Effect of Timolol on Optineurin Aggregation in Transformed Induced Pluripotent Stem Cells Derived From Patient With Familial Glaucoma

Satoshi Inagaki,1,2 Kazuhide Kawase,3 Michinori Funato,2 Junko Seki,2 Chizuru Kawase,2 Kazuki Ohuchi,1,2 Tsubasa Kameyama,1,2 Shiori Ando,1,2 Arisu Sato,1,2 Wataru Morozumi,3 Shinsuke Nakamura,1 Masamitsu Shimazawa,1 Daisuke Iejima,4 Takeshi Iwata,4 Tetsuya Yamamoto,3 Hideo Kaneko,2 and Hideaki Hara1

1Molecular Pharmacology, Department of Biofunctional Evaluation, Gifu Pharmaceutical University, Gifu, Japan
2Department of Clinical Research, National Hospital Organization, Nagara Medical Center, Gifu, Japan
3Department of Ophthalmology, Gifu University Graduate School of Medicine, Gifu, Japan
4Division of Molecular and Cellular Biology, National Institute of Sensory Organs, National Hospital Organization Tokyo Medical Center, Tokyo, Japan

Correspondence: Masamitsu Shimazawa, Molecular Pharmacology, Department of Biofunctional Evaluation, Gifu Pharmaceutical University, 1-25-4 Daigaku-nishi, Gifu 501-1196, Japan; Shimazawa@gifu-pu.ac.jp.

1 and KK contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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PURPOSE. To determine a chemical agent that can reduce the aggregation of optineurin (OPTN) in cells differentiated from induced pluripotent stem cells obtained from a patient with normal-tension glaucoma (NTG) caused by an E50K mutation in the OPTN gene (OPTNE50K-NTG).

METHODS. Retinal ganglion cells (RGCs) were created from induced pluripotent stem cells derived from a healthy individual (wild-type [WT]-iPSCs) and from a patient with NTG due to OPTNE50K (E50K-iPSCs) mutation. The death of the induced RGCs was evaluated by counting the number of TUNEL- and ATH5-positive cells. Axonal growth was determined by measuring the axonal length of TUJ1-positive cells. OPTN aggregation was assessed by measuring the OPTN-positive area by immunofluorescence and by Western blotting. Autophagic flux assay was investigated by determining the light chain 3 (LC3)B-II/LC3B-I ratio and p62 expression by Western blotting.

RESULTS. The results showed OPTNE50K aggregation, activation of astrocytes, reduction in the number of RGCs, and enhancement of apoptotic cell death in the in vitro OPTNE50K model of NTG. Timolol was found to reduce the OPTNE50K-positive area and decreased the insoluble OPTNE50K, suggesting that it has the potential of reducing the OPTNE50K aggregation. Timolol also increased the ATH5-positive cells, decreased TUNEL-positive cells, increased the LC3B-II/LC3B-I ratio, and decreased the expression of p62. These findings suggest that timolol might enhance autophagic flux, leading to reduced OPTNE50K aggregation.

CONCLUSIONS. Timolol should be considered a potential therapeutic agent specific to OPTNE50K-NTG because it can reduce the OPTNE50K aggregation in E50K-iPSCs-RGCs by enhancing autophagic flux and neuroprotective effects.

Keywords: glaucoma, induced pluripotent stem cells, retinal ganglion cells, optineurin, timolol, autophagy

Glaucoma is characterized by a progressive loss of retinal ganglion cells (RGCs) and their axons, resulting in a constriction of the visual field and eventual blindness.1 Primary open-angle glaucoma (POAG) is the most common type of glaucoma, and it is classified into two subtypes: high-tension glaucoma (HTG) and normal-tension glaucoma (NTG). The intraocular pressure (IOP) in eyes with NTG is not elevated above normal levels at any time.

Genetic mutations of the optineurin (OPTN) gene have been identified in patients with NTG.2,3 The OPTN protein consists of 577 amino acids including a coiled-coil domain, leucine zipper domain, light chain 3 (LC3) autophagy marker interacting motif, ubiquitin-binding domain, and zinc finger domains.4 These domains allow the OPTN gene to control various physiological functions. For example, the OPTN protein interacts with autophagy-associated proteins such as TANK-binding kinase (TBK1) and with membrane vesicular trafficking proteins such as TBC1D17 and Rab8.4,5,6 Earlier studies have shown different OPTN mutations including E50K, OPTNE50K, and M98K, OPTNM98K.7,8 The OPTNE50K mutation was the first to be identified as the cause of familial NTG, and patients with this mutation had severe signs and symptoms of glaucoma. In the OPTNE50K mutation, glutamine is replaced by lysin at amino acid 50, and this results in an increase in the interaction of the OPTN protein and TBK1. This then increases the aggregation of OPTN protein in the Golgi apparatus, leading to
a loss of cellular homeostasis. The aggregation of OPTN protein in the Golgi apparatus of the RGCs can lead to their death followed by degeneration of the axons of the RGCs.

