increased to 27.2 ± 4.8 cells/mm compared to 3.9 ± 1.1 cells/mm in control retinas that underwent a sham operation (Fig. 2F). Ecel1-positive cells were colocalized with RBPMS (an RGC marker), suggesting that Ecel1 was expressed in the RGCs (Fig. 2G).

Inhibiting Axonal Transport in Mice Promoted Retinal Ecel1 Induction

To determine more details of the mechanism of Ecel1 induction in mice, we performed qRT-PCR in retinas that underwent vinblastine administration to inhibit axonal transport or NMDA administration to induce excitotoxicity. Both chemicals induced and promoted RGC damage, as confirmed by the reduction of Thy-1.2 and Rbpms, which are known to be RGC markers (Figs. 3A, 3B, 3D, 3E). In a manner similar to optic nerve crush, vinblastine treatment strongly promoted the mRNA expression of Ecel1 (Fig. 3C). On the other hand, NMDA damage did not induce Ecel1 expression (Fig. 3F).

Ecel1 Knockdown Promoted RGC Loss in Mice After Optic Nerve Crush

To evaluate the potential role of Ecel1 induction in RGC protection, we used an AAV2-CRISPR/Cas9 system to perform genome editing and induce Ecel1 knockdown. First, we designed four different guide RNA sequences targeting the second exon; these are shown by the arrowheads and are identified by name: 228s, 331s, 385s, and 586s (Fig. 4A). Next, to confirm that AAV2 infection was effective, we performed the intravitreal coinjection of AAV2-mCherry and AAV2-CRISPR/Cas9-CFP136. Four weeks after injection, we observed that mCherry-positive cells were present in the retinas of the mice, spread evenly throughout the GCL, and AAV2 infected the RGCs (Supplementary Fig. S1). To confirm the effectiveness of genome editing in vivo, we performed cell sorting to purify mCherry-positive purified RGCs. The data from a T7E1 assay indicated that AAV2-CRISPR/Cas9-Ecel1-331s and 385s were effective at introducing genomic mutations in mCherry-positive purified RGCs. The data from a T7E1 assay indicated that AAV2-CRISPR/Cas9-Ecel1-331s and 385s were effective at introducing genomic mutations in mCherry-positive purified RGCs. The data from a T7E1 assay indicated that AAV2-CRISPR/Cas9-Ecel1-331s and 385s were effective at introducing genomic mutations in mCherry-positive purified RGCs. The data from a T7E1 assay indicated that AAV2-CRISPR/Cas9-Ecel1-331s and 385s were effective at introducing genomic mutations in mCherry-positive purified RGCs. The data from a T7E1 assay indicated that AAV2-CRISPR/Cas9-Ecel1-331s and 385s were effective at introducing genomic mutations in mCherry-positive purified RGCs. The data from a T7E1 assay indicated that AAV2-CRISPR/Cas9-Ecel1-331s and 385s were effective at introducing genomic mutations in mCherry-positive purified RGCs. The data from a T7E1 assay indicated that AAV2-CRISPR/Cas9-Ecel1-331s and 385s were effective at introducing genomic mutations in mCherry-positive purified RGCs. The data from a T7E1 assay indicated that AAV2-CRISPR/Cas9-Ecel1-331s and 385s were effective at introducing genomic mutations in mCherry-positive purified RGCs. The data from a T7E1 assay indicated that AAV2-CRISPR/Cas9-Ecel1-331s and 385s were effective at introducing genomic mutations in mCherry-positive purified RGCs. The data from a T7E1 assay indicated that AAV2-CRISPR/Cas9-Ecel1-331s and 385s were effective at introducing genomic mutations in mCherry-positive purified RGCs. The data from a T7E1 assay indicated that AAV2-CRISPR/Cas9-Ecel1-331s and 385s were effective at introducing genomic mutations in mCherry-positive purified RGCs. The data from a T7E1 assay indicated that AAV2-CRISPR/Cas9-Ecel1-331s and 385s were effective at introducing genomic mutations in mCherry-positive purified RGCs. The data from a T7E1 assay indicated that AAV2-CRISPR/Cas9-Ecel1-331s and 385s were effective at introducing genomic mutations in mCherry-positive purified RGCs.

