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Parkin overexpression protects retinal ganglion cells against glutamate excitotoxicity

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Purpose: To investigate the role of parkin in regulating mitochondrial homeostasis of retinal ganglion cells (RGCs) under glutamate excitotoxicity.

Methods: Rat RGCs were purified from dissociated retinal tissue with a modified two-step panning protocol. Cultured RGCs were transfected with parkin using an adenovirus system. The distribution and morphology of mitochondria in the RGCs were assessed with MitoTracker. The expression and distribution of parkin and optineurin proteins were measured with western blot analysis and immunofluorescence. Cytotoxicity of RGCs was evaluated by measuring lactate dehydrogenase (LDH) activity. Mitochondrial membrane potential was determined with the JC-1 assay. The expression of Bax and Bcl-2 were measured with western blot analysis.

Results: In the presence of glutamate-induced excitotoxicity, the number of mitochondria in the axons of the RGCs was predominantly increased, and the mitochondrial membrane potential in RGCs was depolarized. The expression of the parkin and optineurin proteins was upregulated and distributed mostly in the axons of the RGCs. Overexpression of parkin stabilized the mitochondrial membrane potential of RGCs, decreased cytotoxicity and apoptosis, attenuated the expression of Bax, and promoted the expression of optineurin under glutamate excitotoxicity.

Conclusions: Overexpression of parkin exerted a significant protective effect on cultured RGCs against glutamate excitotoxicity. Interventions to alter the parkin-mediated mitochondria pathway may be useful in protecting RGCs against excitotoxic RGC damage.

Glaucoma, the leading cause of irreversible blindness, is a neurodegenerative disease characterized by retinal ganglion cell (RGC) loss [1]. Glutamate excitotoxicity has been implicated as an important pathophysiological mechanism in glaucomatous neurodegeneration. Growing evidence indicates that glutamate excitotoxicity contributes to alteration of mitochondrial dynamics, leading to mitochondrial dysfunction and cellular death in neurodegenerative disorders, including glaucoma [2-5]. However, the molecular mechanisms underlying these effects are poorly understood.

Loss-of-function mutations within the PARK2 locus, which encodes the protein parkin, are the most common causes of autosomal recessive Parkinson disease [6]. Parkin has been shown to be neuroprotective against a variety of toxic stressors in cell culture and in vivo [7]. Moreover, parkin has recently been implicated in the mitochondrial quality-control pathway to induce the removal of damaged mitochondria via mitophagy [8]. When the mitochondrial membrane potential is depolarized, parkin is recruited to the outer mitochondrial membrane, leading to the parkin-mediated ubiquitination of mitochondrial membrane proteins and facilitating mitophagy [9]. Optineurin is an autophagy receptor [10], which is actively recruited to ubiquitinated mitochondria downstream of parkin. Current evidence suggests a mitochondrial function for parkin and a neuroprotective role, which may be interrelated. Parkin is present in all main neuronal types of the rodent retina, and the protein level of parkin is especially prominent in the ganglion cell layer [11]. Nevertheless, the pathophysiological relation between parkin and RGCs under excitotoxic stress has not been reported. Therefore, the aim of the present study was to investigate the role of parkin in regulating mitochondrial homeostasis of RGCs under glutamate excitotoxicity.

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METHODS

Animals, isolation, purification, and culture of RGCs: All procedures concerning animals were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and under protocols approved by the Animal Ethics Committee of the Eye and ENT Hospital of Fudan University. Retinal tissues from 2- to 3-day-old Sprague-Dawley rats were isolated and placed in a 6-cm Petri dish containing Earle's Balanced Salt Solution (EBSS; Gibco, Grand Island, NY), according to the methods described by

Winzeler et al. [12]. Briefly, the tissues were placed at 37 °C for 20 to 30 min in minimum essential media (MEM; Gibco) containing 5 mg/ml of papain (Worthington Biochemical, Lakewood, NJ), 10 U/ml of DNase I (Sigma-Aldrich, St. Louis, MO), and 0.24 mg/ml of L-cysteine (Sigma-Aldrich). To yield a suspension of single cells, the tissues were then added to MEM containing 0.1% ovomucoid (Worthington), 0.1% bovine serum albumin (BSA, Sigma-Aldrich), and 1% DNase I (4 mg/ml). The suspension was centrifuged at 200 ×g for 10 min after settling in the tube for 2 min. Then the retinal suspension was resuspended in MEM containing 0.5 mg/ml BSA and filtered through Nitex mesh (pore size 40 µm; BD Falcon, Franklin Lakes, NJ) twice.

RGCs were purified from dissociated retinal tissue with a two-step panning protocol, essentially as previously described [13,14], with minor modifications. The retinal suspension was incubated in two anti-rat-macrophage panning plates (Millipore Corp, Billerica, MA; 15 µl in 7.5 ml of 1 mM Tris buffer, pH 9.5 at 4 °C overnight) at 37 °C for 40 min, and each plate was shaken every 20 min. The nonadherent cells were transferred to two anti-rat-Thy1.1 panning plates (Abcam, Cambridge, MA; 15 µl in 7.5 ml of 1 mM Tris buffer, pH 9.5 at 4 °C overnight) at 37 °C for 1 h, and each plate was shaken every 20 min. Then the plates were washed three times with Dulbecco's PBS (1X; 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$ -$6H_2O$, 137.9 mM NaCl, 2.67 mM KCl, 8.06 mM Na_2HPO_4 -$7H_2O$, 1.47 mM KH_2PO_4, pH 7.4; D-PBS, Gibco) and swirled moderately vigorously to dislodge nonadherent cells. Each plate was incubated at 37 °C for 2 min with EBSS media containing 0.25% trypsin (Gibco). Immediately following treatment, DMEM (Gibco) media with 30% fetal bovine serum (Gibco) was added to each plate to stop the trypsin. After centrifugation at 200 ×g for 5 min, the cells were seeded on glass coverslips that had been coated with 0.01% poly-D-lysine (Sigma-Aldrich).