The NTG caused by the OPTNE50K mutation is dominantly inherited and has an onset at a younger age than sporadic NTG. The current treatment for OPTNE50K-NTG is surgery or topical medications that reduce the IOP, but an effective treatment for OPTNE50K-NTG eyes has not been established.

Thus, the purpose of this study was to determine a chemical agent that will lower the IOP and reduce the aggregation of OPTN in the RGCs. To accomplish this, we studied induced pluripotent stem cells (iPSCs) that were obtained from a patient with OPTNE50K-NTG. These iPSCs were transformed to cells with biochemical markers of RGCs, and we tested whether timolol, a β-adrenergic receptor (β-AR) antagonist, would reduce the aggregation of OPTN in these cells. Our results showed that timolol reduced the OPTNE50K aggregation in transformed iPSCs obtained from a patient with OPTNE50K-NTG. This was accomplished by an increase in the autophagic flux leading to an enhancement of axonal outgrowth.

**MATERIALS AND METHODS**

**Ethics Statement**

The research followed the tenets of the Declaration of Helsinki. Informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study. The procedures used for the pathologic analyses and establishment of patient-derived iPSCs including human gene analyses were approved by the Ethics Review Committee of the National Hospital Organization, Nagara Medical Center, Gifu University and Gifu Pharmaceutical University. The established induced human stem cells were handled according to the Revisions of the Guidelines for Clinical Research using Human Stem Cell from the Ministry of Health, Labor, and Welfare of Japan.

**Differentiation of iPSCs Into Retinal Ganglion Cells in Culture**

The isolated iPSCs (Table 1) were cultured in primate embryonic stem (ES) cell medium (ReproCELL, Kanagawa, Japan) supplemented with 4 ng/mL basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA) and 500 U/mL penicillin/streptomycin (PS; Life Technologies, Carlsbad, CA, USA). The iPSC colonies were cultured in 5% CO₂ at 37°C and passed every 7 days. A reported modified protocol that mimics the normal general RGCs was used. In the first stage, a serum-free floating culture of embryoid body (EB)-like aggregates with the quick reaggregation (SFEbq) method was used as previously described in detail. We exposed 9000 single iPSCs with 2 μM dorsomorphin (Sigma-Aldrich Corp., St. Louis, MO, USA), 10 μM SB431542 (SB; Cayman, San Diego, CA, USA), and 10 μM Rho-associated coiled-coil forming kinase inhibitor, Y-27632 (Wako, Osaka, Japan). This exposure was performed in the EB medium, which consisted of Dulbecco’s modified Eagle’s medium (DMEM/F12 (Life Technologies), 20% knockout serum replacement (KSR, Life Technologies), 1% nonessential amino acids (NEAA), 0.1% 2-mercaptoethanol, and 500 U/mL PS. Eight days after the neuronal induction using the SFEbq method, the aggregates were transferred into Matrigel-coated plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). On the following day, the culture medium was changed to a differentiation medium consisting of DMEM/F12, 1% N2 supplement (Life Technologies), B27 supplement (Life Technologies), L-glutamine (Life Technologies), 500 U/mL PS, 2 μM dorsomorphin, 10 ng/mL human Dickkopf1 (h-Dkk1; R&D Systems), 10 ng/mL insulin-like growth factor-1 (IGF-1, R&D Systems), and 10 ng/mL bFGF for a further 7 days. Sixteen days after the neuronal induction, the neuronal precursor cells were cultured in a differentiation medium containing 10 μM N-(3,5-difuorophenyl)(acetyl)-L-alanyl-2-phenylglycine-1,1-dimethyl ester (DAPT; Tocris Bioscience, Avonmouth, UK). Twenty-three days after the neuronal induction, all cells except the EB were released by Accutase (Life Technologies) and collected.

The cells were reseeded into Matrigel-coated plates with the addition of 2 ng/mL acidic fibroblast growth factor (aFGF; R&D Systems) to the differentiation medium. After the reseeding, the percentage of ATH5-positive cells (RGC marker) was 93.16 ± 0.46%, and that of TUJ1-positive cells (neuronal marker) was 28.33 ± 5.88%. At all differentiated stages, the medium was changed every 2 or 3 days.

