

# Identification of the Stress Which Causes Optineurin Aggregation

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## Abstract

Glaucoma is a common neurodegenerative disease that can cause blindness and occurs worldwide. Currently, lowering intraocular pressure is the only therapy available to protect retinal ganglion cells (RGCs). However, this therapy does not prevent RGC death in all patients. Therefore, new therapeutic approaches for glaucoma are urgently required, and neuroprotection of RGCs is a focus for many researchers. Optineurin (OPTN) is one of the normal tension glaucoma (NTG) relative genes, while mutant OPTN can form a characteristic aggregation, causing RGC death. Hence, elucidation of the mechanism of OPTN aggregation might provide a clue to help understand RGC death. To examine whether non-mutant OPTN could also aggregate, we pharmacologically induced some glaucoma-related stresses, such as endoplasmic reticulum (ER) stress, glutamate toxicity, activation of TNF- $\alpha$  signaling, mitochondrial dysfunction, and autophagic flux impairment. Our results showed that ER stress, TNF- $\alpha$  signaling, and autophagic flux are involved in OPTN aggregation. Furthermore, our data indicated that increased ER stress, activation of TNF- $\alpha$  signaling, and impaired autophagic flux induce OPTN aggregation, suggesting that OPTN aggregation might be an important therapeutic target not only for familial NTG with mutated OPTN but also for patients with glaucoma more generally.

## Keywords

Glaucoma, Induced Pluripotent Stem Cells, Retinal Ganglion Cells, Optineurin, Protein Aggregation

## 1. Introduction

Glaucoma is characterized by the progressive loss of retinal ganglion cells (RGCs) and their axons, and is one of the leading causes of irreversible blindness worldwide [1]. Primary open-angle glaucoma (POAG) is the most common type of glaucoma. POAG is classified into two subsets: high tension glaucoma (HTG) and normal tension glaucoma (NTG). The only therapy currently available for POAG is the lowering of intraocular pressure (IOP). However, some individuals develop glaucoma when their IOP is in the normal range [2] [3]. In addition, NTG is the major subtype that occurs in patients with glaucoma in Japan [4]. It has been suggested that glaucoma is caused not only by high IOP but also by other, unknown risk factors in its pathology [5].

In research undertaken to discover the complex mechanisms of glaucoma pathology, genetic and genomic studies have accelerated the discovery of genes that contribute to glaucoma. It is reported that optineurin (*OPTN*) gene mutations, such as E50K ( $OPTN^{E50K}$ ) and M98K ( $OPTN^{M98K}$ ), are associated with NTG, and that patients with these mutations exhibited severe glaucoma symptoms [6]-[11]. The *OPTN* protein plays multiple roles in various processes, such as autophagic flux [12] [13], where it is an important regulator, and NF- $\kappa$ B signaling [14] [15]. However, it has been reported that both  $OPTN^{E50K}$  and  $OPTN^{M98K}$  mutations result in protein aggregation [16] [17], and that  $OPTN^{E50K}$  interacts more strongly with TBK1 and causes autophagic flux dysregulation, leading to RGC degeneration [17] [18] [19] [20] [21]. Hence, we explored the use of chemical agents to reduce *OPTN* aggregation, and found that timolol has the potential to decrease *OPTN* aggregation and shows neuroprotective effects in familial NTG patients with  $OPTN^{E50K}$  iPSC-derived RGCs (E50K-iPSCs-RGCs) [20]. This work demonstrated that *OPTN* aggregation, which is the common phenotype among  $OPTN^{E50K}$  and  $OPTN^{M98K}$ , might be an important pathology and a potentially useful therapeutic target in cases of familial glaucoma caused by *OPTN* mutation.

Hence, we examined whether *OPTN* aggregation might result not only from *OPTN* mutation but also following stress associated with RGC death in some glaucoma pathologies.

## 2. Materials and Methods

### 2.1. Ethical Considerations

The research followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all participants after explaining to them the nature and possible consequences of the study. The procedures used for the pathological 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, Japan. The established human iPSCs 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.