We found that 4 days after optic nerve crush, Ecel1 protein levels and the number of Ecel1-positive cells was significantly reduced in the retinas that had undergone injection with AAV2-
CRISPR/Cas9-Ecel1, in comparison with control retinas injected with AAV2-CRISPR/Cas9-CFP (Figs. 4D–G). Next, we compared the number of surviving RGCs in animals that had or had not undergone Ecel1 knockdown to determine whether Ecel1 promoted RGC survival after optic nerve injury. We found that in the whole retina, FG-positive cells were significantly reduced in retinas injected with AAV2-CRISPR/Cas9-Ecel1 (AAV2-CRISPR/Cas9-Ecel1-331: 829 ± 6138 cells/mm², AAV2-CRISPR/Cas9-Ecel1-385: 894 ± 171 cells/mm²), in comparison with control retinas injected with AAV2-CRISPR/Cas9-CFP136 (1123 ± 178 cells/mm²) (Figs. 4H, 4I). On the other hand, FG-positive cells in uninjured retina treated with AAV2-CRISPR/Cas9-Ecel1 injection mice were not different in control mice (Figs. 4J, 4K). Consistently, transcriptional expression of RGC markers were also reduced in retinas with AAV2-CRISPR/Cas9 in comparison with control retinas injected with AAV2-CRISPR/Cas9-CFP136 (Fig. 4L). In addition, we measured the photopic VEP to evaluate the electrophysiological response and determine the relationship between RGC survival and visual function in mice treated with AAV2-CRISPR/Cas9 after optic nerve crush. In the mice injected with AAV2-CRISPR/Cas9-Ecel1-331 or -385, the P1 and N1 waveforms were weak (Fig. 4M). We calculated the amplitude and found that P1-N1 amplitude tended to decrease, but did not significantly change in the three CRISPR/Cas9 groups at a stimulus intensity of 2.0 log cd·s/m² (P = 0.1 for AAV2-CRISPR/Cas9-CFP-136 versus -Ecel1-331 and AAV2-CRISPR/Cas9-CFP-136 versus -Ecel1-385; Fig. 4N). Specifically, the baseline-N1 amplitude in the mice injected with AAV2-CRISPR/Cas9-Ecel1-331 was significantly lower than in the mice injected with AAV2-CRISPR/Cas9-CFP-136 (as controls) at a stimulus intensity of 2.0 log cd·s/m² (P = 0.018; Fig. 4O). In the mice injected with AAV2-CRISPR/Cas9-Ecel1-385, the baseline-N1 amplitude also showed a tendency to be lower than in the mice injected with AAV2-CRISPR/Cas9-CFP-136 (as controls) at a stimulus intensity of 2.0 log cd·s/m² (P = 0.07; Fig. 4O).

**DISCUSSION**

**Role of Ecel1 in the RGCs of Mice After Optic Nerve Injury**

This study analyzed the role of Ecel1 in protecting the RGCs after optic nerve crush. We found that the induction of Ecel1...
peaked 4 days after optic nerve crush and that Ecel1 was induced by axonal flow, not by NMDA-induced RGC damage. Our previous work showed that the time course of Ecel1 induction was correlated with changes in the number of dead, Sytox orange–positive RGCs. Furthermore, Ecel1-positive RGGs were not colocalized with TUNEL (Supplementary Fig. S3), and Ecel1 knockdown with a CRISPR/Cas9 system significantly promoted the loss of RGCs and reduced electro-physiological visual function after optic nerve crush. Taken together, these findings suggest that the induction of Ecel1 may be part of the neuroprotective mechanism of the mouse retina.