**Antibodies**

All the antibodies for biochemical analysis were purchased from the companies listed in Table 2 and used as the indicated dilutions.

**Immunocytochemistry**

After washing the plated cells with PBS, they were fixed in 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan) for 20 minutes at 4°C and then washed again with PBS. The cells were then blocked against nonspecific labeling with 5% donkey serum and 0.1% Triton X-100 (Nacalai Tesque) in PBS for 30 minutes at 4°C. The cells were washed with PBS and incubated with the primary antibodies overnight at 4°C, followed by labeling with the appropriate secondary antibody tagged with a fluorescent dye. The nuclei were stained with Hoechst 33342 (Life Technologies). The secondary antibodies were Alexa Fluor-labeled antibodies including 594 donkey anti-rabbit, 594 donkey anti-goat IgG, 594 donkey anti-mouse IgG,

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**Table 1. Information About iPSCs**

<table>
<thead>
<tr>
<th>iPSC Line</th>
<th>Age</th>
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<tr>
<td>WT-iPSCs #1 (201B7)</td>
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<td>Female</td>
<td>Caucasian</td>
</tr>
<tr>
<td>WT-iPSCs #2 (201B6)</td>
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<td>Caucasian</td>
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</tr>
<tr>
<td>E50K-iPSCs #2</td>
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<td>Male</td>
<td>Mongolian</td>
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</table>

**Table 2. Sources of Antibodies Used This Study and Dilutions Used**

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<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>anti-ATH5</td>
<td>Chemicon International (Billerica, MA, United States)</td>
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<tr>
<td>anti-THY-1</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA, United States)</td>
<td>1:50</td>
</tr>
<tr>
<td>anti-BRN3</td>
<td>Santa Cruz Biotechnology</td>
<td>1:50</td>
</tr>
<tr>
<td>anti-SM25</td>
<td>Biologend (San Diego, CA, USA)</td>
<td>1:2000</td>
</tr>
<tr>
<td>anti-OPTN</td>
<td>Cayman</td>
<td>1:200</td>
</tr>
<tr>
<td>anti-GM130</td>
<td>Becton, Dickinson and Company</td>
<td>1:500</td>
</tr>
<tr>
<td>anti-β-AR</td>
<td>Santa Cruz Biotechnology</td>
<td>1:50</td>
</tr>
<tr>
<td>anti-LC3B</td>
<td>Cell Signaling Technology (Danvers, MA, United States)</td>
<td>1:1000</td>
</tr>
<tr>
<td>anti-p62</td>
<td>Cell Signaling Technology</td>
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</tr>
<tr>
<td>anti-β-actin</td>
<td>Sigma-Aldrich Corp.</td>
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<tr>
<td>anti-FoxO3a</td>
<td>Cell Signaling Technology</td>
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</tr>
<tr>
<td>anti-GFAP</td>
<td>Cell Signaling Technology</td>
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</table>
488 donkey anti-mouse IgG, 488 donkey anti-rabbit IgG, and 488 donkey anti-goat IgG (Life Technologies; dilution for all secondary antibodies was 1:1000).

For the assays of ATH5-positive and TUNEL-positive cells, the numbers of cells that were positive for these markers and for HOECHST 33342 were counted with BIOREVO BZ-9000 Analysis Software (Keyence, Osaka, Japan), an image processing software program. For the assays of the OPTN-positive areas, the stained areas were measured with the same software. The axon length of the TUJ1-positive cells was measured. These cell count and area and length measurements were performed on 10 images/well. The data from three independent experiments were analyzed statistically.

**Terminal Deoxynucleotidyl Transferase Nick-End Labeling (TUNEL) Staining Assays**

The TUNEL assays were performed 50 days after the beginning of differentiation. After washing the plated cells with PBS, they were fixed in 4% paraformaldehyde for 20 minutes at 4°C and then washed again with PBS. The cells were then incubated in 0.1% citrate buffer at room temperature for 1 hour, and after washing with PBS, they were incubated in the In Situ Cell Death Detection kit with fluorescein (Roche Diagnostics, Mannheim, Baden-Württemberg, Germany) at 37°C for 1 hour. After washing again with PBS, the nuclei were stained with HOECHST 33342 at room temperature for 15 minutes and washed again with PBS. The specimens were blocked by 5% donkey serum and 0.1% Triton X-100 in PBS for 30 minutes at 4°C. Then, ATH5 staining was performed as described.

