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Original Article

PINK1 Deficiency Aggravates the β-amyloid-attenuated Mitophagy-lysosomal Degradation in PC12 Cells

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Abstract

Background and objective: PTEN-induced putative kinase 1 (PINK1) is a mitochondrial kinase that regulates mitophagy. PINK1-deficient mAPP mice display low LC3B levels, and PINK1 overexpression enhances autophagy and increases the expression level of lysosome-associated membrane protein 1 (LAMP1). The present study evaluated whether altered PINK1 expression could modulate β-amyloid (Aβ)-treated mitophagy in PC12 cells, a simple cellular model to simulate pathological changes in neurodegenerative diseases *in vitro*.

Methods: PC12 cells were transfected with PINK1 siRNA for 48 h, and then incubated with 20 μM $A\beta_{25–35}$ for 24 h. The relevant protein expression was determined by immunofluorescence, immunoelectron microscopy, and Western blot. Mitochondrial membrane potential (MMP) was tested by JC-1-based confocal fluorescent imaging.

Results: Following $A\beta_{25–35}$ treatment, PINK1 silencing significantly decreased the levels of LC3B, Parkin, and LAMP1 as well as Parkin in mitochondria, p62 degradation, but increased OPTN and Parkin expression in PC12 cells, relative to that of the control PC12 cells. Furthermore, PINK1 silencing decreased MMP in PC12 cells.

Conclusions: PINK1 deficiency deteriorated the blockade of the $A\beta_{25–35}$-induced mitophagy-lysosome pathway in PC12 cells. Aβ-treated PC12 cells might be a valuable cellular model to evaluate PINK1-mediated mitophagy and bioactive compound screening.

Introduction

Mitochondria are important organelles that regulate cell survival, proliferation, apoptosis, calcium storage, energy, and lipid metabolism.[1] Mitochondrial dysfunction preferentially affects the brain and heart, which require much energy. Alzheimer's disease (AD) is a neurodegenerative disease. In AD patient brains, the activity of mitochondrial enzymes significantly decreases, accompanied by mitochondrial DNA mutations.[2–5] β-Amyloid (Aβ) can disrupt the electron transport chain in mitochondria, causing DNA fragmentation and chromatine condensation, producing apoptosis-inducing factor, and finally inducing cell apoptosis.[6]

Mitophagy is a specialized form of autophagy that eliminates damaged and dysfunctional mitochondria to promote the structural and functional integrity of mitochondria and has been identified as a key regulator of AD.[7–11] During AD, the dynamics of mitophagy are significantly altered; however, the role of dynamic changes in the mitophagy-lysosomal pathway remains controversial.[12] Mitophagy occurs in rodent models induced by Aβ and is characterized by mitophagosome accumulation and lysosomal activation.[7,13,14] Of note, Aβ can trigger a defect in the mitophagy-lysosomal pathway by causing lysosomal dysfunction, which results in the abnormal accumulation of mitophagosomes and substrates.[15] The PTEN-induced putative kinase 1 (PINK1)/Parkin

Keywords: β-Amyloid; PINK1; Mitophagy; PC12 cells.

Abbreviations: AD, Alzheimer's disease; Aβ, β-amyloid; LAMP1, lysosome-associated membrane protein 1; LC3, light chain 3; MMP, mitochondrial membrane potential; mtDNA, mitochondrial DNA; NDP52, a 52 kDa nuclear dot protein; OPTN, optineurin; p62/SQSTM1, sequestosome 1; PINK1, PTEN-induced putative kinase 1.

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pathway mainly regulates mitophagy. Dysfunctional mitochondria can promote PINK1 accumulation in the outer membrane of damaged mitochondria, which leads to Parkin phosphorylation and ubiquitination degradation.[16] Subsequently, optineurin (OPTN), a 52 kDa nuclear dot protein, and sequestosome 1 (p62/SQSTM1) are recruited to the damaged outer mitochondrial membrane and interact with autophagy-resident protein microtubule-associated protein light chain 3 (LC3). Phagocytes amalgamate and fuse into a complete ring, separating each damaged mitochondrion into a mitophagosome. Finally, mitophagosomes fuse with lysosomes and are degraded by proteolytic enzymes (e.g., cathepsin B and cathepsin D) in lysosomes.[8] PINK1-deficient mAPP mice display low LC3B levels, and PINK1 overexpression enhances autophagy and increases the expression level of lysosome-associated membrane protein 1 (LAMP1).[17] This study evaluated whether PINK silencing could modulate the effect of $A\beta_{25-35}$ on the mitophagy-lysosome pathway in PC12 cells, which is an excellent cellular model.

