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δ-Opioid Receptor Activation Attenuates Hypoxia/MPP$^+$-Induced Downregulation of PINK1: a Novel Mechanism of Neuroprotection Against Parkinsonian Injury

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Abstract

There is emerging evidence suggesting that neurotoxic insults and hypoxic/ischemic injury are underlying causes of Parkinson's disease (PD). Since PTEN-induced kinase 1 (PINK1) dysfunction is involved in the molecular genesis of PD and since our recent studies have demonstrated that the δ-opioid receptor (DOR) induced neuroprotection against hypoxic and 1-methyl-4-phenyl-pyridimium (MPP$^+$) insults, we sought to explore whether DOR protects neuronal cells from hypoxic and/or MPP$^+$ injury via the regulation of PINK1-related pathways. Using highly differentiated rat PC12 cells exposed to either severe hypoxia (0.5–1% O_2) for 24–48 h or varying concentrations of MPP$^+$, we found that both hypoxic and MPP$^+$ stress reduced the level of PINK1 expression, while incubation with the specific DOR agonist UFP-512 reversed this reduction and protected the cells from hypoxia and/or MPP$^+$-induced injury. However, the DOR-mediated cytoprotection largely disappeared after knocking down PINK1 by PINK1 small interfering RNA. Moreover, we examined several important signaling molecules related to cell survival and apoptosis and found that DOR activation attenuated the hypoxic and/or MPP$^+$-induced reduction in phosphorylated Akt and inhibited the activation of cleaved caspase-3, whereas PINK1 knockdown largely deprived the cell of the DOR-induced effects. Our novel data suggests a unique mechanism underlying DOR-mediated cytoprotection against hypoxic and MPP$^+$ stress via a PINK1-mediated regulation of signaling.

Keywords Parkinson's disease · Cytoprotection · δ-Opioid receptor · PINK1 · Akt · Caspase-3

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease and affects approximately 1% of the world's population [1]. Pathologically, PD is characterized by the progressive loss of dopaminergic neurons in the substantia nigra, degeneration of the nigrostriatal pathway, and depletion of striatal dopamine [2]. These pathological changes are responsible for the clinical symptoms of PD patients such as hypokinesia, resting tremor, and autonomic dysfunction [3]. Although the etiopathogenesis of PD seems to be complex, alterations in mitochondrial homeostasis have long been regarded as a key mechanism in most PD cases and this is demonstrated by human tissue studies, as well as by genetic and neurotoxin models [4–7].

A well-maintained, balanced mitochondrial network is indispensable for neuronal signaling, plasticity, and transmitter release [8, 9]. The interest in mitochondrial alterations linked to PD greatly increased when it became evident that some PD-associated gene products have direct or indirect impacts on mitochondrial integrity. Mutations in the genes encoding

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PTEN-induced kinase 1 (PINK1) and Parkin are identified in autosomal recessive parkinsonism [4, 6, 10], and emerging evidence establishes the important role of PINK1 for promoting mitochondrial homeostasis. It has been reported that the overexpression of PINK1 can protect cells from oxidative stress-induced apoptosis by suppressing cytochrome c release from the mitochondria [11]. Several study groups also claimed that PINK1 interacts with Parkin to regulate mitochondrial dynamics by recruiting Parkin into the mitochondria from the cytosol and initiating mitophagy [6, 12–14]. In vivo, PINK1 KO mice exhibited similar symptoms to those seen in PD, including mitochondrial dysfunction in dopaminergic neurons, reduced dopamine overflow, and impaired corticostriatal synaptic plasticity [15, 16]. Altogether, a growing consensus suggests that the mitochondrial deficit induced by the loss of function of PINK1 is a major contributor to the pathogenesis of PD [6, 7, 17].