**Glial Fibrillary Acidic Protein (GFAP) Expression Assay**

Using our procedure to differentiate RGFs from iPSCs, we did not detect GFAP-positive astrocyte. However, in this assay, we extended the number of days of culture from thirty-four days to fifty days and without collecting and reseeding migrating cells from embryoid bodies by accutase in twenty-three days after the RGCs induction from iPSCs. By modifying the protocol for transforming iPSCs into RGCs as mentioned above we were able to evaluate the GFAP-positive area using immunocytochemistry.

**Drug Assays**

Timolol was obtained from LKT Laboratories (St. Paul, MN, USA). Timolol was dissolved in dimethyl sulfoxide (DMSO) at 10 mM and stocked at −20°C. For treatment with timolol, timolol was serially diluted with differentiation medium. For treatment, differentiation medium was exchanged for the total amount of the medium not containing DMSO. Considering that 0.01% DMSO might not influence OPTNE50K aggregation, cell viability, or autophagic flux, we compared the NT group with the 0.01% DMSO treatment group; 0.01% DMSO did not influence OPTNE50K aggregation, cell viability, or p62 expression in E50K-iPSCs-RGCs (Supplementary Figs. S7A–S7F). Therefore, 0.01% DMSO in the timolol treatment group would not influence OPTNE50K aggregation, cell viability, or p62 expression in E50K-iPSCs-RGCs. For treatment with timolol, timolol treatment group would not influence OPTNE50K aggregation, cell viability, or p62 expression in E50K-iPSCs-RGCs (Supplementary Figs. S7A–S7F). Therefore, 0.01% DMSO in the timolol treatment group would not influence OPTNE50K aggregation, cell viability, or p62 expression in E50K-iPSCs-RGCs.

**RESULTS**

**Transformation of iPSCs Into Cells Positive for Retinal Ganglion Cell Markers**

We first examined whether the iPSCs derived from a control individual (wild-type [WT]-iPSCs, 20IB7 line14) and a patient with glaucoma from an E50K mutation of the optineurin gene (E50K-iPSCs) can be transformed into RGCs using a reported protocol13 with modifications (Fig. 1A). We adopted the SFEBq protocol, with the medium changed every 3 days. For the autophagic flux assay, the cells were exposed to timolol, NF449 (Cayman), and gallein (Tocris Bioscience) for 6 or 24 hours at 34°C after the induction of the transformation. The treated cells were washed with PBS and collected for each of the following assays. For Western blotting, the cells were seeded at 90,000 cells/well in 24-well plates, 360,000 cells/well in 6-well plates, and 5000 cells/well in 96-well plates.

**Western Blot Analyses**

At the end of the culture period, samples were washed with PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris hydrochloride, 150 mM sodium chloride, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Igepal CA-630 and protease (Sigma-Aldrich Corp.) and phosphatase inhibitor (Sigma-Aldrich Corp.) cocktail. The lysates were centrifuged at 11,000g for 10 minutes, and the supernatant (Sup.) and pellet fraction (Ppt.) were separated. The protein concentration was determined by the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL, USA) with bovine serum albumin as the standard. Equal volumes of the lysate and sample buffer containing 20% 2-mercaptoethanol (Wako) were mixed, and the proteins were separated using a 12% SDS-polyacrylamide gel electrophoresis (Wako) or 15% SDS-polyacrylamide gel electrophoresis (Wako). The separated proteins were then transferred onto a polyvinylidene difluoride membrane (PVDF; Immobilon-P; Merck KGaA, Darmstadt, Germany). The immunoreactive bands were made visible with Immunostar LD (Wako), and the intensities of the bands were determined by ImageQuant LAS 4000 (Fuji XEROX, Minato-ku, Tokyo, Japan).

**Statistical Analyses**

Unpaired t-tests were used to determine the significance of differences in two samples. ANOVA followed by Dunnett’s test or Bonferroni test was used to compare the means in multigroup analyses. Data are presented as the means ± standard deviations. The level of statistical significance was set at P < 0.05.

**Establishment of In Vitro Disease Model Using E50K-iPSCs**

To establish an in vitro OPTNE50K-NTG model, we used the transformed iPSCs derived from a control individual (WT-iPSCs-
RGCs #1 and #2) and from a patient with OPTNE50K-NTG (E50K-iPSCs-RGCs #1 and #2). The phenotypes of the generated cells were determined by immunostaining analysis as previously reported. The results showed that the E50K-iPSCs-RGCs #1 and #2 that were ATH5-positive were significantly reduced and the number of TUNEL-positive RGCs was increased by a dysfunction of OPTNE50K. In addition, the GFAP-positive astrocytes were activated in E50K-iPSCs #1 (Figs. 2P–R). These findings suggested that the OPTNE50K mutation promoted apoptosis of the transformed RGCs (Figs. 2A–J).