Our study investigated changes in the patterns of the mitophagy-lysosomal pathway in $A\beta_{25-35}$-treated PC12 cells following PINK1 silencing. The results provided evidence that PINK1 deficiency enhanced the blockade of the $A\beta_{25-35}$-induced mitophagy-lysosome pathway. Aβ-treated PC12 cells might be a valuable cellular model to evaluate the PINK1-mediated mitophagy and bioactive compound screening.

Materials and methods

Cell cultures and treatment

$A\beta_{25-35}$ (A4559, Sigma Aldrich, USA) was dissolved in sterile saline at a concentration of 1 mg/mL and incubated at 37 °C for 7 days. PC12 cells were obtained from the National Infrastructure of Cell Line Resource (Beijing, China). PC12 cells were cultured in RPMI-1640 medium that contained 10% fetal bovine serum in a humidified cell incubator with an atmosphere of 5% CO_2 at 37 °C.

PC12 cells (3×10^5 cells/well) were cultured on glass coverslips in 6-well plates for 12 h and transfected with control siRNA or PINK1 siRNA (50 nM) (Dharmacon, USA) using Lipofectamine 2000 (Invitrogen, USA). Two days later, the PC12 cells were treated with 20 μM $A\beta_{25-35}$ for 24 h and the cells were collected for subsequent experiments.

Mitochondria isolation

Mitochondria from individual groups of cells were extracted using a mitochondrial extraction kit (C3606, Beyotime, China), according to the manufacturer's instructions.

Immunofluorescence

The different groups of PC12 cells (1×10^5 cells/well) were cultured on glass coverslips for 12 h and treated as described previously. The cells were labeled with MitoTracker (M22426, Invitrogen, USA) at 37 °C for 20 m. The cells were fixed with 4% formaldehyde buffer and incubated for 60 m in blocking solution, followed by permeabilization. The cells were probed with antibodies against LC3B (1:500, PM036, Medical & Biological Laboratories, Japan), Parkin (1:100, 2132S, Cell Signaling Technology, USA) and LAMP1 (1:100, ab24170, Abcam, USA) overnight at 4 °C. After washing, the cells were incubated with fluorescent secondary antibodies for 1 h at room temperature and nuclearly stained with DAPI (1:10,000, 4083S, Cell Signaling Technology, USA) for 10 m at 37 °C. Finally, the fluorescent signals were examined under a fluorescence microscope (Nikon, Japan).

Immunoelectron microscopy

The different groups of cells were fixed with immunoelectron microscope fixative solution at room temperature for 5 m, and further fixed with 2% paraformaldehyde and 0.2% glutaraldehyde in sodium carbonate buffer (pH 7.4) at 4 °C for 12 h. After being washed with phosphate buffer solution (PBS) at room temperature three times, the cells were treated with 0.5% osmium tetroxide at 4 °C for 1 h, washed with PBS three times, dehydrated in gradient absolute ethanol, and embedded in acrylic resin. The samples were subjected to ultrathin sections (70 nm) and stained with rabbit primary antibody against p62/SQSTM1 at 4 °C overnight. The sections were washed three times for 10 m each time in PBS and sealed with electron microscope diluent for 20 m. Subsequently, the sections were incubated with gold-labeled goat anti-rabbit antibody for 2 h at room temperature, washed ten times for 10 m each in PBS, washed three times in distilled water (30 s), stained and dried at room temperature. The sections were visualized by transmission electron microscopy (TEM, hatachht7700) at 80 kV, and images were obtained.

Western blotting

Different groups of cells were harvested and lyzed in lysis buffer. After centrifugation, the concentrations of proteins were determined. The cell lysates (30–40 μg/lane) were separated on 8% or 10% gradient SDS–PAGE gels and electronically transferred to PVDF membranes. After blocking with 5% skim milk in Tris HCl buffer and tween20 solution, the membranes were incubated with primary antibodies, including Parkin (1:1,000, 2132S, Cell Signaling Technology), OPTN (1:1,000, 10837-1-AP, Proteintech, China), VDAC1 (1:1,000, WL02790, Wanleibio, China), and β-actin (1:1,000, sc-47778, Santa Cruz Biotech, USA). The bound antibodies were detected with HRP-conjugated secondary antibodies and visualized with an enhanced chemiluminescence kit (Kangwei Biotechnology, China). The relative levels of interesting proteins were quantified by ImageJ software and calculated by normalizing control β-actin or VDAC1.