Differences in RGC Loss Between AAV2-CRISPR/Cas9-Ecel1 and Ecel1 KOTg Mice After Optic Nerve Injury

This study revealed that knockdown of Ecel1 with the AAV2-CRISPR/Cas9 system promoted RGC loss after optic nerve injury. However, previous work suggested that Ecel1 deletion has tended to reduce RGCs but did not significantly affect the survival of injured RGCs in Ecel1 KO Tg mice. One possible explanation is that the permanent deletion of Ecel1 in Ecel1 KO Tg mice might change the expression of other genes or signaling pathways to compensate for the loss of Ecel1. In the retina of Ecel1 KO Tg mice, no significant difference was seen in the number of RGCs or the axonal projections of the optic nerve to the superior colliculus and lateral geniculate nucleus. However, Ecel1 is expressed in the embryonic neural retina. Thus, the retinal network surrounding the RGCs might somehow be changed. Our AAV2-CRISPR/Cas9 system was able to delete Ecel1 protein from the RGCs specifically, after the completion of retinal development. Key differences in the design of these two studies might have caused these differing results, and further study will be needed to elucidate the mechanism of these phenomena in more detail.

Neuroprotective Mechanisms of Ecel1 Against RGC Death in Mice After Optic Nerve Injury

To investigate in detail the neuroprotective mechanism of Ecel1 induction, we attempted to determine whether Ecel1 could prevent cell death induced by oxidative stress and ER stress, which are associated with RGC death after optic nerve injury. In fact, we found that Ecel1 overexpression did not prevent cell death by H2O2-induced oxidative damage or tunicamycin-induced ER stress in vitro and that it did not have a toxic effect in the RGCs in vivo without optic nerve crush (Supplementary Figs. S4, S5). A previous study showed that RGC death involved apoptosis through ceramide signaling after optic nerve crush. Another report showed that Ecel1 overexpression inhibited C2 ceramide-induced apoptosis in vitro. These studies suggest that the induction of Ecel1 has a neuroprotective effect and can prevent the activation of signaling pathways involving ceramide-induced apoptosis, but not those involving oxidative stress or ER stress, in the RGCs of mice after optic nerve crush. Additionally, Ecel1 overexpression in the retina did not increase the transcriptional levels of antioxidant enzymes, such as SOD1, SOD2, and Gpx4 in mice (Supplementary Fig. S5G), in contrast with previous results.

**Figure 3.** Ecel1 expression was strongly induced in damaged RGCs treated with vinblastine but not in those treated with NMDA. Relative expression levels of Thy-1.2 (A), Rbpms (B), and Ecel1 (C) in vinblastine-treated retinas, and Thy1 (D), Rbpms (E), and Ecel1 (F) in NMDA-treated retinas, were determined with quantitative RT-PCR and normalized to the Gapdh level. Error bars denote SD (vinblastine; n = 7-8, NMDA; n = 6-10 for each group at each time point). Vin, vinblastine. *P < 0.05, **P < 0.01, ***P < 0.001.
Ecel1 knockdown with the CRISPR/Cas9 system promoted RGC loss after optic nerve crush. (A) Structure of the mouse Ecel1 gene; the 2nd exon is magnified. The mouse Ecel1 gene has 18 exons and 17 introns. The translation start codon (ATG) is located on the second exon. The guide RNA sequences for Ecel1 and for CFP as a control are shown in the box. (B) T7 Endonuclease 1 (T7E1) assay revealing genome editing at the Ecel1 locus in sorted mCherry-expressing cells from AAV2-mCherry and AAV2-CRISPR/Cas9-injected retinas. Asterisks indicated indel formation. (C) Mutation pattern sequencing of Ecel1 locus. (D) Immunoblotting with an anti-Ecel1 antibody in retinas injected with AAV2-CRISPR/Cas9 4 days after optic nerve crush. (E) Quantification of Ecel1 protein levels normalized with β-actin in (D) (n = 3 each). (F) Immunostaining images for Ecel1 with AAV2-CRISPR/Cas9-Ecel1 treatment 4 days after optic nerve crush. (G) Histogram showing the number of Ecel1-positive cells in the GCL 4 days after optic nerve crush and treatment with AAV2-CRISPR/Cas9. (H) Representative images of FG-labeled RGCs 7 days after optic nerve crush in mice.
from in vitro studies. This suggests that Ecel1 in the retina does not induce the production of antioxidant enzymes and that in contrast to in vitro findings, there is no protection of the RGCs after optic nerve crush in retinas overexpressing Ecel1. Alternatively, it may be that even if these antioxidant enzymes were produced in the RGCs of mice, detecting their genetic expression might be difficult in whole retinal samples because of the low population of RGCs in the retina. Another possibility is that RGC death due to Ecel1 knockdown might have been caused by the loss of RGC regenerative capacity after axonal injury. The supply of growth factor from the brain to the RGCs via axonal transport is believed to prevent RGC damage. In particular, brain-derived neurotrophic factor is known to have a powerful neuroprotective effect against RGC damage, such as occurs in optic nerve crush and ischemia/reperfusion models. Kaneko et al. clearly showed that Ecel1 has the potential to induce axonal regeneration of the RGCs after optic nerve crush. However, we could not find any protective effect against oxidative stress or ER stress in vitro (Supplementary Fig. S4). This result also suggests that the mechanisms of Ecel1 RGC protection may be indirect, acting through the promotion of axonal regeneration.