## 2.2. Differentiation of Induced Pluripotent Stem Cells (iPSCs) into Retinal Ganglion Cells (RGCs) in Culture

In this study, we used the 201B7 iPSC line. This line was provided by Kyoto University [22]. The culture of isolated iPSCs and the production of embryoid bodies using the quick reaggregation (SFEBq) method was performed as previously described [22] [23] [24]. To create mimics of normal RGCs, we used a previously described, modified protocol [20] [25]. Briefly, the day after the culture was started, the culture medium was changed to a differentiation medium consisting of Dulbecco's Modified Eagle Medium/F12 (Invitrogen), 1% N2 supplement (Invitrogen), B27 supplement, L-glutamine, 500 U/ml penicillin/streptomycin (Invitrogen), 2  $\mu$ M dorsomorphin (Sigma-Aldrich), 10 ng/ml human Dickkopf 1 (R & D Systems, Minneapolis, USA), 10 ng/ml insulin-like growth factor-1 (R & D Systems), and 10 ng/ml bFGF (R & D Systems), and cultured for 7 days in 5% CO<sub>2</sub> at 37°C. Neuronal precursor cells were cultured in differentiation medium containing 10  $\mu$ M N-[(3)[5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethyl ester (DAPT; Tocris Bioscience, Avonmouth, UK) for a further 7 days to retinal ganglion cell lineage as previous report [26]. For the final stage, the neuronal precursor cells were cultured with the addition of 2 ng/ml acidic fibroblast growth factor (R & D Systems) to the differentiation medium for 11 days. The medium was changed every 2 or 3 days for all differentiation stages. Finally, all cells except the embryoid bodies were reseeded onto Matrigel-coated plates.

## 2.3. 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) and phosphatase inhibitor (Sigma-Aldrich) cocktails. The lysates were centrifuged at 12,000 g for 10 min at 4°C. The protein concentration was determined using a bicinchoninic acid assay protein assay kit (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 5% - 20% SDS-polyacrylamide-gel electrophoresis (Wako). The separated proteins were then transferred onto a polyvinylidene difluoride

**Table 1.** Antibodies: All antibodies for the biochemical analysis were purchased from the following companies and used at the following dilutions.

Antibody	Source	Dilution
anti-OPTN	Cayman	1:200
anti-TBK1	Cell Signaling Technology	1:1000
anti- $\beta$ -actin	Sigma Aldrich	1:2000
anti-LC3B	Cell Signaling Technology	1:1000
Anti-p62	Cell Signaling Technology	1:1000

membrane (PVDF, Immobilon-P; Merck KGaA, Darmstadt, Germany). The immunoreactive bands were made visible using ImmunoStar<sup>®</sup> LD, and the intensities of the bands were determined by ImageQuant LAS 4000. For the OPTN aggregation assay, we followed a previously reported protocol [17]. Briefly, we lysed cells using TNE buffer and centrifuged the lysates at 15,000× g for 15 min. Then, we separated the supernatant and precipitated the insoluble pellet fraction (TNE insoluble fraction), and equal volumes of the lysate were used for the assay. Finally, we used appropriate antibodies (Table 1).

#### 2.4. Evaluation of Various Compounds That Induce Stress in WT-iPSCs-RGCs

All compounds tested were purchased from the companies and used at the dilutions shown (Table 2). In this assay, the vehicle group contained 0.1% DMSO. We treated WT-iPSCs-RGCs with each chemical or protein for 24 h.

#### 2.5. Statistical Analyses

Data are presented as means ± standard error of the means (SEMs). Unpaired Student's *t*-tests were used to determine if there were significant differences between two samples. ANOVA, followed by Dunnett's test or the Bonferroni test, was used to compare means in multigroup analyses. The level of statistical significance was set at  $P < 0.05$ .

### 3. Results

#### Identification of Stresses That Induce OPTN Aggregation

To evaluate whether OPTN might aggregate in WT-iPSCs-RGCs following the induction of pharmacological stresses related to RGC degeneration, we evaluated OPTN protein levels in the TNE buffer insoluble fraction, as previously described [17]. RGC death in patients with glaucoma or experimental animal models is related to elevated endoplasmic reticulum (ER) stress [27] [28] [29], increased excitatory glutamate [30] [31], TNF- $\alpha$  signaling [32] [33] [34] [35], oxidative stress [36], mitochondrial dysfunction [21] [36] [37], and abnormal autophagic flux [38] [39] [40]. Based on this knowledge, we treated WT-iPSCs-RGCs with one of six compounds (Table 2) for 24 hours to explore if any of the agents