Mitochondrial membrane potential test

After treatment, the cells were incubated with JC-1 (10 μmol/L) (C2006, Beyotime, China) at 37 °C for 10 m in the dark. The cells were analyzed by confocal fluorescent imaging under a fluorescence microscope (Nikon, Japan).

Statistical analysis

The data were analyzed using SPSS 26.0 (USA). Statistical significance was determined using one-way ANOVA followed by Fisher's least significant difference (LSD) multiple-comparisons test or Dunnett's T3 test. Experimental data were represented as the mean ± standard error. A p-value <0.05 was considered statistically significant.

Results

Effect of PINK1 deficiency on Aβ$_{25–35}$-induced mitophagy in PC12 cells

To evaluate the effect of PINK1 deficiency on mitophagy, PC12 cells were transfected with control siRNA or PINK1 siRNA (50 nM) for 48 h. Transfection with PINK1 siRNA efficiently reduced PINK1 expression by nearly 80% in PC12 cells (data not shown). Subsequently, the cells were incubated with 20 μM Aβ$_{25–35}$ for 24 h. Anti-LC3B antibody and MitoTracker (a marker of mitochondria) were used for double immunostaining. Confocal microscopy displayed that the Aβ$_{25–35}$ treatment increased the colocalization coefficients of LC3-positive vesicles with mitochondria in PC12 cells (Fig. 1a, b). In addition, compared with that in Aβ$_{25–35}$-treated cells, the colocalization coefficients of LC3 with mitochondria induced by Aβ$_{25–35}$ treatment were markedly reduced in PINK1 silenced cells (Fig. 1a, b). Immunoelectron microscopy further revealed that p62/SQSTM1 degradation decreased in PINK1-silenced cells (Fig. 1c).

Effect of PINK1 deficiency on the Parkin-mediated mitophagy-lysosomal degradation in Aβ$_{25–35}$-treated PC12 cells

PINK1 accumulates in the outer membrane of damaged mitochondria and phosphorylates Parkin, leading to its ubiquitination degradation. OPTN is recruited to the damaged outer mitochondrial membrane. PINK1 silencing increased OPTN and Parkin levels in whole cell lysates but decreased Parkin levels in mitochondria of PC12 cells following Aβ$_{25–35}$ treatment (Fig. 2a, b). Compared with the controls, immunofluorescence displayed that the Mander's overlap coefficient of Parkin-positive vesicles and mitochondria increased, but lower levels of colocalization between the lysosomal LAMP1 and mitochondria in PINK1-silenced PC12 cells following Aβ$_{25–35}$ treatment (Fig. 2c–f). Similarly, the colocalization of Parkin and mitochondria significantly decreased, and colocalization of LAMP1 and mitochondria was almost absent in PINK1-silenced PC12 cells following Aβ$_{25–35}$ treatment (Fig. 2c–f). These results supported that PINK1 deficiency inhibited mitophagy and deteriorated the Aβ$_{25–35}$-attenuated mitophagy-lysosomal pathway in PC12 cells.

Effect of PINK1 deficiency on the Aβ$_{25–35}$-induced mitochondrial dysfunction

Autophagic flux blockade can increase the accumulation of damaged mitochondria and further aggravate mitochondrial dysfunction. The Aβ$_{25–35}$ treatment reduced JC-1 red/green fluorescence ratios, a hallmark of decreased MMP and PINK1 silencing slightly further decreased MMP in PC12 cells (Fig. 3a, b). A significantly abnormal mitochondrial distribution was observed in AD patients and animal models. Of note, while the mitochondria (marked by MitoTracker) were evenly distributed in the cytoplasm of control cells, PINK1 silencing disturbed the distribution of mitochondria and caused abnormal mitochondrial locations in the nuclei of PC12 cells following Aβ$_{25–35}$ treatment (Fig. 3c).

