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Optineurin E50K triggers BDNF deficiency-mediated mitochondrial dysfunction in retinal photoreceptor cell line

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Abstract

Optineurin (OPTN) mutations are linked to glaucoma pathology and E50K mutation shows massive cell death in photoreceptor cells and retinal ganglion cells. However, little is known about E50K-mediated mitochondrial dysfunction in photoreceptor cell degeneration. We here show that overexpression of E50K expression triggered BDNF deficiency, leading to Bax activation in RGC-5 cells. BDNF deficiency induced mitochondrial dysfunction by decreasing mitochondrial maximal respiration and reducing intracellular ATP level in RGC-5 cells. However, BDNF deficiency did not alter mitochondrial dynamics. Also, BDNF deficiency resulted in LC3-mediated mitophagosome formation in RGC-5 cells. These results strongly suggest that E50K-mediated BDNF deficiency plays a critical role in compromised mitochondrial function in glaucomatous photoreceptor cell degeneration.

Keywords

Glaucoma; Optineurin; E50K mutation; BDNF; Mitochondrial dysfunction; RGC-5 cell

1. Introduction

Primary open angle glaucoma (POAG) is characterized by a slow and progressive degeneration of retinal ganglion cells (RGCs) and optic nerve damage that lead to vision loss

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Conflicts of interest

All authors declare no conflict of interests.

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[1]. Although controversial [2, 3], there are several studies that have demonstrated that loss of photoreceptors is associated with patients with POAG and experimental glaucoma [4-6]. Nevertheless, the pathophysiological mechanisms of glaucomatous photoreceptor cell degeneration are currently not well characterized.

Among the various mutations of *optineurin (OPTN)*, the E50K is the most prevalent mutant form that is associated with POAG [7]. OPTN is a highly expressed protein in the retina and has ubiquitous effects. In particular, it has been shown to be involved in the maintenance of Golgi organization [8], regulation of nuclear factor kappa B (NF-κB) signaling [9] and induction of autophagy and/or mitophagy [10,11]. Recent studies, including those from our laboratory, have demonstrated that E50K mutation triggers not only age-related RGC loss but also photoreceptor cell degeneration, resulting in functional visual impairment in transgenic mice [12-15].

There is accumulating evidence demonstrates that BDNF protects not only RGCs but also photoreceptor cells against glaucomatous damage [16-18] and rescues retinal function in a mouse model of glaucoma [19]. Our recent study has demonstrated that both retina of E50K transgenic ($E50K^{-tg}$) mice and RGCs overexpressing E50K *in vitro* induced BDNF deficiency [14]. Of note, BDNF regulates mitochondrial function by changing respiratory efficiency [20]. However, the mitochondrial pathogenic mechanism underlying the E50K mutation-mediated BDNF alteration in glaucomatous photoreceptor cell degeneration remains unclear.

We here report that E50K mutation triggers BDNF deficiency-mediated mitochondrial bioenergetic dysfunction and mitophagosome formation in a photoreceptor cell line, RGC-5 cells.

2. Materials and methods

2.1. Cell culture

RGC-5 cell culture was performed as described previously [21]. Briefly, the cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, US) at 5% CO_2 and 37 °C.

2.2. Transfection of RGC-5 cells

Transfection of RGC-5 cells was performed as described previously [22]with some modifications. Briefly, 100 μl of Amaxa™ Basic Glial cells Nucleofector™ Solution (Lonza, US) was mixed with 8×10^6 cells and 2~4 μg of each plasmid or siRNAs, and then the cells were electroporated using an Amaxa™ Nucleofector II Device (Lonza).

2.3. Cell viability assay and cellular ATP measurement

RGC-5 cells (0.5×10^4 per well) were plated on a 96-well plate. Cell viability was measured using 3-[4, 5-dimethylthiazol-2yl]-2, 5-diphenyl tetrazolium bromide (MTT) and a microplate reader (Spectra MAX; Molecular Devices Corp., US) [23]. The cellular ATP

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level was measured using a luciferase-based assay kit (Promega Corp., US) and a microplate luminometer (GMI Inc., US) [23].