Our research group has previously proven the neuroprotective effects of delta-opioid receptor (DOR) against hypoxic and 1-methyl-4-phenyl-pyridimium (MPP^+) insults, which have been demonstrated as important factors contributing to the pathological process of PD [18–22]. There is also initial evidence to show that DOR may be a beneficial factor against parkinsonism. DOR may reverse methamphetamine-induced loss of dopamine transporters, as well as improve the motor function of rats with parkinsonism. Also, DOR is likely to act as a promising target in Parkinson's disease via hypoxic tolerance [23–26]. However, the mechanism underlying DOR-mediated neuroprotection against parkinsonism is still unknown. As we have a clue for the relationship between DOR and PINK1, a PD-associated gene [21], we conducted this work to investigate whether DOR activity could alter the expression of PINK1 in hypoxic and/or MPP^+ conditions and affect cell injury at the same time. Moreover, we explored the potential molecules involved in PINK1-DOR signaling in survival/apoptosis in the PC12 cells. Our novel data has well elucidated the interaction between DOR and PINK1 as a unique mechanism underlying DOR-mediated cytoprotection against hypoxic and MPP^+ stress.

Materials and Methods

Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM) for cell culture was purchased from Gibco®, Thermo Fisher Scientific (Waltham, MA, USA; Cat: 11995-065). Fetal bovine serum (FBS), MPP^+, and MTT powder for cell viability measurement were purchased form Sigma Chemical Co. (St. Louis, MS, USA; Cat: M2128, 15H467, and D048, respectively). LDH kit for cytotoxicity assay was purchased from Beyotime Biotechnology (Shanghai, China; Cat: C0016). UFP-512, a highly specific and potent DOR agonist, was produced by our research group [27–29]. Naltrindole hydrochloride, a DOR antagonist, was purchased from Tocris Bioscience (Bristol, UK; Cat: 0740). PINK1 small interfering RNA (siRNA) for PINK1 knockdown was purchased from GenePharma Co. (Shanghai, China; Cat: PINK1-homo-786). Anti-PINK1 antibody was purchased from Novus Biologicals (Littleton, CO, USA; Cat: BC100-494). Anti-β-actin antibody, anti-caspase-3 antibody, anti-Akt antibody, anti-phospho-Akt antibody, anti-c-Jun N-terminal kinase (JNK) antibody, anti-phospho-JNK antibody, anti-Erk1/2 antibody, anti-phospho-Erk1/2 antibody, anti-p38 antibody, and anti-phospho-p38 antibody were all purchased from Cell Signaling Technology (Danvers, CO, USA; Cat: 4970, 4691, 4060, 9662S, 9252, 9251, 9102, 9101, 8690, and 9211, respectively).

Cell Cultures and Experimental Groups

Highly differentiated rat PC-12 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China, and were cultured in DMEM with 10% FBS. The differentiated cells were maintained in six-well plates and randomly allocated to normoxia, MPP^+, and hypoxia groups. Under normoxic conditions, cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C. To induce hypoxia, cells were put in a hypoxic chamber (Galaxy 48R, New Brunswick, Edison, NJ, USA) with the O_2 levels being kept strictly at 0.5 or 1% [21]. To induce MPP^+ injury, cells were exposed to 0.5–2.0 mM of MPP^+ at 24 h after cell passage. The DOR agonist (UFP-512, 5 μM) and the DOR antagonist (naltrindole, 1 μM) were added to the culture media immediately before the onset of hypoxia and MPP^+ stress.

The experimental groups were divided as follows: normoxic control (C), DOR activation with UFP-512 (5 μM) without any other treatment (C + U), UFP-512 (5 μM) plus DOR antagonist naltrindole (1 μM) in normoxic conditions (C + U + N), hypoxia (H), DOR activation with UFP-512 (5 μM) in hypoxic conditions (H + U), UFP-512 (5 μM) plus naltrindole (1 μM, a DOR antagonist) in hypoxic conditions (H + U + N), naltrindole (1 μM) in hypoxic conditions (H + N), MPP^+ (M), UFP-512 (5 μM) under MPP^+ insult (M + U), UFP-512 (5 μM) plus naltrindole (1 μM) under MPP^+ insult (M + U + N), and naltrindole (1 μM) under MPP^+ insult (M + N).

Cell Viability Assay

Cell viability was measured using the MTT assay. The cells were planted in 96-well plates (6000 cells per well), and the plates were incubated overnight in a humidified incubator with 5% CO_2 at 37 °C. The wells without cells but containing 200 μl of culture medium were used as the blank control. After treating the cells with drugs under MPP^+ or hypoxic conditions for 24 or 48 h, MTT reagent (20/200 μl per well in the 96-well plates) was added to the cells and the plates were incubated for another 4 h in the incubator with 5% CO_2 before measurement. The absorbance was measured at a

wavelength of 490 nm using a microplate reader (BioTek, Winooski, VT, USA).