**Induction Mechanisms of Ecel1 Expression After Optic Nerve Injury**

This study showed that the inhibition of axonal flow induced Ecel1 expression in the mouse retina. Nerve growth factor (NGF) is a candidate molecule to stimulate Ecel1 expression. Previous research showed that treatment with leukemia inhibitory factor (LIF) and NGF deprivation induced Ecel1 mRNA expression in vitro and in vivo.17 ATF3, c-Jun, and STAT3 are activated downstream after LIF stimulation and NGF deprivation.16 Our previous work revealed that ATF3 and c-Jun were upregulated in the retina of mice after optic nerve crush.3 These studies demonstrate that in mice, supply of NGF into RGCs from the superior colliculus is failed after axonal damage, and this stimulus led to the upregulation and activation of ATF3 and c-Jun in the nuclei of the RGCs, resulting in the gene expression of Ecel1 in the retina. ATF3 is also known as a core transcriptional factor that initiates nerve regeneration and is colocalized with Ecel1. Furthermore, Ecel1 promotes axonal regeneration in mice after optic nerve crush,19 suggesting that injury-induced Ecel1 expression is promoted by NGF deprivation to ameliorate the effects of axonal damage and ceramide-induced apoptosis in RGCs.

In conclusion, we have shown that Ecel1 has a neuroprotective effect after optic nerve crush in the RGCs of mice. Furthermore, our results suggest that the mechanism of this protective effect might involve the signaling pathway of ceramide-induced apoptosis, but not the pathways of oxidative stress or ER stress. Thus, we propose that further study of Ecel1 might provide insight into novel therapeutic targets for the attenuation of RGC damage after optic nerve injury, such as traumatic optic neuropathy.

**Acknowledgments**

The authors thank Yoko Hirata (Gifu University, Japan) for generously providing the mouse HT22 hippocampal cells, Tim Hills for reviewing and editing the language of the manuscript, Junko Sato, Kanako Sakai, and Eriko Kamii for their technical assistance, and the Biomedical Research Unit of Tohoku University Hospital for technical support.

Supported by Grant-in-Aid from the Ministry of Education, Science and Technology of Japan 15K20247 (KS) and 17K16956 (ST).

Disclosure: K. Sato, None; Y. Shiga, None; Y. Nakagawa, None; K. Fujita, None; K.M. Nishiguchi, None; H. Tawarayama, None; N. Murayama, None; S. Maekawa, None; T. Yabana, None; K. Omodaka, None; S. Katayama, None; Q. Feng, None; S. Tsuda, None; T. Nakazawa, None

**References**


11. Nagata K, Kiyuru-Seo S, Maeda M, Yoshida K, Morita T, Kiyama H. Damage-induced neuronal endopeptidase is critical for...