2.4. Measurement of mitochondrial respiration

RGC-5 cells (2×10^4 per well) following 48 h transfection were seeded into Seahorse XF24-well plates approximately 6 h before the measurement. Oxygen consumption rate was measured using an XF24 analyzer (Agilent, US). After measuring the basal respiration, oligomycin (2 μg/ml), an inhibitor of ATP synthesis and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 0.3 μM), the uncoupler, were sequentially added to measure maximal respiration.

2.5. Quantitative real-time RT-PCR.

cDNAs were amplified using MX3000P (Stratagene, US) real-time PCR system with iQ™ SYBR Green super-mix (Bio-Rad, US) and BDNF (Forward; 5'-gcggcagataaaaagactgc-3' and Reverse; 5'-cttatgaatcgccagccaat-3') primers for 40 cycles [initial incubation at 50°C for 2 min and then at 95°C for 10 min, and 40 cycles (95°C for 15 sec, 55°C for 1 min and 72°C for 1 min)]. GAPDH mRNA, an internal control, was amplified along with the target genes, and the *Ct* value of GAPDH was used for normalization [14].

2.6. Western blot analysis

Western blot analysis was performed as described previously [23]. Briefly, RGC-5 cells were harvested and lysed for 30 min on ice with a modified RIPA lysis buffer (150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, 1 mM DTT, 0.5% sodium deoxycholate and 50 mM Tris-Cl, pH 7.6), containing the complete protease inhibitors. Primary antibodies included Bax (6A7; 1:1,000; Santa Cruz Biotechnology, US), BDNF (1:1,000; Santa Cruz Biotechnology), phosphocyclic adenosine monophosphate response element-binding protein (CREB, Serine 133) (1:1,000; Life Technologies, Grand Island, US), cyclophilin D (CypD) antibody (1:1,000; Life Technologies), dynamin-related protein 1 (DRP1) (1:5,000, BD Transduction Laboratories, US), phospho-DRP1 (Serine 616) (1:1,000, Cell Signaling, US), microtubule-associated protein 1A/1B-Iight chain 3 (LC3) (1:3,000; MBL International, US), OPTN (1:1,000; Santa Cruz Biotechnology), total oxidative phosphorylation (OXPHOS) complex (Cx) (containing a mixture of antibodies to CxI-IV and ATP synthase, 1:4000; Life Technologies), optic atrophy type 1 (OPA1) (1:5,000; BD Transduction Laboratories), mitofusin (Mfn)1 and 2 (1:3,000; Abcam), huntingtin (HTT) (1:2,000, Millipore) and actin (1:10,000; Millipore, US). The images were captured and quantified by using ImageQuant™ LAS 4000 system and Image Quant TL 8.1 Software Package (GE Healthcare Bio-Science, US) and the band densities were normalized to the band densities for actin.

2.7. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (Sigma, US) as previously described [24]. Briefly, cells were incubated with LC3 (1:500; MBL International) for 16 h at 4°C and then images were acquired with confocal microscopy (Olympus Fluo View 1000; Olympus, Japan).

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2.8. Transmission electron microscopy

Cells were fixed with 2% paraformaldehyde, 2.5% glutaraldehyde (Ted Pella, US) in 0.15 M sodium cacodylate (pH 7.4) and were prepared as previously described [23, 25], Ultrathin (70nm) sections were evaluated by a FEI spirit transmission EM operated at 120kV equipped with 2048 × 2048 pixel CCD camera.

2.9. Electron microscope tomography

For quantitative analysis, the number of mitochondria was normalized to the total area occupied by axons in each image, which was measured using ImageJ (NIH; http://rsb.info.nih.gov/ij/) [23, 25]. For each reconstruction, a double-tilt series of images at 1-degree tilt increments was collected with a FEI titan intermediate-voltage electron microscope operated at 300 kV and equipped with a 4096 × 4096 pixel CCD camera. The IMOD package was used for rough alignment with the fine alignment and reconstruction performed using the TxBR package.