Lactate Dehydrogenase Assay

Cytotoxicity was quantitatively determined by detecting the activity of lactate dehydrogenase (LDH) in the cell culture medium using the LDH assay kit since LDH leakage is a reliable index of cell injury as shown in our previous work [19–21]. Before taking the measurement, cells were treated as required. The wells without cells but containing 200 μl of culture medium and the wells containing 10% lactate release reagent were set as the blank control and the maximum control, respectively. After centrifuging for 5 min at 400*g*, a 120-μl supernatant per well was transferred from the plates to a new 96-well plate and mixed with the working solution. The new 96-well plate was protected from light and incubated for 30 min at room temperature. The absorbance of the solution was detected by a microplate reader (BioTek, Winooski, VT, USA) at a wavelength of 490 nm. The calculation of % cytotoxicity followed the below equation

$$\%\text{Cytotoxicity} = \frac{[\text{Experimental}(\text{OD490}) - \text{Blank}(\text{OD490})] \times 100}{[\text{Maximum LDH release}(\text{OD490}) - \text{Blank}(\text{OD490})]}$$

siRNA Transfection

The PC12 cells were transfected with PINK1 siRNA constructs or control siRNA. The sequences were as follows: negative control siRNA, 5′-UUC UCC GAA CGU GUC ACG UTT-3′, and PINK1 siRNA, 5′-GCC AUC UUG AAC ACA AUG ATT-3′. Following the manufacturer's instructions (GenePharma Co., Shanghai, China), the siRNA compound was diluted in Opti-MEM and then mixed in a 1:1 ratio with an equal volume of diluted Lipofectamine in Opti-MEM. After incubating the cells for 20 min, the mixture was added directly to the cells. After incubation for 4 h, the medium containing siRNA was removed and the new medium was added at 12 h before the onset of drug treatment. Transfected PC12 cells were tested by Western blot for silencing of PINK1, and the successfully transfected cells were used for the following experiments.

Western Blotting

Cells were lysed at 4 °C using the lysis buffer containing 0.1% protease inhibitor, 0.5% 100 mM PMSF, and 1% phosphatase inhibitor (KeyGen Biotech, Nanjing, China; Cat: KGP2100). Using the BCA protein assay kit, the protein concentration was determined and equal amounts of protein samples were diluted in a 6× sample buffer and run in 10% SDS-PAGE. After proteins were transferred to hydrophobic polyvinylidene difluoride (PVDF) membranes, the membranes were probed by several mAbs. Then, following incubation with HRP-conjugated secondary antibodies for 1 h at room temperature, the membranes were visualized by chemiluminescence exposure using Western Lightening® Chemiluminescence Reagent Plus (PerkinElmer, Boston, MA, USA). Quantitation was performed by densitometry using the NIH Image program (ImageJ).

Statistical Analysis

All data are presented as means ± SEM, and the number of independent experiments performed for each measurement is at least 3. Statistical analysis was processed with one-way ANOVA followed by Bonferroni's multiple comparison tests (Prism 5; GraphPad Software, La Jolla, CA, USA).

Results

DOR Activation Attenuates PINK1 Downregulation Under Both Hypoxic and MPP^+ Insults

Before the onset of the experiment, a series of concentrations of UFP-512 (1–20 μM) were applied to the cultures to investigate the cytoprotective effects of UFP-512 in terms of cells viability and LDH leakage (Fig. 1). We found that 1–5 μM of UFP-512 had a dose-dependent cytoprotective effect on these cells under hypoxic and MPP^+ insults with no further increase in the cytoprotection when increasing the dose beyond 5 μM of UFP-512 (Fig. 1), so we chose 5 μM as the optimized concentration of UFP-512 for subsequent experiments. Our first step to determine whether PINK1 plays a role in DOR-mediated neuroprotection was to examine whether DOR activation could alter the expression of PINK1 in the PC12 cell line. As shown in Fig. 2, there was no significant effect of the DOR agonist and antagonist on PINK1 expression in normoxic conditions (Fig. 2a). However, DOR activation had a major impact on PINK1 expression under hypoxic conditions. Hypoxia at 0.5% of O_2 for 24 h caused a 44.35% reduction in PINK1 expression ($p < 0.01$ vs. the control, $n = 3$, respectively; Fig. 2b), whereas UFP-512 significantly attenuated PINK1 downregulation by 27.16% ($p < 0.05$ vs. H, $n = 3$; Fig. 2b). Furthermore, hypoxia at 1% of O_2 for 48 h led to a more severe decrease in PINK1 expression (64.80% reduction vs. the control level, $p < 0.01$, $n = 3$; Fig. 2c) and was more significantly reversed by the application of the DOR agonist UFP-512 (60.04% increase vs. H, $p < 0.01$, $n = 3$; Fig. 2c).