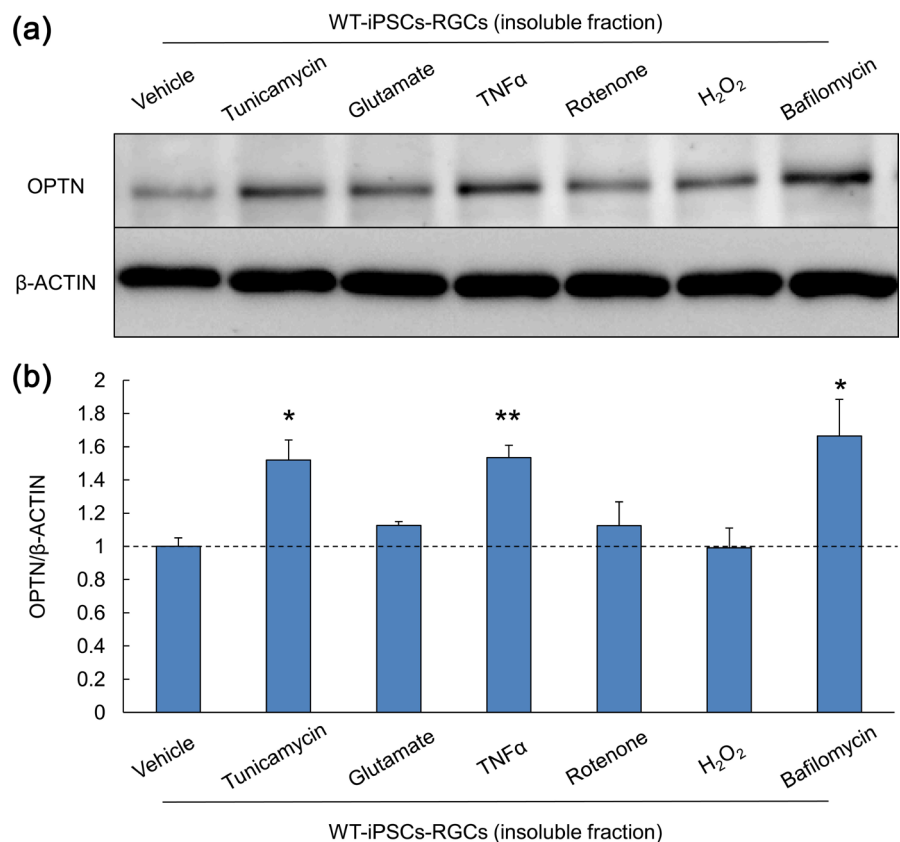
**Table 2.** Compounds that induce stress in WT-iPSCs-RGCs.

Compound	Source	Dilution
Tunicamycin	FUJIFILM Wako	2 $\mu$ g/ml
Glutamate	FUJIFILM Wako	200 $\mu$ M
TNF- $\alpha$	Merck KGaA	12.5 ng/ml
Rotenone	FUJIFILM Wako	0.1 $\mu$ M
H <sub>2</sub> O <sub>2</sub>	FUJIFILM Wako	100 $\mu$ M
Bafilomycin	Tocris Bioscience	100 ng/ml

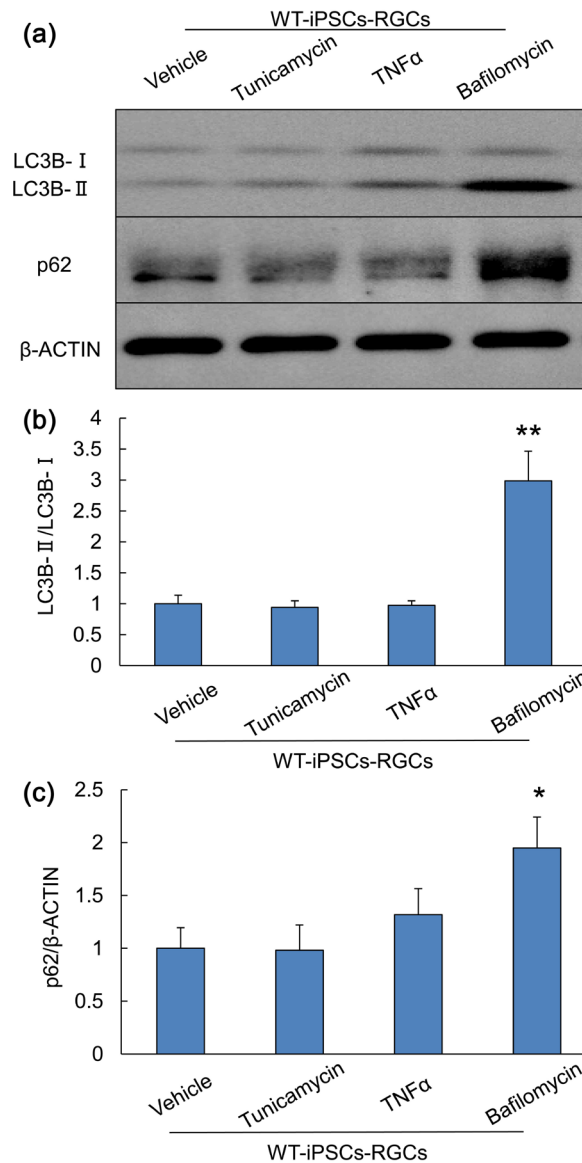
induced OPTN aggregation. In this assay, we evaluated tunicamycin (an ER stress inducer), glutamate, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), rotenone (a mitochondrial dysfunction inducer), H<sub>2</sub>O<sub>2</sub>, and bafilomycin (an autophagolysosome inhibitor). Our previous study demonstrated that bafilomycin induces OPTN aggregation [20], therefore we used it as a positive control. Their concentration is determined as inducing about 20% - 30% of cell death [29] [41] [42] [43] [44]. Tunicamycin, TNF- $\alpha$ , and bafilomycin increased OPTN aggregation in WT-iPSCs-RGCs (Figure 1(a) and Figure 1(b)), suggesting that OPTN aggregation occurs following ER stress, impairment of autophagolysosomes, and in the presence of TNF- $\alpha$ .