2.10. Statistical analysis

Data were presented as the mean ± SD. Comparison of two conditions was evaluated using the unpaired, two-tailed Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Overexpression of OPTN E50K triggers BDNF deficiency in RGC-5 cells

We found that overexpression of E50K significantly decreased BDNF gene expression in RGCs by 44.71 ± 3.29 % and RGC-5 cells by 12.66 ± 4.59 % (Fig. 1A). Consistently, overexpression of E50K significantly decreased BDNF by 0.48 ± 0.03-fold in RGC-5 cells compared with control (Fig. 1B). Since endogenous BDNF can be regulated by CREB [26] and wild-type HTT [27], we also found that overexpression of E50K mutation significantly decreased HTT protein expression by 0.82 ± 0.02-fold and phosphorylation of CREB at serine 133 (p-CREB S133) by 0.81 ± 0.03-fold in RGC-5 cells (Fig. 1B).

3.2. BDNF deficiency activates Bax but does not increase CypD expression in RGC-5 cells

We found that BDNF deficiency significantly increased active Bax protein expression by 1.80 ± 0.25-fold in RGC-5 cells but did not affect the expression levels of total Bax and p-CREB S133 (Fig. 1C), suggesting that p-CREB S133 is an upstream regulator of BDNF. In addition, BDNF deficiency did not alter the expression level of CypD protein, which has a critical role in mitochondrial permeability transition pore opening-mediated apoptosis [28], in RGC-5 cells (Fig. 1C).

3.3. BDNF deficiency results in bioenergetic dysfunction but did not alter mitochondrial dynamics-related protein expression in RGC-5 cells

We found that there were no statistically significant differences in expression levels of total DRP1 and phosphorylation of DRP1 at serine 616, which is related to mitochondrial fission,

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as well as OPA1 and mitofusins 1 and 2, which is related to mitochondrial fusion, in RGC-5 cells transfected with BDNF siRNA (Fig. 2). However, bioenergetics profile analysis showed that BDNF deficiency significantly decreased the maximal respiration by 121 ± 14 pmols/min compared with control cells by 169 ± 2 pmols/min (Fig. 3A). However, there was no statistically significant difference in the basal respiration between the two groups. Consistently, we observed that BDNF deficiency significantly decreased cell viability by 18.5 ± 2.5% (Fig. 3B). In addition, we found that BDNF deficiency significantly decreased the intracellular ATP level by 42.24 ± 1.85 pmol/cell) in RGC-5 cells compared with control cells (49.62 ± 2.07 pmol/cell) (Fig. 3C).

3.4. BDNF deficiency triggers mitophagosome formation in RGC-5 cells

We found that BDNF deficiency significantly increased the expression level of LC3-II protein, a marker for autophagosome formation, by 1.67 ± 0.09-fold and increased LC3 punta formation in RGC-5 cells. However, there was no statistically significant difference in the expression level of p62 protein between the two groups (Fig. 4A and B). In agreement with this finding, representative images from electron tomography analyses displayed mitophagosomes that contained well-defined cristae in the degenerating mitochondria enveloped in autophagosomes in RGC-5 cells transfected with BDNF siRNA (Fig. 4C and D).

4. Discussion

Based on our previous findings of E50K mutation-mediated degeneration of photoreceptors cells as well as BDNF deficiency and mitochondrial dysfunction in the retina of aged E50K^{-tg} mice [13-15], we investigated E50K-mediated BDNF deficiency and its relevance with mitochondrial dysfunction in a photoreceptor cell line, RGC-5 cells. In the present study, we report that overexpression of E50K triggers BDNF deficiency as well as Bax activation, bioenergetic impairment and mitophagosome formation in RGC-5 cells.