Discussion

Mitochondria are cellular energy powerhouses and are responsible for the maintenance of normal cell life. As high-energy cells, neurons are particularly sensitive to damage to mitochondrial function. Multiple studies found that neurons in AD patients have dysfunctional mitochondria, and an age-related decline in mitochondrial function is an early initiating event in AD, which leads to various pathophysiological changes in neurons and contributes to disease progression.[18] Mitophagy can selectively degrade defective or functionally altered mitochondria, and the balance between mitotic phagocytosis and clearance controls mitochondrial homeostasis. An alternation in mitophagy is found in the brains and peripheral tissues of AD patients, which is a new characteristic of AD.[19] When MMP decreases, PINK1 induces Parkin accumulation in mitochondria, mediates VDAC1 ubiquitination, and recruits p62/SQSTRM and LC3.[20–22] A previous study showed that Aβ$_{25–35}$ treatment could enhance mitophagy in rats.[7] Increased LC3II/LC3I ratios and PINK1/Parkin expression have been detected in 6-month-old APP/PS1 transgenic mice.[14] PINK1 accumulated at Braak II and III in AD patients. These indicated that mitophagy activity increases in the early stage of AD to maintain mitochondrial function. Of note, Aβ injury can trigger a defect in the mitophagy-lysosome pathway by causing lysosomal dysfunction; therefore, resulting in the abnormal accumulation of mitophagosomes and substrates.[15,19]

Aβ$_{25–35}$ is a short, highly neurotoxic, and naturally occurring Aβ peptide.[23] Aβ$_{25–35}$ can decrease synaptic function, rapidly accumulate and deposit in mitochondria, inhibit mitochondrial biogenesis, and induce mitochondria-mediated apoptosis.[24–28] A previous study showed that Aβ$_{25–35}$ inhibits PC12 cell proliferation and induces autophagy in neurites, and autophagy stimulators, not inhibitors, significantly attenuate the Aβ-induced neurite degeneration.[29] Therefore, enhanced autophagy appears to be neuroprotective for Aβ$_{25–35}$-treated PC12 cells. However, to the best of our knowledge, the effect of mitophagy on Aβ$_{25–35}$-treated PC12 cells is not fully understood.

Of note, Aβ$_{1-42}$ treatment decreases mitophagy in PC12 cells, based on the relative levels of LC3B, PINK1 and Parkin expression in whole cell lysates, but not mitochondrial extracts.[30] However, their study indicates that Aβ-treated PC12 cells might be a good cellular model to evaluate the PINK1/Parkin-mediated mitophagy. The current study found that the Aβ$_{25–35}$ treatment promoted the accumulation of mitophagosomes and substrates in PC12 cells. Aβ$_{25–35}$ treatment decreased the colocalization of the lysosomal LAMP1 and mitochondria in PC12 cells. These results indicated that Aβ$_{25–35}$ was induced to attenuate autophagic flux in PC12 cells. Furthermore, PINK1 deficiency inhibited the Aβ$_{25–35}$-induced mitophagy and deteriorated the Aβ$_{25–35}$-attenuated autophagic flux, possibly because PINK1 deficiency aggravated lysosomal dysfunction. Autophagic flux blockage can lead to the accumulation of damaged mitochondria, which aggravates mitochondrial dysfunction and forms a vicious cycle. PINK1 silencing significantly further reduced the Aβ$_{25–35}$-decreased MMP in PC12 cells. This MMP loss might release cytochrome c from the mitochondrial intermembrane to the cytosol, further activating caspase-9 and caspase-3 and the mitochondrial apoptosis pathway.[31]

Future directions

More experiments are required to explore the effect of PINK1 on Aβ-treated PC12 cells, including its effect on mitochondrial dynamics and biosynthesis. Further studies are required to investigate whether PINK1 deficiency could modulate AD in transgenic mice.