Purified RGCs were plated at a density of 1×10^6 cells per 24-well plate. Cultures were maintained at 37 °C in humidified atmosphere containing 5% CO_2 and 95% air in Neurobasal medium (Gibco) containing supplemental factors. The RGC yields with this procedure were $26.70 \pm 16.11 \times 10^4$ per retina, and the purity of the RGCs was 84.86 ± 1.97% [14].

Excitotoxicity model: Three days after seeding, the RGCs were exposed to cell culture medium alone (control) or to cell culture medium containing different concentrations of glutamate (25 µM, 100 µM, and 200 µM; Sigma-Aldrich) for 24 h in a 37 °C, 5% CO_2 tissue culture incubator. Treatment of 100 µM NMDA was also applied to a subgroup of RGGs for 24 h in a 37 °C, 5% CO_2 tissue culture incubator.

Plasmid, recombinant adenovirus constructs, and infection: The rat cDNAs of parkin were amplified with PCR. PCR reaction cycle conditions: 1X: 98 °C 3 min, 30X: 98 °C 10 s; 55 °C 15 s; 72 °C 1 min, 1X: 72 °C 10 min. Under the control of the cytomegalovirus (CMV) promoter, a recombinant adenovirus (Ad) plasmid containing the parkin gene (Gene ID:56816, NM_020093.1) was constructed by homogenous recombination in *Escherichia coli*. Then the recombinant plasmid was cotransfected into human embryonic kidney cells (HEK) 293 cells to construct a recombinant adenovirus (pAd/mCMV; Sunbio, Shanghai, China) via the Cre/loxP recombinase system. After several rounds of amplification, adenoviral titers were obtained at 1×10^{10} particles/ml.

The RGCs were infected with the adenovirus that had been diluted in cell culture medium for 48 h. The number of viral particles per cell was ten.

Assessment of cell apoptosis: Apoptosis of RGCs was assessed with Hoechst staining. The RGCs on the coverslips were fixed with 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature, rinsed with PBS, and then were permeabilized with 0.1% Triton X-100 for 20 min at room temperature. Cells were then washed three times and were stained with Hoechst 33,342 (1 µg/ml, Life Technologies, Grand Island, NY) for 10 min at room temperature. Images were taken of randomly using a confocal microscope (Leica SP8, Mannheim, Germany). RGC apoptosis was quantified by having pyknotic nuclei. The number of cells with pyknotic nuclei and the total number of cells were counted. The percentage of apoptotic cells was calculated for each control and experimental condition.

Measurement of mitochondrial membrane potential: Measurement of mitochondrial membrane potential was performed using the JC-1 Assay Kit (Abcam). Cultured RGCs were harvested at the end of the exposure to the drug, incubated with JC-1 (5 µg/ml) dye for 20 min at 37 °C, and then rinsed twice with EBSS. JC-1 fluorescence intensities of red aggregates (hyperpolarization) and green fluorescence monomers (depolarization) were read with a fluorescent plate reader (Infinite M1000; Tecan, Mnnedorf, CH). The maximum excitation wavelength of the JC-1 monomer was 514 nm, and the maximum emission wavelength was 529 nm. The maximum excitation wavelength of the JC-1 aggregates was 585 nm, and the maximum emission wavelength was 590 nm. The ratio of the JC-1 red fluorescence aggregates versus the green fluorescence monomers for each treatment was measured. All experiments were repeated independently at least three times.

Measurement of cytotoxicity: The amount of lactate dehydrogenase (LDH) released from damaged RGCs was measured

using the LDH Cytotoxicity Detection Kit (TaKaRa Biotechnology, Dalian, China). The supernatant of RGC culture is collected and incubated with the kit's reaction mixture. LDH activity is determined via the two-step enzymatic reaction.

Mitochondria distribution: In the presence of glutamate for 24 h in culture, RGCs were loaded with 200 nM MitoTracker Red (Molecular Probes, M7512; Life Technologies) at 37 °C for 30 min and then were washed three times. The RGCs on the coverslips were fixed with 4% PFA in PBS for 10 min at room temperature, rinsed with PBS, and then were permeabilized with 0.2% Triton X-100 for 5 min. Coverslips were observed with a confocal microscope (Leica SP8). Three randomly selected fields from one coverslip were included for statistical analysis, and the experiments were performed in triplicate.

Immunofluorescence analysis: RGCs were fixed with 4% PFA in PBS for 20 min, rinsed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 20 min at room temperature, and then washed three times with PBS. The cells were then blocked with 5% BSA/PBS for 1 h at room temperature and with the primary antibodies against polyclonal rabbit anti-Tubulin antibody (1:800; Abcam) or monoclonal mouse anti-γ-synuclein (1:400; Abcam), polyclonal rabbit anti-parkin (1:200; Abcam), or polyclonal rabbit anti-optineurin (1:50; Abcam) for 16 h at 4 °C. After several washes, the RGCs were incubated with Alexa Fluor 488-conjugated goat immunoglobulin G (IgG) secondary antibody (1:200; Life Technologies) and Fluor cy3-conjugated goat anti-mouse IgG secondary antibody (1:200; Life Technologies) for 1 h at room temperature and then were washed with PBS. The RGCs were counterstained with Hoechst 33,342 (1 μg/ml; Life Technologies) in PBS. Images were captured with a confocal microscope (Leica SP8).