Since the studies of electrophysiology [29, 30] and pattern of color vision loss [31, 32] in patients with POAG suggested that glaucoma damage causes not only degeneration of inner retina, but also impairment of the outer retina, accumulating evidence demonstrates that glaucoma damage is associated with the loss of photoreceptors [4-6, 33, 34]. Specifically, swelling of red- and green-sensitive cones photoreceptors, but not rod photoreceptors, were prominent in the retina tissues from patients with POAG and non-human primates with experimental glaucoma [5]. Furthermore, human eyes with secondary angle-closure glaucoma significantly had reduced the photoreceptor count [6]. Of note, E50K mutation selectively induced cell death in RGC-5 cells, but not in several other cell lines tested, including IMR32, a neuronal cell line [35]. The RGC-5 cell line was originally introduced as an immortalized RGC [36]. However, the re-characterization of this cell line provided the evidence of the identifying it as the 661W photoreceptor cell line, a mouse SV-40 T antigen transformed cone photoreceptor cell line [37-39]. Therefore, we here used RGC-5 cells to determine the effect of the E50K mutation on BDNF expression and mitochondrial dysfunction in photoreceptor cells *in vitro*.

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In the current study, BDNF mRNA and protein expression were found to be reduced in RGC-5 cells overexpressing E50K. BDNF is known to protect photoreceptor cells against light damage or oxidative stress [16, 17], and RGCs [26, 40] against glaucomatous damage. Its protective effect is regulated through the activation of survival signaling pathways such as the extracellular signal-regulated kinases 1/2 and the phosphatidylinositol-3 kinase/Akt pathways in a CREB dependent manner in photoreceptor and RGCs [26, 41, 42]. On the other hand, HTT plays a role in regulating BDNF transcription level [27] and its vesicular transport [43]. Also, HTT directly binds to the C-terminal (amino acids 411–461) region of OPTN [44, 45], suggesting that HTT-mediated OPTN deficiency is associated with the vulnerability of striatal neurons in a mouse model of Huntington's disease [46]. In agreement with these results, the findings of the reduction of HTT, p-CREB S133 and BDNF by overexpression of E50K strongly suggest that HTT/p-CREB S133-mediated BDNF deficiency is an important pathophysiological mechanism in E50K mutation-mediated glaucomatous photoreceptor cell degeneration.

Bax mediates mitochondrial outer membrane permeabilization (MOMP) and contributes to retinal cell susceptibility in glaucomatous neurodegeneration [47, 48]. In aged E50K^{-tg} mice, a significant increase in Bax protein expression was also noted in retinal extracts.[14] In the current study, BDNF deficiency activated Bax in RGC-5 cells, suggesting that increased Bax activity could be associated with the pathophysiological mechanism mediated by E50K to induce photoreceptor cell degeneration. Bax activation is associated with alterations of mitochondrial dynamics in mammalian cells including RGCs [14, 23]. Our previous studies showed that impaired mitochondrial dynamics and bioenergetics triggered mitochondrial dysfunction in experimental glaucoma in vivo and in vitro [23]. However, our current findings surprisingly demonstrated that BDNF deficiency impaired mitochondrial bioenergetics but did not alter mitochondrial dynamics in RGC-5 cells. Collectively, these findings importantly suggest that BDNF deficiency-mediated mitochondrial bioenergetic dysfunction is independently induced without alteration of mitochondrial dynamics in RGC-5 cells. Further studies will be necessary to examine the pathophysiological mechanisms of E50K/BDNF deficiency-mediated mitochondrial dysfunction in glaucomatous photoreceptor cells.

OPTN plays a role as an autophagy receptor via binding to LC3 to remove damaged mitochondria by initiating mitophagosome formation [10, 11]. Overexpression of E50K results in fission-mediated mitochondrial degradation and mitophagy in the axons of the glial lamina in aged E50K^{-tg} mice and cultured RGCs [14]. Our finding of BDNF deficiency-induced mitophagosome formation is correlated with an increase in LC3-II protein expression in RGC-5 cells, confirming that BDNF deficiency is critical to mitochondrial damage and dysfunction. Since p62, a poly ubiquitin-binding protein that serves as an autophagy receptor [49], is not required for the recruitment of LC3 to damaged mitochondria [10], not surprisingly, we also found that BDNF deficiency did not alter the expression level of p62 protein in RGC-5 cells. Although the p62 protein expression has been used as an autophagic marker with LC3-II, it also should be noted that the pattern of p62 protein expression is not always correlated with the alteration of the LC3-II level.