DOR activation also greatly increased the PINK1 expression under MPP^+ stress (Fig. 2d). Following the 24-h 0.5 mM MPP^+ exposure, DOR activation by UFP-512 did not result in

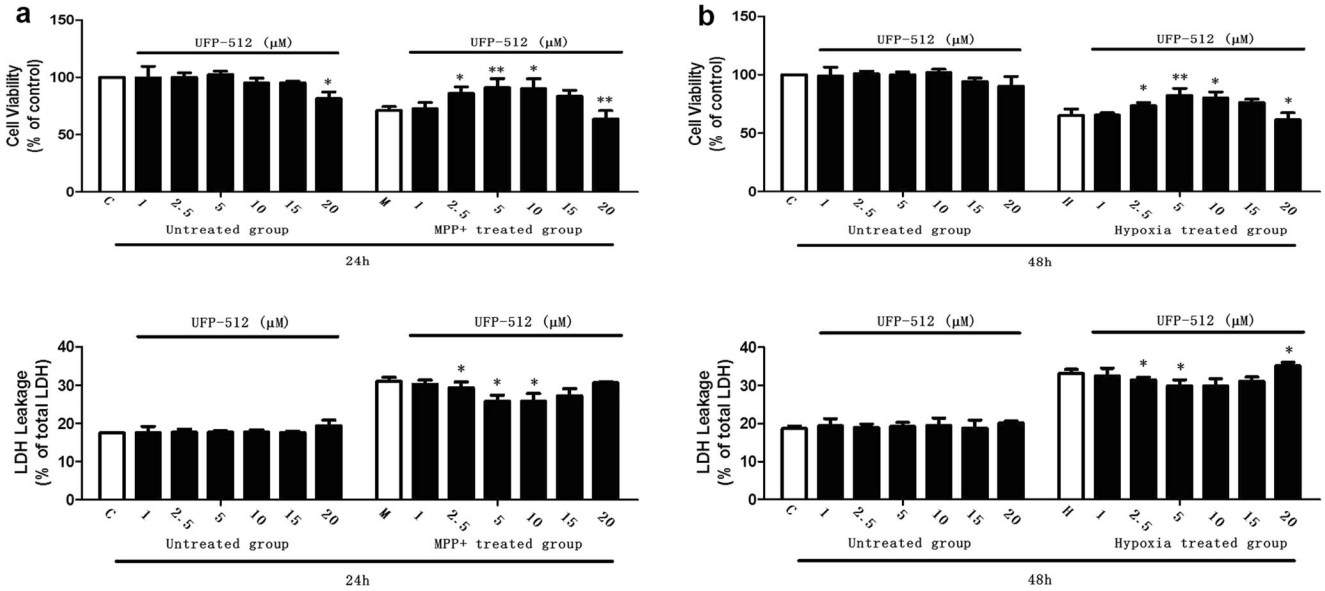


Fig. 1. Dose-response study on UFP-512-induced cytoprotection. **a** PC12 cells were exposed to 1.0 mM MPP^+ for 24 h. A series of concentrations of UFP-512 (1–20 μM) were applied to the culture to investigate the effects of UFP-512 on PC12 cell viability and LDH leakage. C: normoxic control. M: MPP^+. $*p < 0.05$ and $**p < 0.01$ vs. C or M. Note that the PC12 cells treated with 2.5–10 μM of UFP-512 led to a significant increase in cell viability and a decrease in LDH leakage. The neuroprotection mediated by UFP-512 increased with the increase of UFP-512 concentration from 1 to 5 μM, whereas the cell viability and LDH leakage remain unchanged in the concentration of more than 5 μM. **b** PC12 cells were cultured in a hypoxic chamber with the O_2 levels being kept strictly at 1%. A series of concentrations of UFP-512 (1–20 μM) were applied to the culture to investigate the effects of UFP-512 on PC12 cell viability and LDH leakage in hypoxic conditions. C: normoxic control. H: hypoxia. $*p < 0.05$ and $**p < 0.01$ vs. C or H. Note that the cell viability was significantly increased after the cells were treated with 2.5–10 μM of UFP-512, and the LDH leakage respectively declined after the same treatment