To confirm that OPTNE50K mutation will cause its aggregation in the Golgi apparatus of the transformed RGCs, we determined the ratio of the OPTN-positive areas to that in the nuclei. The results showed that the OPTN area/nucleus ratio was significantly reduced in E50K-iPSCs-RGCs #1 and #2 compared to WT-iPSCs-RGCs and E50K-iPSCs (Fig. 2C).

**Figure 1.** Transformation of induced pluripotent stem cells (iPSCs) obtained from a normal individual and from an individual with normal-tension glaucoma due to an E50K mutation of the optoneurin gene. (A) Schematic of transformation of iPSCs to cells that are positive to retinal ganglion cell markers. (B) Beginning with the start of adhesive cultures on D34, axons grow out radially from the mass of new cell bodies. Scale bar: 200 μm. (C) Immunostaining using RGC markers, ATH5, BRN3, and THY1, mature neuron marker (SMI-32). Scale bars: 50 μm.
FIGURE 2. Comparisons of WT-iPSCs-RGCs and E50K-iPSCs-RGCs. (A–D) Comparisons of the number of RGCs derived from WT-iPSCs #1 and #2 and E50K-iPSCs #1 and #2 using RGC marker (ATH5). (E) Quantification of the number of surviving RGCs derived from WT-iPSCs #1 and #2 and E50K-iPSCs #1 and #2 by analyzing the number of ATH5-positive cells. Data are the means ± standard error of the means (SEM), n = 3 (*P < 0.05 versus WT-iPSCs-RGCs #1 and #2). (F–I) TUNEL-positive cell proportion in differentiated WT-iPSCs-RGCs #1 and #2 and E50K-iPSCs-RGCs #1 and #2. (J) Quantification of the number of apoptotic RGCs derived from WT-iPSCs #1 and #2 and E50K-iPSCs #1 and #2 by analyzing of TUNEL-positive cells. Data are the means ± SEM, n = 3 (**P < 0.01 versus WT-iPSCs-RGCs #1 and #2). (K–N) Comparisons of forming OPTN aggregation derived from WT-iPSCs #1 and #2 and E50K-iPSCs-RGCs #1 and #2 around Golgi apparatus detected by GM130. (O) Quantification of the OPTN area/nuclei ratio in differentiated WT-iPSCs-RGCs #1 and #2 and E50K-iPSCs-RGCs #1 and #2. Data are the means ± SEM, n = 4 (***P < 0.001 versus WT-iPSCs-RGCs #1 and #2). (P, Q) Immunostaining of GFAP, active astrocyte marker. Scale bars: 50 μm. (R) Quantification of GFAP area. Data are the means ± SEM, n = 4 (*P < 0.05 versus WT-iPSCs-RGCs #1). Scale bars: 50 μm.
noradrenaline (NAd; Figs. 3A, 3B). 20 We also found that the binding immunoglobulin protein (Bip), which is an ER stress marker, was increased in the E50K-iPSCs-RGCs in our culture system (Supplementary Fig. S1).

Effect of Timolol on OPTNE50K Aggregation and Neuroprotection of Transformed iPSCs

To determine a chemical agent that can reduce the degree of OPTNE50K aggregation, we screened different chemicals using the in vitro OPTNE50K-NTG model. We selected chemical compounds such as memantine, brimonidine, latanoprost, lomerizine, and timolol. Some agents are used as IOP-lowering chemical compounds, such as brimonidine, latanoprost, lomerizine, and timolol, and memantine and lomerizine are used as therapeutic agents for other diseases. Memantine was originally licensed for Alzheimer’s disease, thus we evaluated whether memantine would be therapeutic agents for glaucoma in this study. The results showed that timolol, a \( \beta \)-AR antagonist, improved the OPTNE50K aggregation most strongly (Supplementary Fig. S2). In addition, E50K-iPSCs-RGCs #1 were found to express \( \beta_1 \)-AR and dopamine \( \beta \)-hydroxylase (DBH), which are enzymes that convert dopamine to noradrenaline (NAd; Figs. 3A, 3B). 20 We also found that the level of DBH expression was increased in E50K-iPSCs-RGCs #1 (Figs. 3C, 3D).