Western blot analysis: RGCs (n = 4 per group) were mixed with RIPA buffer (Beyotime, Shanghai, China). Each sample (10 μg) was separated with polyacrylamide gel electrophoresis (PAGE) and electrotransferred on polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% nonfat dry milk at room temperature for 1 h, incubated with polyclonal rabbit anti-parkin (1:1,000; Abcam), polyclonal rabbit anti-optineurin (1:200; Abcam), polyclonal rabbit anti-Bcl-2 (1:500; Abcam), monoclonal rabbit anti-Bax (1:1,000; Abcam), polyclonal rabbit anti-OPA1 (1:1,000; Abcam), and polyclonal rabbit anti-GAPDH (1:2000; Yesen, Shanghai, China) in primary antibody dilution (Beyotime) at 4 °C overnight. The membranes were rinsed with 1X Tris-buffered saline/Tween 20 (TBST; Worthington) several times, incubated with peroxidase-conjugated goat anti-rabbit IgG (1:5,000; Jackson Laboratories, West Grove, PA), and then developed using chemiluminescence detection (Super-Signal™ West Femto Substrate Trial Kit, Thermo Fisher, Waltham, MA). Chemiluminescent images were captured using a Kodak Image Station 4000MM PRO (Carestream, Rochester, NY) and analyzed with Image J (National Institutes of Health).

Statistical analysis: Experiments were repeated at least three times. Different sets of cultures were used in each experiment. Data were expressed as mean ± standard deviation (SD). One-way ANOVA and the Bonferroni *t* test were used to evaluate the study results. A p value of less than 0.05 was considered statistically significant.

RESULTS

Effects of glutamate on cultured RGCs: The RGCs were identified by their morphology and immunocytochemical staining. The RGCs had characteristic long neurites connecting each other as the duration of the culture increased. The purity of the RGCs was verified with immunocytochemical costaining of γ-synuclein and tubulin (Figure 1).

Applications of increasing concentrations (25–200 μM) of glutamate caused a dose-dependent increase in apoptosis of RGCs compared to the control group ($p<0.01$, Figure 2A). The JC-1 ratio was also statistically significantly decreased ($p<0.05$, Figure 2B), suggesting that glutamate excitotoxicity depolarized the mitochondrial membrane potential and induced apoptosis in RGCs.

Increased expression of the parkin and optineurin proteins is mostly distributed in the axons of RGCs under glutamate excitotoxicity: Mitochondria in RGCs were stained with MitoTracker Red and visualized under a confocal microscope. In the control RGCs, mitochondria with a tubular mitochondrial network were mainly distributed around the nucleus in the cell body; few mitochondria in the axon were observed. In response to glutamate excitotoxicity, small spherical mitochondria were observed around the nucleus. More mitochondria in the axons of the RGCs were visualized (Figure 3A,B).

Parkin and optineurin were mainly expressed in the cell bodies of cultured RGCs. In the excitotoxicity model, parkin and optineurin immunoreactivity was greater in the axons of the RGCs than that in the control RGCs (Figure 4). Western blot analysis showed that a dose-dependent increase in the levels of parkin ($p<0.01$) and isoforms L ($p<0.01$) and S2 ($p<0.05$) of the optineurin protein and the levels of isoforms L ($p<0.05$) and S ($p<0.05$) of the OPA1 protein (Figure 5) were observed in the excitotoxicity model of RGCs; no statistically significant change ($p>0.05$) was observed in the level of isoform S1 of the optineurin protein.

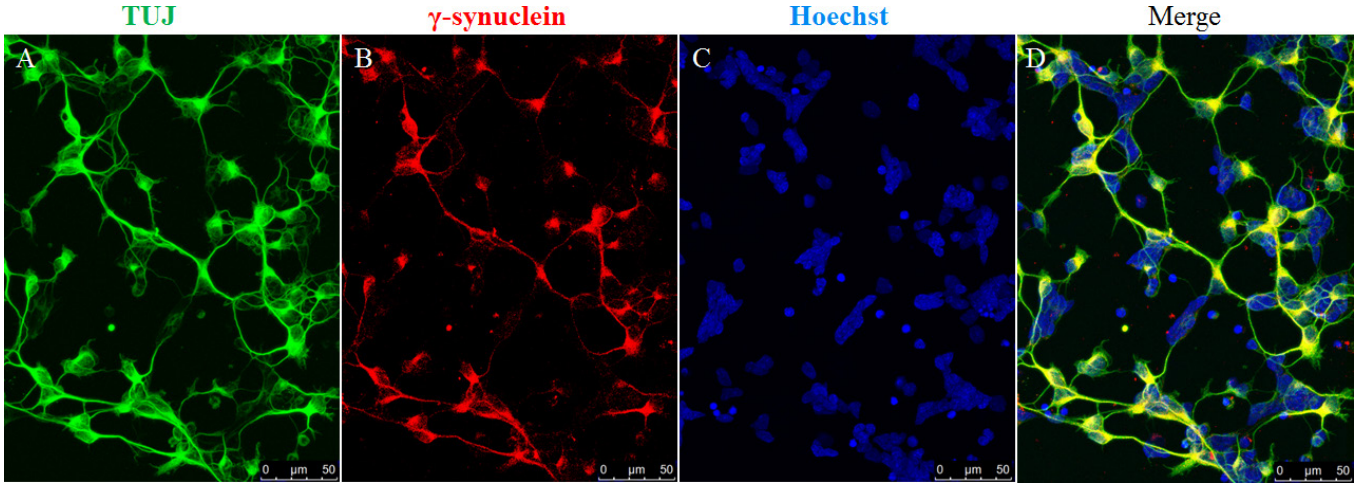

Figure 1. RGC identification. Immunofluorescence of retinal ganglion cells (RGCs) 2 days after seeding in culture showed costaining of TUJ1 (green; **A**) and γ-synuclein (red; **B**). **C**: Hoechst nuclear staining. **D**: The merged images. Scale bar = 50 μm.