any significant changes in the PINK1 relative density. However, after raising the concentration of MPP^+ to 1.0 mM, the application of UFP-512 significantly reversed the reduction in PINK1 expression (from 80.93% in 1.0 mM MPP^+ stress alone to 103.31% in 1.0 mM MPP^+ plus DOR activation, $p < 0.01$, $n = 3$; Fig. 2d), and similar results were observed when the cells were exposed to 2.0 mM MPP^+. The relative density of PINK1 in cells treated with 2.0 mM MPP^+ also increased by DOR activation from 50.41% in cells exposed to 2.0 mM MPP^+ stress alone to 74.16% in cells treated with 2.0 mM MPP^+ plus DOR agonist UFP-512 ($p < 0.05$, $n = 3$; Fig. 2d).

These observations were further confirmed by the addition of the DOR antagonist naltrindole along with UFP-512 to the medium. Compared with the data from the cells treated with the DOR agonist alone, the addition of naltrindole completely reversed the UFP-512-induced changes in the PINK1 expression under both hypoxic and MPP^+ conditions (Fig. 2).

PINK1 Knockdown Reduced PC12 Cell Survival and DOR-Mediated Cytoprotection Against Hypoxia and MPP^+ Stress

To further determine the interaction between DOR and PINK1, we transfected the PC12 cells with PINK1 siRNA. This transient transfection caused a significant reduction in PINK1 proteins by 58.1% in comparison to that of the control siRNA-transfected cells (Fig. 3). As depicted in Fig. 4a, both hypoxia and MPP^+ induced significant cell injury, and PINK1 knockdown aggravated the injury characterized by a severe reduction in cell density. Morphologically, the cells became shrunken and round. The addition of UFP-512 largely reduces the injury in the cells transfected with negative control siRNA but had an undetectable effect on the cells transfected with PINK1 siRNA.

PINK1 knockdown also led to a significant decrease in cell viability and an increase in LDH leakage in both *normal* (normoxic) and hypoxia/MPP+ conditions, especially in the hypoxia and MPP^+ groups (Fig. 4). We found that the transfection with PINK1 siRNA attenuated the DOR-mediated cytoprotection against hypoxic insults. At 48 h of hypoxia, as shown in Fig. 4b (top panel), the DOR agonist significantly increased cell viability in the cells transfected with control siRNA; however, this increase was diminished by the knockdown of PINK1 (− 54.97%, $p < 0.01$ vs. the control siRNA without PINK1 knockdown; $n = 3$; Fig. 4b).

Under the conditions of 1.0 mM MPP^+ insults for 24 h, successful knockdown of PINK1 also caused a major loss of the DOR-mediated cytoprotection, i.e., a reduction of 44.99% in cell viability compared to that of the control siRNA group ($p < 0.01$ vs. MPP^+ plus DOR activation in the control siRNA-treated group, $n = 3$; Fig. 4c, top panel). The DOR-mediated cytoprotection was further inhibited in