#### Identification of the Mechanism Involved in OPTN Aggregation

To investigate the mechanism underlying OPTN aggregation, we first evaluated autophagic flux and TBK1 expression, because OPTN<sup>E50K</sup> impairs autophagolysosome and interacts strongly with TBK1, leading to aggregation of OPTN [45]. We found that bafilomycin increased the ratio of LC3B-II/LC3B-I (autophagosome markers, Figure 2(a) and Figure 2(b)) and the expression of p62 (an autophagolysosome marker, Figure 2(a) and Figure 2(c)), suggesting that bafilomycin impaired autophagic flux, leading to OPTN aggregation (Figure 4). However, tunicamycin and TNF- $\alpha$  had no influence on the LC3B-II/LC3B-I ratio or p62



**Figure 1.** Identification of the stress of OPTN aggregation in WT-iPSCs-RGCs. (a) Typical images showing western blotting analysis of TNE-buffer insoluble (aggregated) OPTN protein levels; (b) Quantitative data showing OPTN protein levels. Data are means  $\pm$  SEMs, n = 7 (\*\* $P$  < 0.01, \* $P$  < 0.05 vs. vehicle, Student's  $t$ -test).

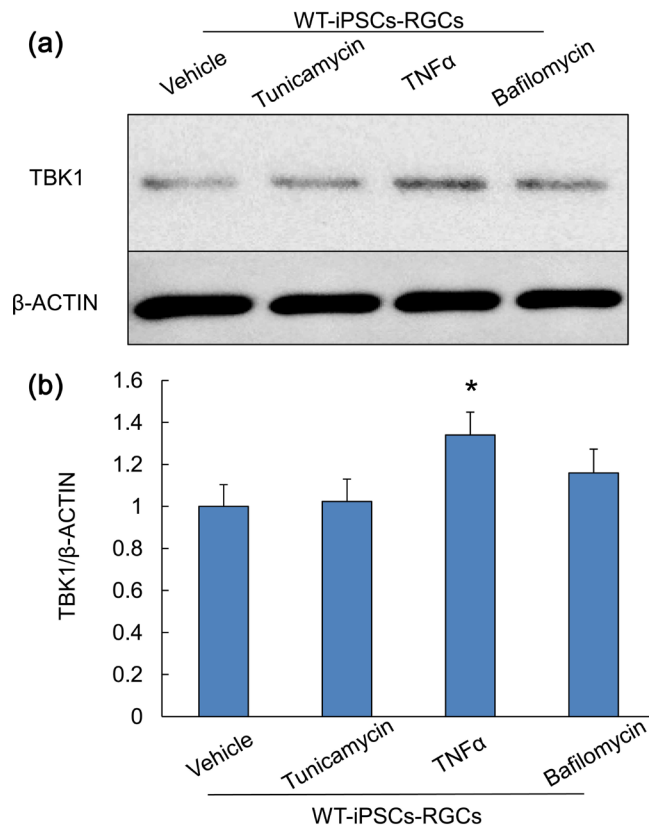


**Figure 2.** Autophagic flux assay with tunicamycin, TNF $\alpha$ , and bafilomycin. (a) Typical images showing western blotting analysis of LC3B-II, LC3B-I, and p62 protein levels; (b) Quantification of protein expression of LC3B-II, LC3B-I ratio, and p62. Data are means  $\pm$  SEMs,  $n = 7$  (\*\* $P < 0.01$ , \* $P < 0.05$  vs. vehicle, Student's  $t$ -test).