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In summary, the present study demonstrates for the first time that E50K-mediated BDNF deficiency induces mitochondrial damage and bioenergetic dysfunction, but does not alter mitochondrial dynamics in the photoreceptor cells. Therefore, a combinational therapeutic strategy that blocks E50K mutation and boosts BDNF may promote photoreceptor cell survival against glaucomatous retinal damage.

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Highlights

- E50K mutation triggers BDNF deficiency in RGC-5 cells.
- BDNF deficiency results in mitochondrial bioenergetic dysfunction in RGC-5 cells.
- BDNF deficiency induces mitophagosome formation in RGC-5 cells.

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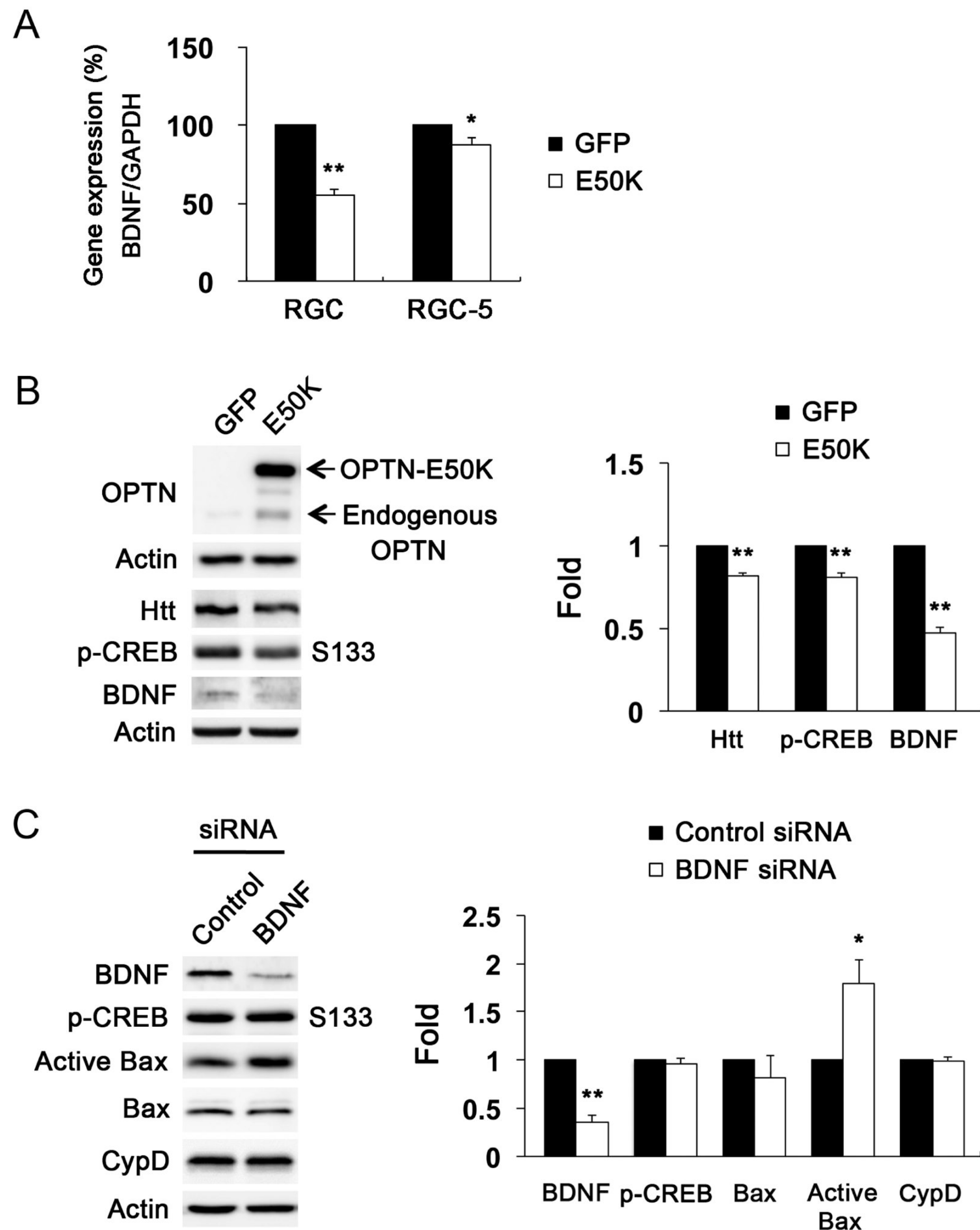