Parkin overexpression has a positive influence on RGC viability in the absence of glutamate: Transfection efficiency was measured by detecting the levels of parkin with immunoblotting after transfection for 2 days. As shown in Figure 6, parkin expression was significantly increased in the Ad-parkin-transfected groups compared with that in the Ad null transfected groups ($p<0.01$). This result confirmed that these procedures led to overexpression of parkin in RGCs.

Compared with the Ad null transfected RGCs, Ad-parkin-transfected RGCs showed that the cytotoxcity as measured with LDH activity was decreased ($p<0.05$), the protein level of Bcl-2 was increased ($p<0.01$), and the protein levels of isoforms L ($p<0.05$) and S ($p<0.05$) of OPA1 were increased. No statistically significant differences were observed for the JC-1 ratio ($p>0.05$), Bax ($p>0.05$), and optineurin ($p>0.05$) protein levels. These results suggest that parkin overexpression has a positive influence on RGC viability.

Impacts of parkin overexpression on RGCs under glutamate excitotoxicity: The Ad null transfected RGCs had a JC-1 ratio of 0.78 ± 0.03, 0.79 ± 0.03, and 0.76 ± 0.05 for the 25 μM, 100 μM, and 200 μM concentrations of glutamate, respectively, whereas the JC-1 ratio of the Ad-parkin-transfected RGCs was 0.88 ± 0.03, 0.88 ± 0.03, and 0.82 ± 0.04, respectively. These results imply that overexpression of parkin can increase the level of mitochondrial membrane potential under glutamate excitotoxicity ($p<0.05$, Figure 7A).

Compared with the Ad null transfected RGCs, the mean MitoTracker intensity was significantly increased in the Ad-parkin-transfected RGCs ($p<0.01$). There were less number of small spherical mitochondria around the nucleus,

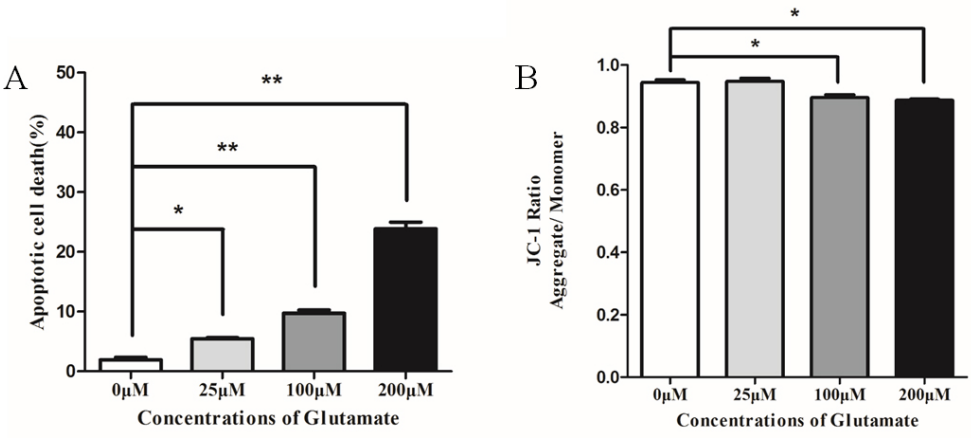

Figure 2. Effects of glutamate on mitochondrial membrane potential and apoptosis in cultured RGCs. **A**: The quantitative analysis of apoptotic cell death indicated that glutamate caused a dose-dependent increase in apoptosis of retinal ganglion cells (RGCs). **B**: The JC-1 ratio was significantly decreased in the 100 μM and 200 μM glutamate groups. n=3, *$p<0.05$ compared with the control group. **$p<0.01$ compared with the control group. Scale bar = 200 μm.