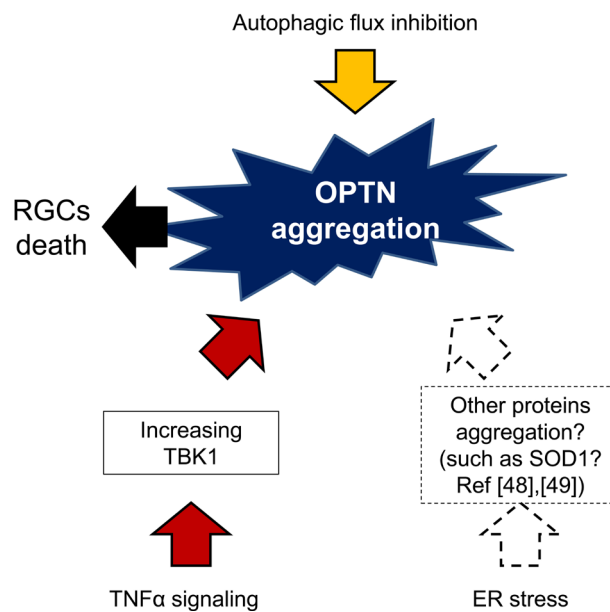
expression, suggesting that they had no influence on autophagic flux in this assay (Figures 2(a)-(c)). Therefore, we next evaluated the influence of stress on TBK1 expression (Figure 3). Although tunicamycin and bafilomycin had no effect on TBK1 expression, TNF- $\alpha$  had the potential to increase TBK1 expression (Figure 3(a) and Figure 3(b)), suggesting that TNF- $\alpha$  increased TBK1 expression, leading to OPTN aggregation (Figure 4).

#### 4. Discussion

A previous study found that OPTN<sup>E50K</sup>, a mutated form of OPTN, aggregates, and that this aggregation might cause the death of RGCs [45]. In this study, we



**Figure 3.** TBK1 protein assay with tunicamycin, TNF $\alpha$ , and bafilomycin. (a) Typical images showing western blotting analysis of TBK1 protein levels; (b) Quantification of protein expression of TBK1. Data are means  $\pm$  SEMs, n = 7 (\* $P$  < 0.05 vs. vehicle, Student's  $t$ -test).



**Figure 4.** The scheme of OPTN aggregation formation. OPTN aggregation was produced by tunicamycin-induced ER stress, TNF $\alpha$ -induced increased TBK1 expression, and bafilomycin-induced autophagic flux impairment. In this figure, the arrows showing broken line means speculation based on the previous reports Ref. [48] and [49].

demonstrated that OPTN<sup>WT</sup> aggregation was also caused by ER stress, TNF $\alpha$  signaling activation, and autophagy dysfunction. Considering that these stresses have been linked with glaucoma pathology, in both patients and experimental models, we suggest that OPTN aggregation might occur in RGCs without OPTN mutation. This could be a very important finding, as it implies that OPTN aggregation might possibly be involved not only in patients with familial glaucoma with OPTN but also patients with glaucoma who lack OPTN<sup>E50K</sup>. Based on this, it is important to elucidate the mechanism by which OPTN aggregates. Our previous study suggested that promoting autophagic flux decreased OPTN aggregation [20]. Thus, the promotion of autophagic flux could be a therapeutic target. TNF $\alpha$  also stimulated OPTN aggregation by increasing the expression of TBK1. A previous report suggested that TBK1 is present downstream of the TNF $\alpha$  receptor [46]. Thus, attenuation of TNF $\alpha$  signaling-induced increases in TBK1 expression might also be a therapeutic target to prevent OPTN aggregation. We were unable to identify the mechanism behind tunicamycin-induced OPTN aggregation because autophagic flux and TBK1 expression did not change. Tunicamycin induces ER stress via the inhibition of protein glycosylation. However, it has been reported that OPTN is not glycosylated [47]. Hence, tunicamycin might not directly influence OPTN conformation. Tunicamycin is also known to induce protein aggregation of SOD1 without its mutation [48]. In addition, OPTN has the potential to bind aggregated proteins such as mutant SOD1 and huntingtin, and OPTN deficiency accelerates the aggregation of these proteins [49]. Therefore, when tunicamycin treatment produces aggregated proteins, such as SOD1 and huntingtin, OPTN might interact with these proteins, causing their aggregation (Figure 4). However, further studies are needed to demonstrate this hypothesis.

Our study demonstrated that glaucoma-related stresses can lead to aggregation of non-mutated OPTN. This knowledge might be important to elucidate novel pathologies of glaucoma.

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### Conflicts of Interest

The authors declare that they have no conflicts of interest.

### Author Contributions

SI, MS, and HH designed the experiments. SI, JS, CK, KO, SA, performed the experiments. MF, SN, MS, HK, and HH supervised the study. SI wrote the manuscript. All authors discussed the results and commented on the manuscript.



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