We examined whether timolol might be a therapeutic agent for OPTNE50K-NTG because of the increase of DBH in E50K-iPSCs-RGCs #1 and the earlier evidence that timolol has neuroprotective effects. 21,22 First, we examined whether timolol can reduce OPTNE50K aggregation. BX795 was used as a positive control because it is a TBK1 inhibitor that is known to reduce OPTN aggregation. 9 The results showed that timolol reduced the degree of OPTNE50K aggregation in a dose-dependent manner in E50K-iPSCs-RGCs #1 (Figs. 3E, 3F).

To confirm that timolol was specific not only for the cell line tested, we differentiated another clone of the E50K-iPS cell line (E50K-iPSCs #1-2), this cell line is derived from the same patient as E50K-iPSCs #1 but another clone which was produced in the process of establishing iPSCs via a different method. We found that timolol also reduced OPTNE50K aggregation in this E50K-iPS RGC cell line (Fig. 3G).

Moreover, timolol and BX795 reduced OPTNE50K aggregation in E50K-iPSCs-RGCs #2 (Supplementary Fig. S2).

It has been reported that OPTNE50K is insoluble and forms aggregates that accumulate in the precipitated fraction (Ppt.) in the RIPA buffer (Fig. 3H, lower). 9 Nonaggregated OPTNE50K was detected in the supernatant fraction in RIPA buffer (Fig. 3H, upper). We found decreased levels of insoluble OPTNE50K and increased levels of soluble OPTNE50K in E50K-iPSCs-RGCs #1 after exposure to timolol and BX795 (Figs. 3H–J). This suggested that timolol will decrease the amount of insoluble OPTNE50K, leading to improving OPTNE50K aggregation.

It has been reported that timolol has neuroprotective effects, 21,22 and our results showed that timolol increased the number of ATH5-positive RGCs and decreased TUNEL-positive RGCs in the E50K-iPSCs-RGCs #1 (Figs. 3K–N) and E50K-iPSCs-RGCs #2 (Supplementary Fig. S3). However, timolol did not show these effects on WT-iPSCs-RGCs #1 and #2 (Supplementary Fig. S4). These results suggest that timolol might show a protective effect mainly on pathologic RGCs such as E50K-iPSCs-RGCs #1 and #2.

Moreover, we analyzed the effect of timolol on neurite outgrowth in E50K-iPSCs-RGCs #1.

Timolol and BX795 enhanced TUJ1-positive neurite outgrowth in E50K-iPSCs-RGCs #1 (Supplementary Figs. S5A, S5B), but they did not change the number of TUJ1-positive cells (Supplementary Fig. S5C).

Mechanisms of Timolol Effect on OPTNE50K Aggregation

OPTNE50K interacted strongly with TBK1 leading to the formation of aggregates, whereas inhibition of TBK1 enhanced the aggregation. 9 Therefore, we investigated whether timolol could inhibit TBK1 by Western blot analysis. Our results showed that timolol did not inhibit TBK1 expression (Supplementary Fig. S6).

Next, we considered the autophagic flux of E50K-iPSCs-RGCs #1 as another mechanism for forming OPTNE50K aggregates. It has been reported that OPTNE50K caused an inhibition of autophagic flux, which would suggest that an inhibition of autophagic flux might cause OPTNE50K aggregation. 9 Our results showed that inhibition of autophagic flux by bafilomycin enhanced OPTN aggregation in WT-iPSCs-RGCs #1 (Supplementary Figs. S7A–S7E). Moreover, the LC3B-II/LC3B-I ratio and p62 expression were increased in E50K-iPSCs-RGCs #1 (Figs. 4A–C), suggesting that autophagic flux is impaired in E50K-iPSCs-RGCs, especially autophagolysosome formation.

Next, we investigated whether timolol can enhance autophagic flux. Our results showed that the LC3B-II/LC3B-I ratio was increased although p62 was not increased at 6 hours after timolol exposure in E50K-iPSCs-RGCs #1 (Figs. 4D–F). In addition, p62 was significantly decreased at 24 hours after timolol exposure in E50K-iPSCs-RGCs #1 (Figs. 4G, 4I).