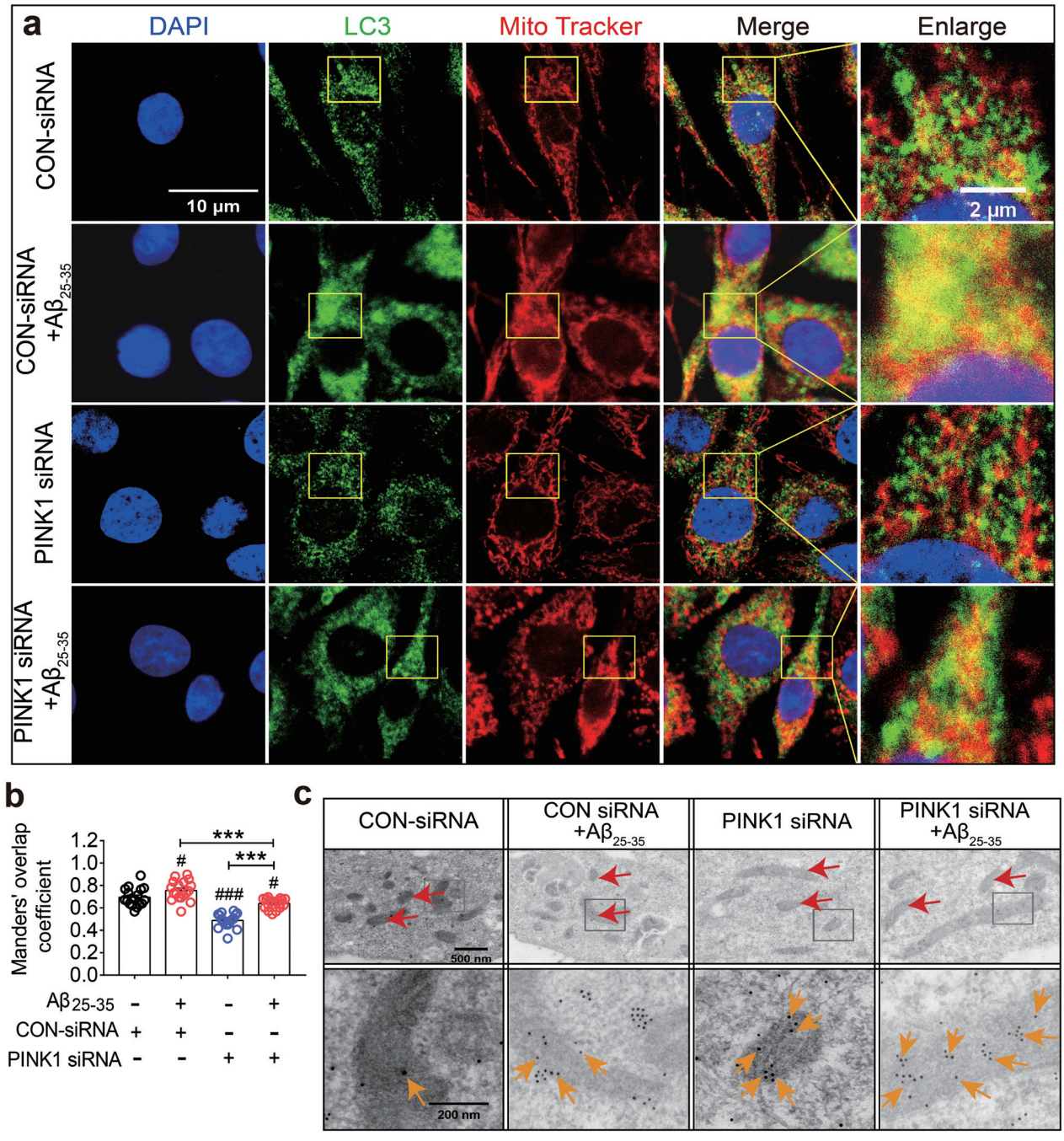

Fig. 1. Effect of PINK1 deficiency on Aβ25–35-induced mitophagy. PC12 cells were transfected with PINK1 siRNA and CON-siRNA for 48 h and incubated with $A\beta_{25-35}$ for 24 h. (a) immunofluorescence analysis of the colocalization of the autophagic LC3 (green) with the mitochondria (marked by MitoTracker, red) in PC12 cells. The nuclei were stained with DAPI (blue); (b) quantitative analysis of the Mander's overlap coefficient of LC3 with MitoTracker in PC12 cells; and (c) immunoelectron microscopy images displayed the colocalization of p62 (yellow arrow) with mitochondria (red arrow). n = 3 per group. LC3, light chain 3; PINK1, PTEN-induced putative kinase 1.

Conclusions

The current study provided evidence that PINK1 deficiency deteriorated the $A\beta_{25-35}$-inhibited mitophagy-lysosomal pathway in PC12 cells. Aβ-treated PC12 cells might be used as an excellent cellular model to evaluate PINK1-mediated mitophagy and bioactive compound screening.

Acknowledgments

None.