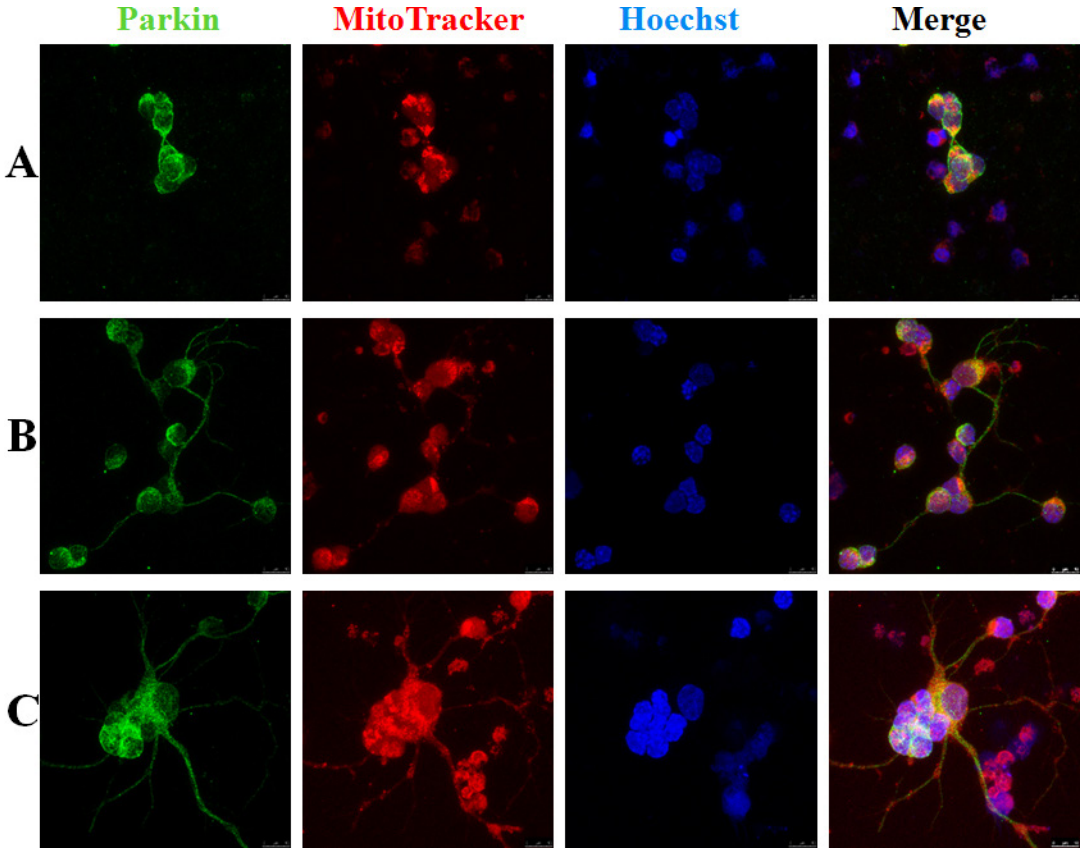

Figure 3. Mitochondrial distribution and morphology in RGCs stained with MitoTracker Red. **A**: In the control retinal ganglion cells (RGCs), mitochondria with a tubular mitochondrial network were mainly distributed in the cell body; few mitochondria in the axons were observed. **B**: Glutamate treatment induced much more mitochondria in the axons of the RGCs, the number of small spherical mitochondria was increased. **C**: Overexpression of parkin increased the number of mitochondria in the RGCs and reduced glutamate-induced mitochondrial fragmentation. Scale bar = 10 μm.

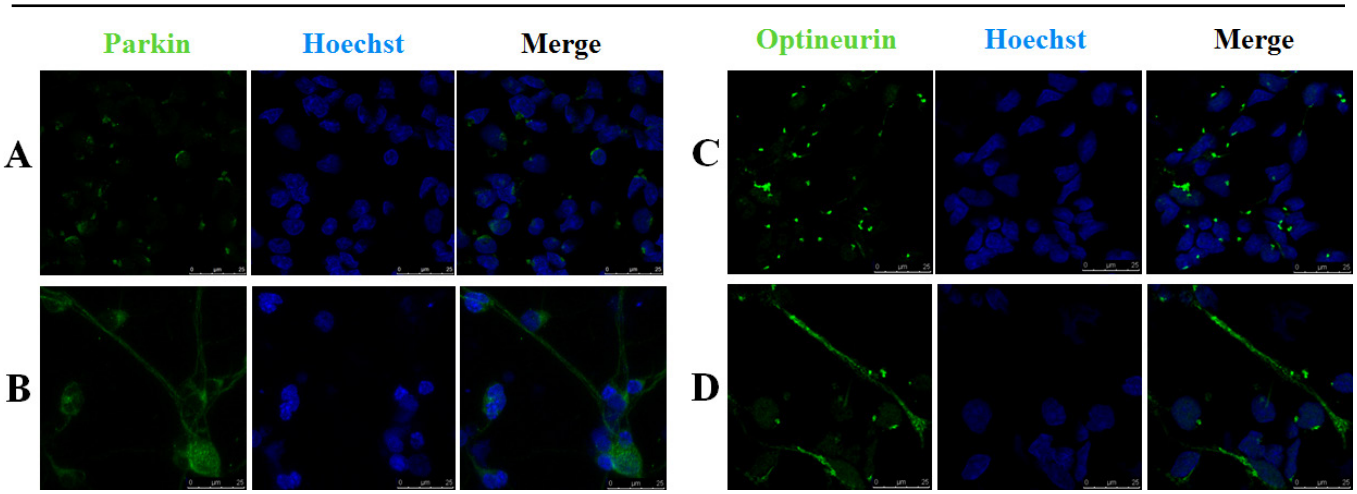

Figure 4. Immunofluorescence analysis of parkin and optineurin in the control and 100 μM glutamate groups. Compared with the control group (**A**, **C**), parkin and optineurin immunoreactivity was greater in the axons of the retinal ganglion cells (RGCs) under glutamate treatment (**B**, **D**). Scale bar = 25 μm.