Mechanisms of Timolol Enhancing Autophagic Flux

Our results showed that the E50K-iPSCs-RGCs expressed \( \beta_1 \)-AR and DBH (Figs. 3A, 3B), which confirmed that timolol might function as a \( \beta \)-agonist in our in vitro OPTNE50K-NTG model. Accordingly, we investigated whether timolol enhanced the autophagic flux through \( \beta \)-AR. There are G-protein-dependent pathways and \( \beta \)-arrestin-dependent pathways in the \( \beta \)-AR signaling pathway. 23,24 We first focused on the G-protein signaling pathways using NF449, a G\( _\text{z} \) protein inhibitor, and gallein, a G\( _\beta \gamma \) protein inhibitor, to determine whether an inhibition of the signaling pathway could enhance the autophagic flux. 25,26 The result showed that p62 was decreased at 24 hours by NF449 and gallein exposure in E50K-iPSCs-RGCs #1 (Figs. 5D–G). Thus, it is speculated that timolol might promote autophagic flux through decreasing intracellular G\( _\beta \gamma \) protein factor, 17,18 because timolol exposure is known to increase FoxO3a, 29 and an overexpression of FoxO3a has the potential of promoting autophagic flux. 30 Thus, we evaluated whether timolol exposure would increase
Figure 3. Effect of timolol on E50K-iPSCs-RGCs. (A) Expression of β1-AR in E50K-iPSCs-RGCs. Scale bars: 50 μm. (B) Expression of DBH in E50K-iPSCs-RGCs. Scale bars: 50 μm. (C, D) Western blot analysis of DBH expression of WT-iPSCs-RGCs #1 and E50K-iPSCs-RGCs #2. Data are the means ± SEM, n = 5 or 6. (E, F) Improvement of OPTNE50K aggregation by timolol in a concentration-dependent manner, using BX795 as a positive control in E50K-iPSCs-RGCs #1. Data are the means ± SEM, n = 4 (**P < 0.01). (G) The improvement of OPTNE50K aggregation on RGCs derived from another clone of E50K-iPSCs #1-(2). Data are the means ± SEM, n = 4 (*P < 0.05). (H–J) Western blot analysis of OPTNE50K aggregation using timolol and BX795. OPTNE50K aggregation was detected in the precipitated fraction (Ppt.) after supernatant (Sup.) collection. Timolol and BX795 exposure was able to restore the OPTNE50K from Ppt. fraction to the Sup. fraction. Data are the means ± SEM, n = 6 to 8 (*P < 0.05). (K) Immunostaining of ATH5-positive RGC analysis using timolol. Scale bars: 50 μm. (L) Immunostaining of TUNEL-positive RGCs using timolol and BX795. (M) Quantification of the ATH5-positive cells in differentiated E50K-iPSCs-RGCs #1. *P < 0.05. (N) Quantification of the TUNEL-positive cells in differentiated E50K-iPSCs-RGCs #1. *P < 0.05.
FoxO3a. The results showed that timolol increased the expression FoxO3a (Figs. 5H, 5I).

Next, we examined the effect of timolol on the β-arrestin signaling pathway. Some β-agonists are known to influence the β-arrestin–dependent signaling pathways, and carvedilol, which is a β-agonist, has the potential of enhancing autophagosome formation. Our results showed that timolol exposure did not affect the β-arrestin signaling pathway (Supplementary Fig. S8).

Figure 4. Enhancement of autophagic flux by timolol in E50K-iPSCs-RGCs. (A–C) Western blot analysis of LC3B (LC3B-II: autophagosome marker) and p62 (autophagolysosome marker) and quantification of LC3B-II/LC3B-I and p62 in WT-iPSCs-RGCs and E50K-iPSCs-RGCs. Data are the means ± SEM, n = 4 or 5 (*P < 0.05, **P < 0.01). (D–F) Western blot analysis of LC3B and p62 in E50K-iPSCs-RGCs 6 hours after timolol and BX795 exposure. (G–I) Western blot analysis of LC3B and p62 in E50K-iPSCs-RGCs 24 hours after timolol and BX795 exposure. Data are the means ± SEM, n = 8 (*P < 0.05). N.S., not significant.

Autophagic Flux on Motor Neurons Derived From OPTNE50K-iPSCs

The OPTNE50K mutation is known to cause the death of RGCs, leading to glaucoma. There are some reports that while other OPTN mutations, such as OPTNQ398X and OPTNE478G, cause amyotrophic lateral sclerosis (ALS), OPTNE50K does not induce ER stress or form aggregation in NSC-54 cells, a motor neuron-like hybrid cell line. We asked why motor
neurons (MNs) fail to form OPTNE50K aggregation. Inhibition of autophagic flux by OPTNE50K is identified as one of the factors leading to RGC death.9,16,17 Therefore, we evaluated autophagic flux on WT-iPSCs-MNs and E50K-iPSCs-MNs. MNs were differentiated by an earlier protocol using HB9,35 a MN marker (Supplementary Figs. S9A, S9B), in WT and E50K-iPSCs-MNs. Our results showed an increase in the LC3B-II/LC3B-I ratio and decrease in the p62 expression (Supplementary Figs. S9C–S9F).