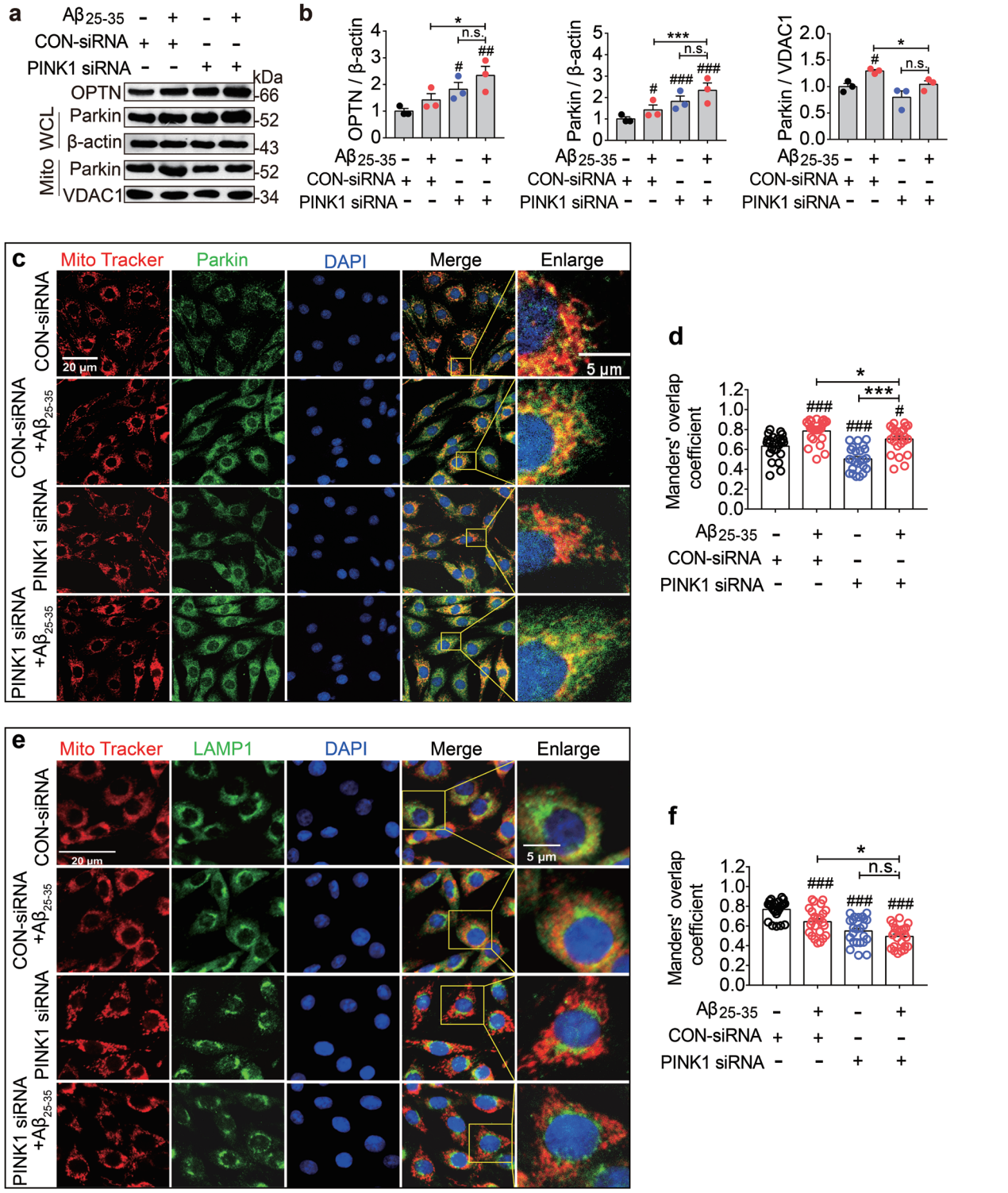

Fig. 2. Effect of PINK1 deficiency on the Parkin-mediated mitophagy-lysosomal degradation in Aβ$_{25–35}$-treated PC12 cells. (a, b) Western blot and quantification of the relative levels of OPTN, Parkin (in whole cell lysate), and Parkin (in mitochondria) from the indicated homogenates of PC12 cells. β-actin and VDAC1 served as loading controls; (c) immunofluorescence analysis of the colocalization of Parkin (green) with mitochondria (red) in PC12 cells. The nuclei were stained with DAPI (blue); (d) quantitative analysis of the Mander's overlap coefficient of Parkin with MitoTracker in PC12 cells; (e) immunofluorescence analysis of the colocalization of the lysosomal LAMP1 (green) with the mitochondria (red) in PC12 cells; (f) quantitative analysis of the Mander's overlap coefficient of LAMP1 with MitoTracker in PC12 cells. All results are expressed as the mean ± SEM (n = 3 per group). #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ versus the CON-siRNA group; *$p < 0.05$, ***$p < 0.001$. LAMP1, lysosome-associated membrane protein 1; OPTN, optineurin; PINK1, PTEN-induced putative kinase 1; VDAC, Voltage-dependent anion channel.