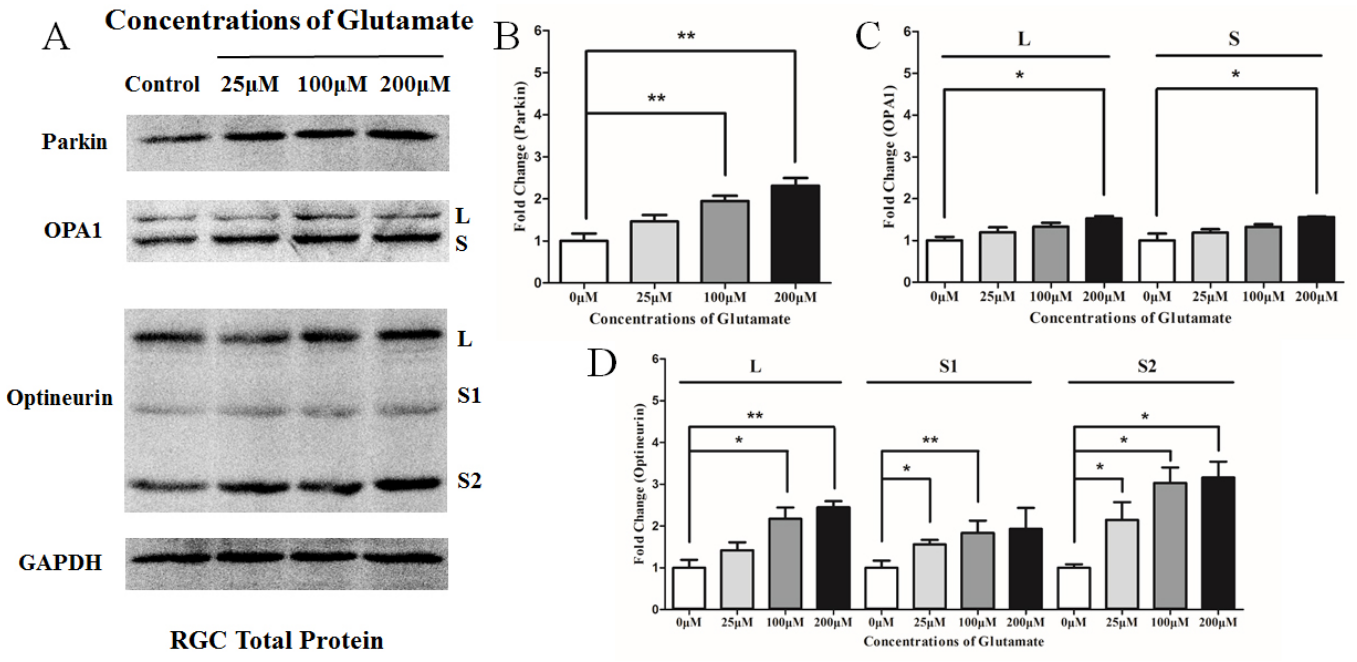

Figure 5. Western blot analysis of the parkin, optineurin, and OPA1 proteins in RGCs under increasing concentrations of glutamate treatment. Compared with the control group (no glutamate treatment), increased expression of the parkin protein (**B**), isoforms L and S2 of the optineurin protein (**D**), and isoforms L and S of the OPA1 protein (**C**) were observed. n=3, *p<0.05. **p<0.01 compared with the control group.

and a tubular mitochondrial network was partially restored (Figure 3B,C).

Cytotoxicity was evaluated by measuring LDH activity. Parkin overexpression significantly decreased the LDH activity of RGCs in different concentrations and durations of glutamate treatment ($p<0.05$, Figure 7B, Appendix 1). The quantitative analysis of apoptotic cell death showed that Ad-parkin-transfected RGCs had less apoptotic cell death than the Ad null transfected RGCs in the glutamate treatment ($p<0.05$, Figure 7C). These results indicated that overexpression of parkin can protect RGCs under glutamate excitotoxicity.

Compared with the Ad null transfected RGCs, the protein levels of Bax were lower in the Ad-parkin-transfected groups in the 25 μM, 100 μM, and 200 μM glutamate excitotoxicity model ($p<0.05$, Figure 6A,C), while the protein level of Bcl-2 was higher in the Ad-parkin-transfected in 25 μM glutamate group ($p<0.05$, Figure 6A,D). No statistically significant change was observed in the expression of Bcl-2 in the 100 μM and 200 μM ($p>0.05$) glutamate groups.

Moreover, when compared to the Ad null transfected RGCs, the Ad-parkin-transfected RGCs showed that the level of isoform S of the OPA1 protein was increased in the 25 μM glutamate group ($p<0.05$). However, the levels of isoforms L ($p<0.05$) and S ($p<0.05$) of the OPA1 protein were decreased in the 100 μM glutamate group, and the level of isoform L ($p<0.05$) of the OPA1 protein was decreased in the 200 μM glutamate group (Figure 6A,E).

Regarding optineurin expression, the Ad-parkin-transfected RGCs showed that the level of isoform S1 of the optineurin protein was increased in the 25 μM, 100 μM, and 200 μM glutamate groups ($p<0.05$). The level of isoform L of the optineurin protein was increased in the 25 μM and 100 μM glutamate groups ($p<0.05$). The level of isoform S2 of the optineurin protein was increased in the 100 μM and 200 μM glutamate groups ($p<0.05$, Figure 6A,F).

To further validate the neuroprotective of parkin, experiments on N-methyl-D-aspartate (NMDA)-induced excitotoxicity were performed. The results showed that overexpression of parkin stabilized the mitochondrial membrane potential of the RGCs, decreased cytotoxicity and apoptosis, attenuated the expression of Bax, and promoted optineurin expression under NMDA treatment (Appendix 2 and Appendix 3).

DISCUSSION

These results demonstrate that in response to a stressor linked to neurodegeneration (glutamate excitotoxicity), increased expression of the parkin and optineurin proteins is mainly distributed in the axons of cultured RGCs. The overexpression of parkin stabilized the mitochondrial membrane potential of