DISCUSSION

The results showed that we were able to develop a human in vitro model of OPTNE50K-NTG as reported previously.9,16,17 Therefore, we evaluated autophagic flux on WT-iPSCs-MNs and E50K-iPSCs-MNs. MNs were differentiated by an earlier protocol using HB9,35 a MN marker (Supplementary Figs. S9A, S9B), in WT and E50K-iPSCs-MNs. Our results showed an increase in the LC3B-II/LC3B-I ratio and decrease in the p62 expression (Supplementary Figs. S9C–S9F).

Using this model, we found that 1 μM timolol, a β-AR antagonist, significantly reduced the OPTNE50K aggregation. The concentration of timolol in the vitreous humor can be elevated to approximately 50 nM by topical application of timolol.36 Hence, a higher concentration of timolol would be necessary to reduce the OPTNE50K aggregation. Nevertheless, these results suggest that timolol has the potential of a direct neuroprotective effect on RGCs. In addition, BX795, which also reduces OPTNE50K aggregation, had neuroprotective effects on E50K-iPSCs-RGCs. These findings suggest that an agent that reduces OPTNE50K aggregation could prevent E50K-iPSCs-RGCs from its toxic effects.

We also found that OPTN aggregation was enhanced by bafilomycin, which inhibited autophagolysosomy, and that autophagic flux was reduced in E50K-iPSCs-RGCs. These findings suggested that the OPTNE50K-induced autophagic flux impairment might result in the formation of OPTNE50K...
aggregation. Therefore, we suggest that promoting autophagic flux will decrease the OPTNE50K aggregation and its intracellular toxicity. This suggestion is supported by the finding that autophagic flux was enhanced in E50K-iPSCs-MNs. These results indicate that the enhancement of autophagic flux could reduce the formation of OPTNE50K aggregation and its intracellular toxicity. Although it has not been reported that OPTNE50K induces motor neuronal death, other mutations such as OPTNQ398X and OPTNE478G lead to MN diseases. These findings suggest that the OPTNE50K-induced autophagic flux impairment could not be evoked in MNs but can in RGCs. Therefore, improving OPTNE50K-induced autophagic flux impairment should be important for avoiding RGC disorders.

Our results also showed that timolol might enhance autophagic flux leading to improvements of OPTNE50K aggregation. Considering that β-AR couples with Gα and Gβγ protein, we investigated the effect of Gα protein inhibitor NF449 and Gβγ protein inhibitor gallein on autophagic flux. Exposure to NF449 and gallein enhanced autophagolysosomy as had timolol exposure. From these findings we speculate that timolol might promote autophagic flux by inhibiting β-AR (Fig. 6).

We investigated the Gα and Gβγ protein downstream factors, especially cAMP and FoxO3a. Our results showed that cAMP inhibited autophagic flux and timolol exposure increased FoxO3a, which enhances autophagic flux. These results suggested that timolol enhanced autophagic flux by decreasing cAMP and increasing FoxO3a. Interestingly, the level of DBH was increased in E50K-iPSCs-RGCs, suggesting that most of the NAd might be contained in the E50K-iPSCs-RGCs in the culture medium. Considering these results, excessive NAd and stimulation of β-AR signaling might inhibit autophagic flux.

It has been suggested that the molecular mechanism of OPTNE50K-induced autophagic flux impairment is the OPTNE50K inactivation of Rab8, a small GTPase that is involved in vesicular traffic, because of enhanced recruiting of TBC1D17, a GTPase-activating protein. Although it is not clear that the interactions of OPTNE50K with Rab8 and TBC1D17 are involved in autophagic flux, TBC1D17 knockdown improves OPTNE50K-induced autophagic flux impairment. This would suggest that regulation of TBC1D17 may be important for autophagic flux in eyes with OPTNE50K-NTG.

In conclusion, our results showed that timolol can reduce OPTNE50K aggregation and axonal degeneration by enhancing autophagic flux. However, timolol does not significantly prevent visual field defects in some patients. One of the reasons might be that the concentration of timolol reaching the RGCs by topical application may be too low. Considering our results, further improvement in the movement of timolol into the retina by a drug delivery system might be expected to increase the clinical therapeutic effects of timolol.

**Figure 6.** Model showing steps involved in improving OPTNE50K aggregation through promoting autophagic flux. Timolol inhibits β-adrenergic receptor and Gα/βγ-protein inhibition-induced autophagic flux. Promoting autophagic flux contributes to improving the OPTNE50K aggregation.
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