Figure 1.

Overexpression of OPTN E50K triggers BDNF deficiency and Bax activation in RGC-5 cells (A) Real-time RT-PCR analysis of BDNF mRNA expression in cultured RGCs and RGC-5 cells overexpressing E50K. Data were normalized by GAPDH expression. (B) Protein expression of OPTN, HTT, p-CREB S133 and BDNF in RGC-5 cells overexpressing E50K. (C) Protein expression of BDNF, p-CREB S133, active Bax, total Bax and CypD in RGC-5 cells transfected with BNDF siRNA. For each determination, the actin level was normalized to a value of 1.0. Data are shown as the mean ± S.D. **P*<0.05; ***P*<0.01 compared with the control group.

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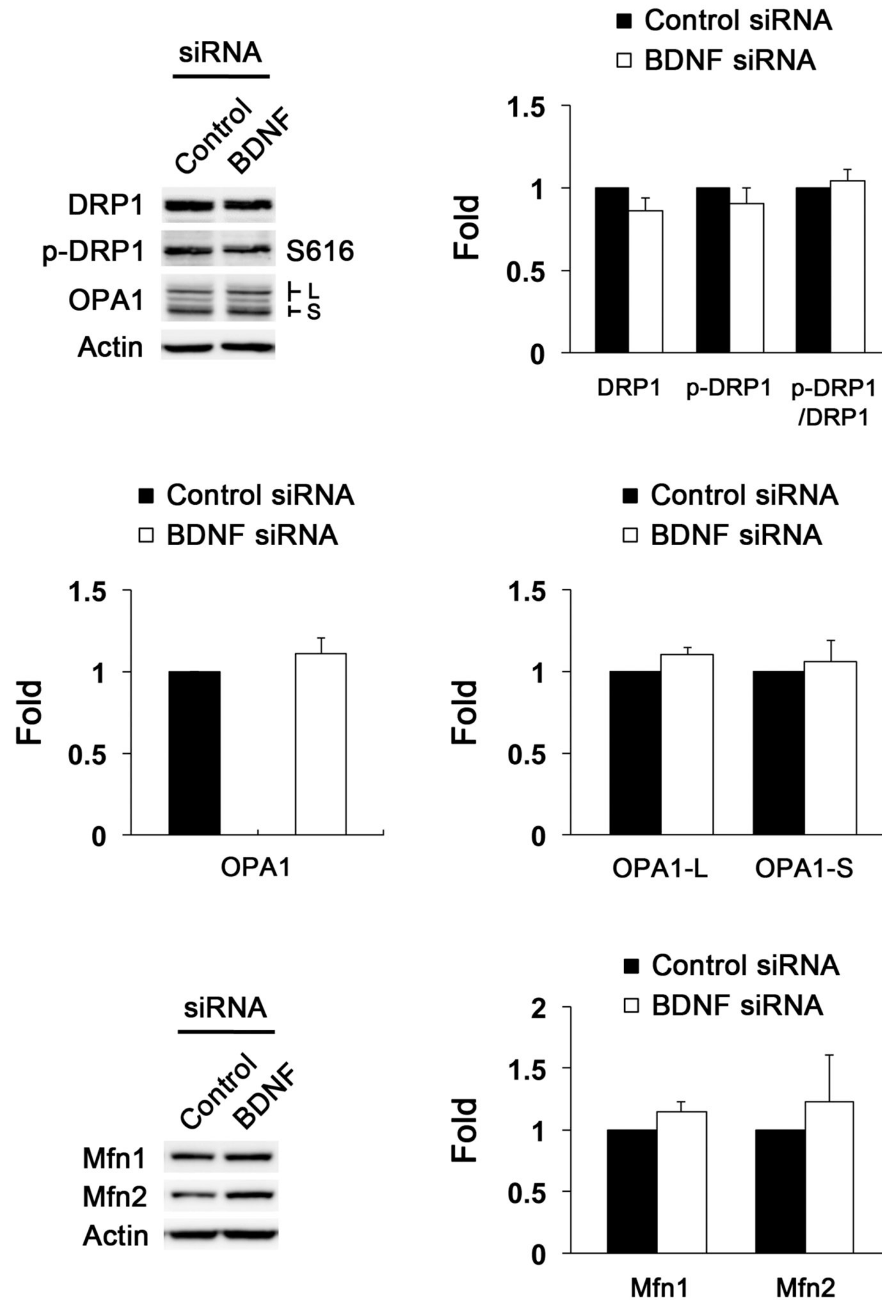