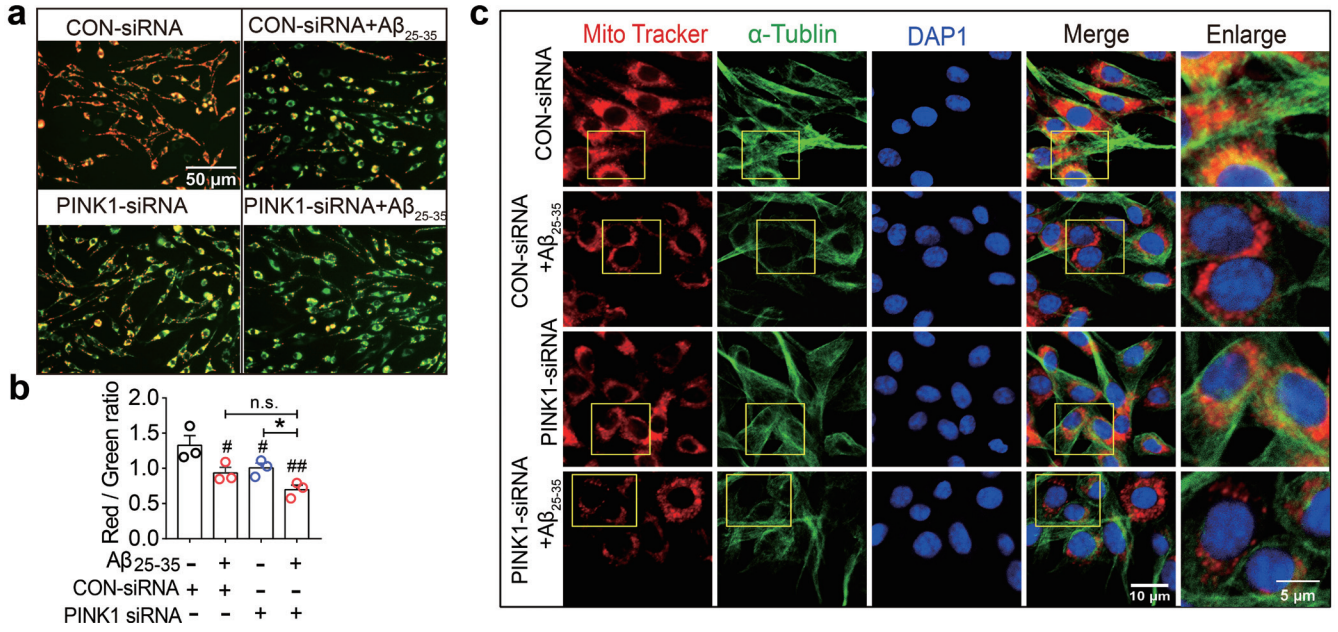

Fig. 3. Effect of PINK1 deficiency on $A\beta_{25-35}$-induced mitochondrial dysfunction. Confocal microscopy analysis of MMP, the red/green ratios. (a) after JC-1 staining; (b) representative images of JC-1 signaling; (c) immunofluorescence analysis of the distribution of mitochondria in PC12 cells, using MitoTracker (red), an antibody against α-tubulin (green), and nuclei DAPI (blue). All results are expressed as the mean ± SEM (n = 3 per group). #$p < 0.05$, ##$p < 0.01$ versus the CON-siRNA group; *$p < 0.05$. MMP, Mitochondrial membrane potential; PINK1, PTEN-induced putative kinase 1.

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Conflict of interest

Peng Liu has been an editorial board member of *Journal of Exploratory Research in Pharmacology* since January 2022. Yong-Qiang Xue is an employee of Suzhou Xishan Zhongke Drug Research & Development Co., Ltd. The authors have no other conflicts of interest related to this publication.

Author contributions

XW and PL conceived the project and designed the experiments. XW performed the experiments and data analysis with the help of YX, HZ and YY. XW and PL were responsible for the manuscript writing. All authors have made a significant contribution to this study and approved the final manuscript.

Data sharing statement

No additional data are available.

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