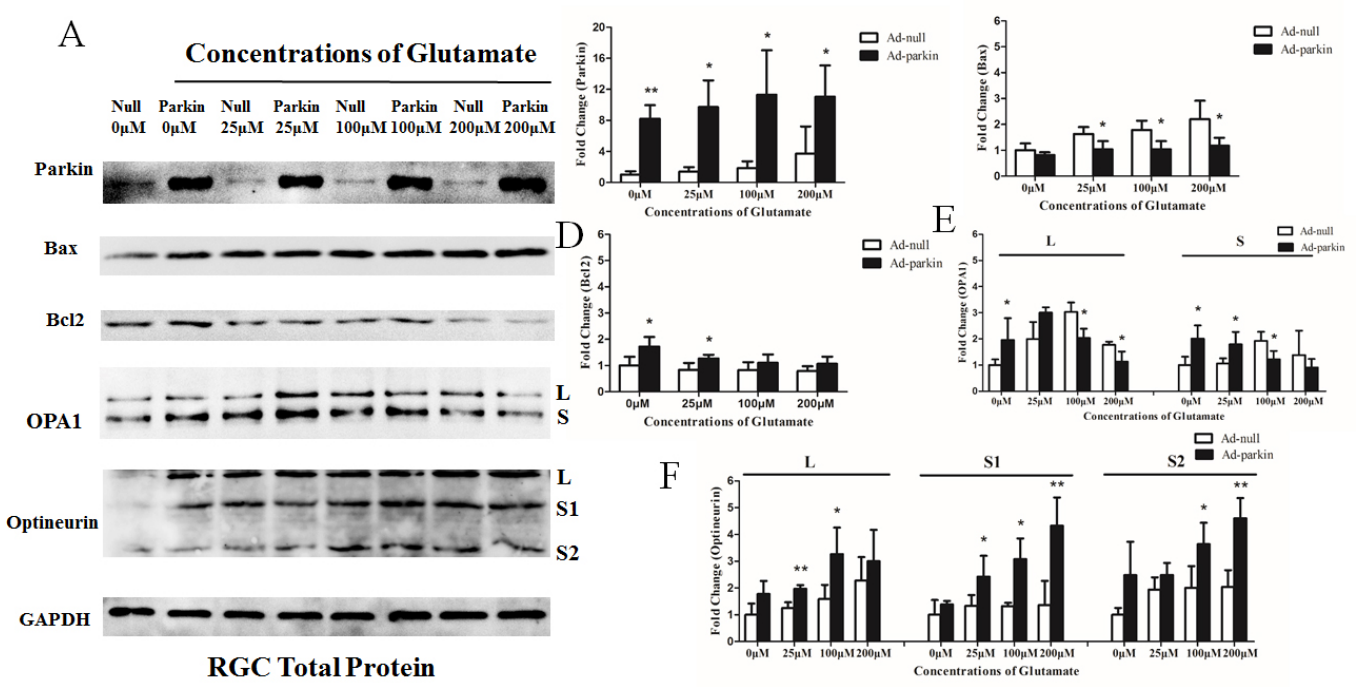

Figure 6. Western blot analysis of the parkin, Bax, Bcl-2, optineurin, and OPA1 proteins in parkin-transfected RGCs under increasing concentrations of glutamate treatment. Compared with Ad null transfected retinal ganglion cells (RGCs), parkin expression in Ad-parkin-transfected RGCs was significantly increased in all concentrations of glutamate treatment (**B**). The expression of Bax in the Ad-parkin-transfected RGCs was decreased in the 25 μM, 100 μM, and 200 μM glutamate groups **(C**), and the expression of Bcl-2 was increased in the control and 25 μM glutamate groups (**D**). Expression of isoform S of the OPA1 protein in Ad-parkin-transfected RGCs was increased in the control and 25 μM glutamate group, while expression of isoform L of the OPA1 protein was decreased in the 100 μM and 200 μM glutamate group (**E**). Expression of isoform S1 of the optineurin protein in Ad-parkin-transfected RGCs was increased in the 25 μM, 100 μM, and 200 μM glutamate groups, expression of isoform L of the optineurin protein was increased in the 25 μM and 100 μM glutamate groups, and expression of isoform S2 of the optineurin protein was increased in the 100 μM and 200 μM glutamate groups (F). n=4, *p<0.05 compared with Ad null transfected groups. **p<0.01 compared with Ad null transfected groups.

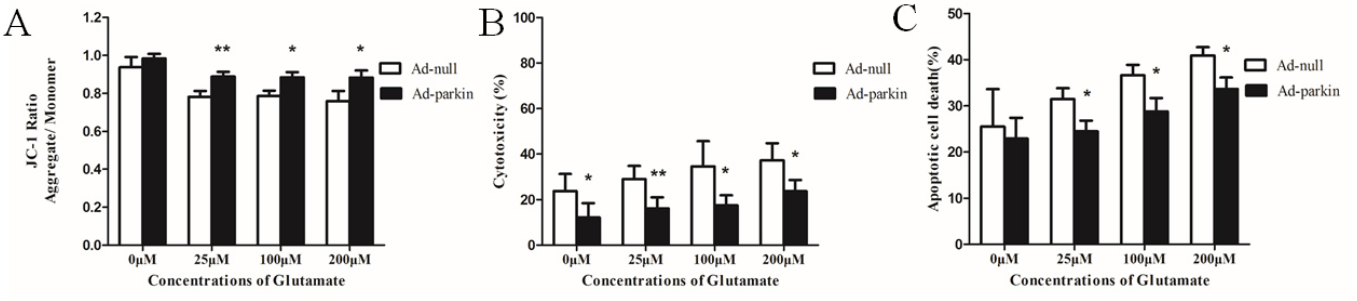

Figure 7. Effects of the overexpression of parkin on mitochondrial membrane potential, cytotoxicity, and apoptosis in cultured RGCs. Compared with the Ad null transfected retinal ganglion cells (RGCs), the Ad-parkin-transfected RGCs showed a higher level of mitochondrial membrane potential (**A**), a lower level of cytotoxicity (**B**), and less apoptotic cell death (**C**) under glutamate excitotoxicity. n=4, *p<0.05 compared with Ad null transfected groups. **p<0.01 compared with Ad null transfected groups.

RGCs, decreased cytotoxicity and apoptosis, attenuated the expression of Bax, and promoted optineurin levels.

Parkin is a 465-amino acid protein that is expressed in multiple tissues and functions as an E3 ubiquitin ligase [15]. In line with an exceptional role of parkin in exerting neuroprotection, expression of the parkin gene is upregulated in various stress paradigms [16,17]. The present study also showed that the expression of parkin was upregulated in cultured RGCs and overexpression of parkin exerted a significant protective effect on RGCs under glutamate excitotoxicity. In addition, the number of mitochondria in the axons of the RGCs was predominantly increased, and upregulated parkin and optineurin proteins were distributed mostly in the axons of the RGCs under glutamate excitotoxicity. Interestingly, a recent study reported that parkin-mediated mitophagy in response to focal damage occurs in distal neuronal axons instead of at the soma [18]. As axonal damage precedes RGC apoptosis in glaucomatous optic neuropathy, it is possible that RGC axons are the sites where parkin-mediated neuroprotection plays key roles against glutamate excitotoxicity.