Figure 2.

BDNF deficiency results in bioenergetic dysfunction but did not alter mitochondrial dynamics-related protein expression in RGC-5 cells. Protein expression of DRP1, p-DRP1 S616, OPA1, and Mfn1 and 2 in RGC-5 cells transfected with BNDF siRNA. For each determination, the actin level was normalized to a value of 1.0. Data are shown as the mean ± S.D.

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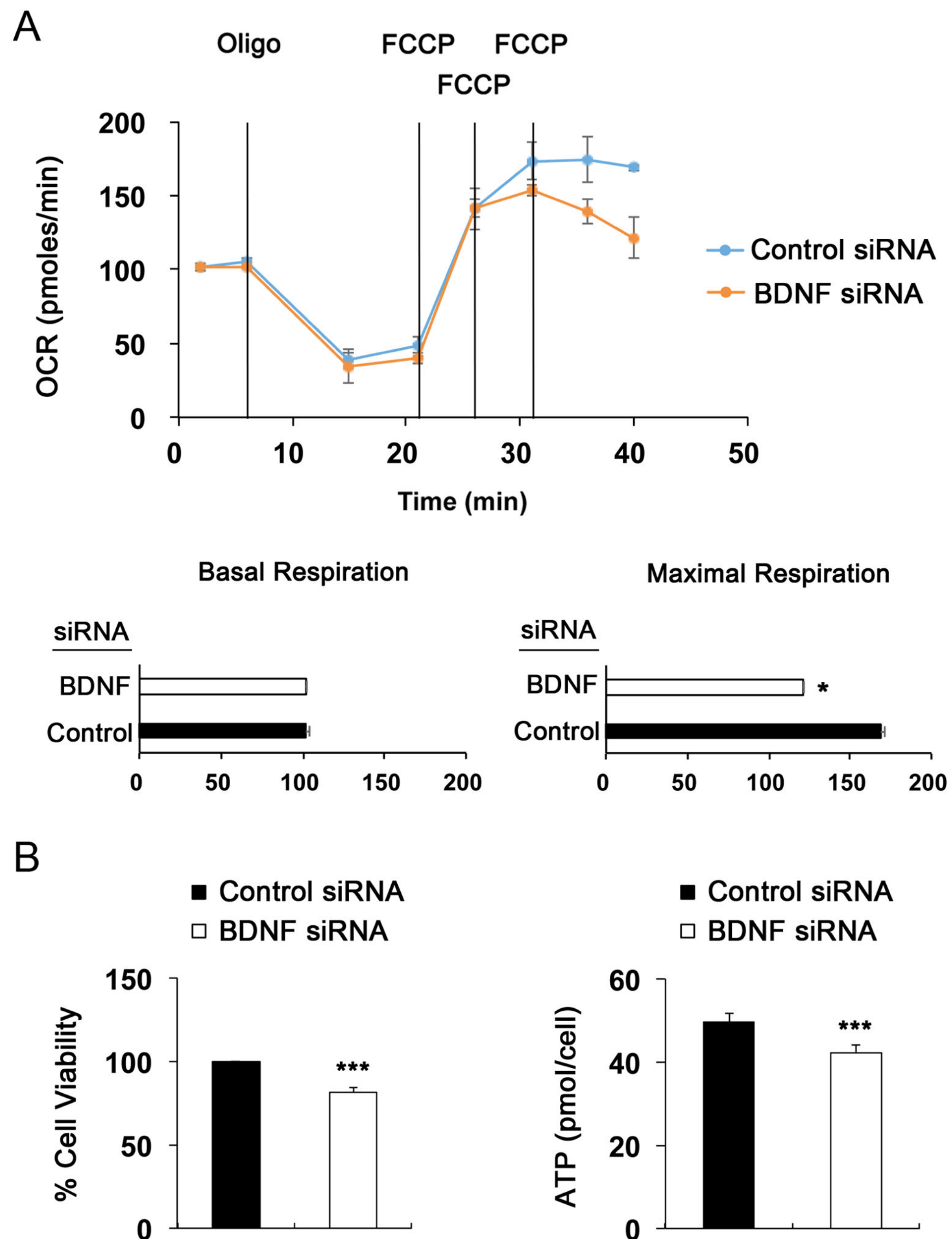

Figure 3.

BDNF deficiency results in bioenergetic dysfunction in RGC-5 cells. OCR changes in RGC-5 cells transfected with BDNF siRNA. Oligomycin A and FCCP were sequentially added at the indicated time point. Basal respiration indicates the starting basal OCR and the value which was set to 100%. Maximum respiration represents the ratio between FCCP uncoupled OCR and basal OCR. (B) Cell viability and cellular ATP production in RGC-5 cells transfected with BDNF siRNA. Data are shown as the mean ± S.D. * P<0.05; *** P,<0.001 compared with the control group.

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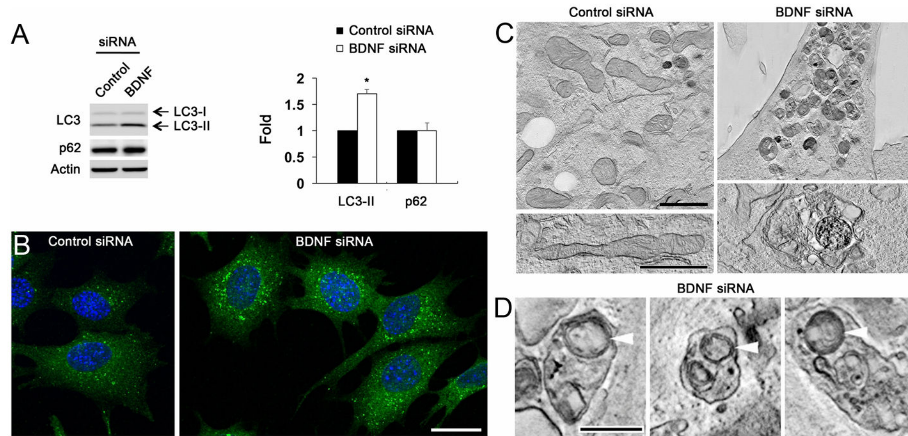

Figure 4.

BDNF deficiency triggers mitophagosome formation in RGC-5 cells. (A) Protein expression of p62 and LC3 in RGC-5 cells transfected with BDNF siRNA. For each determination, the actin level was normalized to a value of 1.0. Data are shown as the mean ± S.D. *$P<0.05$ compared with the control group. (B) LC3 immunoreactivity (green) and puncta formation in the cytoplasm of RGC-5 cells transfected with BDNF siRNA. (C and D) Slices through tomographic volumes show several examples of mitophagosome formation in the cytoplasm of RGC-5 cells transfected with BDNF siRNA. Arrowheads indicate damaged mitochondria engulfed by autophagic membranes (D). Scale bars, 10 μm (B); 500 nm (C and D).