The BCL-2 family of proteins constitutes a critical intracellular checkpoint in the mitochondrial pathway of apoptosis. Antiapoptotic Bcl-2 and proapoptotic Bax are two essential proteins of the Bcl-2 family. Bcl-2 inhibits the release of cytochrome C and ultimately inhibits apoptosis while activation of the Bax protein stimulates the release of cytochrome c from mitochondria and promotes apoptosis [19]. We provided in vitro evidence that overexpression of parkin attenuates the expression of Bax and partially increases the expression of Bcl-2 in RGCs under glutamate excitotoxicity. Previous studies have suggested that Bax is the primary substrate responsible for the antiapoptotic effects of parkin. Ubiquitination of Bax by parkin prevents stress-induced translocation of Bax to the mitochondria [20,21] although Bcl-2, whose C-terminus is directly bounded by parkin, is monoubiquitinated and is then steadily upregulated by parkin [22]. The present data are consistent with the notion that the antiapoptotic effect of parkin on RGCs is mediated by regulating the function of Bax and Bcl-2.

To understand the mechanism involved in the protective activity of parkin in RGCs, we further studied two parkin downstream proteins. Optineurin is an autophagy receptor [10], which is actively recruited to ubiquitinated mitochondria, and induces autophagosome formation around mitochondria [23]. The present study showed that overexpression of parkin induced significant upregulation of the optineurin protein in RGCs under glutamate stress. Moreover, OPA1 is a dynamin-related GTPase located in the mitochondrial intermembrane space that maintains the cristae structure, promotes mitochondria fusion, and prevents apoptosis [3]. Increased expression of OPA1 may protect against RGC death in glaucomatous optic neuropathy [24]. Muller-Rischart et al. [25] reported that parkin has no prosurvival activity in OPA1-deficient SH-SY5Y cells, suggesting OPA1 is an essential downstream mediator of the stress-protective activity of parkin. The present study showed that overexpression of parkin induced upregulation of the OPA1 protein in RGCs in the control and low glutamate concentration groups, while expression of OPA1 was decreased by overexpression of parkin in the high glutamate concentration group. Taken together, these data support the speculation that parkin may protect neurons via different pathways depending on the severity of the mitochondrial damage [19]. It is plausible that OPA1 may mediate the protective effect of parkin in RGCs under moderate stress with minor mitochondrial dysfunction, while parkin-dependent mitophagy may be promoted to protect RGCs when the mitochondria are irreversibly damaged by severe stress.

Regarding the vector for parkin gene delivery, the present data showed that the transfection efficiency of adenovirus in cultured RGCs was much higher than that of adenoassociated viruses (AAVs), lentiviruses, and liposomes. AAVs have been widely used in experimental and clinical studies to deliver genes to the eye, due to the excellent safety profile, low immunogenicity, and ability to efficiently target various retinal layers [26]. One of the major limitations of an AAV is its slow onset of expression owing to the second strand synthesis required for transcription [27]. A major drawback of adenovirus vectors is the immune reactions that the vectors can elicit. However, adenovirus vectors have several advantages in in vitro experiments, such as high transfer efficiency in non-dividing cells [28], fast onset of expression, and minimal risks of insertional mutagenesis [27].

In summary, these findings demonstrate that overexpression of parkin acted at the mitochondrial level and exerted a significant protective effect on RGCs against glutamate excitotoxicity. Further study is needed to elucidate the molecular mechanism involved in mitochondrial function for parkin in RGCs and its neuroprotective role. Interventions to alter the parkin-mediated mitochondria pathway may be useful in protecting RGCs against excitotoxic RGC damage.

APPENDIX 1.

Effects of parkin overexpression on cytotoxicity under different duration of 100 μm glutamate treatment. Compared with Ad null transfected RGCs, Ad-parkin-transfected RGCs showed lower level of cytotoxicity under different duration of 100 μm glutamate treatment (12 h, 24 h, 48 h and 72 h). n=3,

*p<0.05 compared with Ad null transfected groups. To access the data, click or select the words "Appendix 1."

APPENDIX 2.

Effects of parkin overexpression on mitochondrial membrane potential, cytotoxicity and apoptosis in 100 µM NMDA 24 h treatment. Compared with Ad null transfected RGCs, Ad-parkin-transfected RGCs showed higher level of mitochondrial membrane potential (A), lower level of cytotoxicity (B), less apoptotic cell death (C) under NMDA-induced stress. *p<0.05 compared with Ad null transfected groups. n=3,**p<0.01 compared with Ad null transfected groups. To access the data, click or select the words "Appendix 2."

APPENDIX 3.

Western blot analysis of parkin, Bax, Bcl2, optineurin and OPA1 proteins in parkin transfected RGCs in 100 µM NMDA 24 h treatment. Compared with Ad null transfected RGCs, parkin expression in Ad-parkin-transfected RGCs was significantly increased under NMDA-induced excitotoxicity (B). Bax expression in Ad-parkin transfected RGCs was decreased (C), and Bcl2 expression was increased in NMDA treatment (D). No statistically significant differences were observed for protein levels of isoform L and S of OPA1 between Ad-parkin transfected RGCs and Ad null transfected RGCs in NMDA treatment (E). The protein level of isoform S1 and S2 of optineurin were increased in Ad-parkin transfected RGCs, no significant change was observed for isoform L of optineurin (F). n=3,*p<0.05 compared with Ad null transfected groups. **p<0.01 compared with Ad null transfected groups. To access the data, click or select the words "Appendix 3."

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