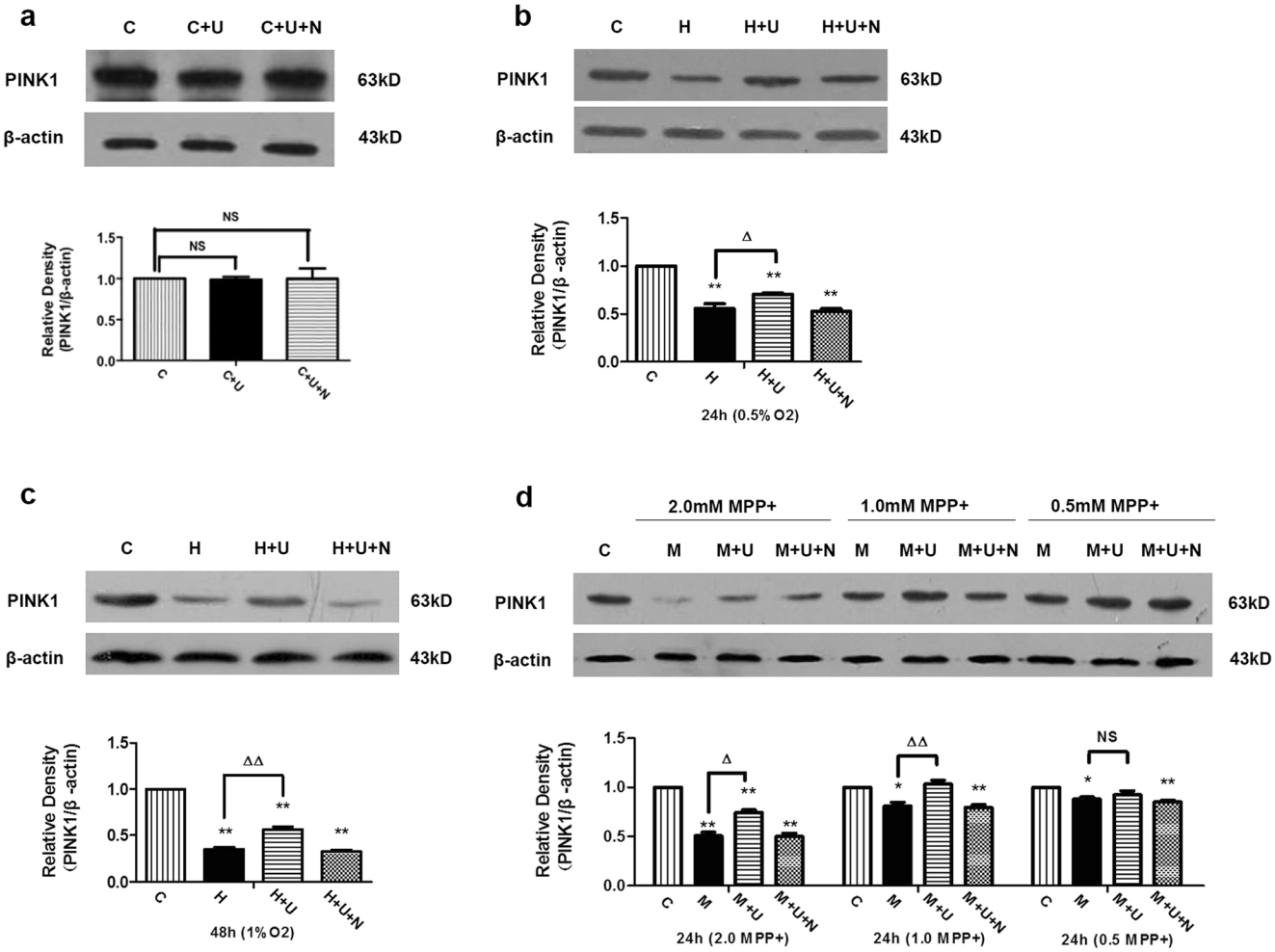


Fig. 2. Effects of DOR activation/inhibition on PINK1 expression under normoxic/hypoxic/MPP$^+$ insults in PC12 cells. **a** The PC12 cells were maintained in six-well plates without any treatment. C: normoxic control. C + U: DOR agonist UFP-512 in normoxic conditions. C + U + N: UFP-512 plus DOR antagonist naltrindole in normoxic conditions. $N = 3$ for each group. NS: not significant. Note that there was no significant change among these three groups, suggesting that the effects of the DOR agonist and DOR antagonist on PINK1 expression are negligible. **b** PC12 cells were exposed to hypoxia at 0.5% O_2 for 24 h. C: normoxic control. H: hypoxia. H + U: DOR was activated using UFP-512 in hypoxic conditions. H + U + N: PC12 cells were treated with UFP-512 plus naltrindole at the same time in hypoxic conditions. $N = 3$ for each group. $^{**}p < 0.01$ vs. control. $^{\Delta}p < 0.05$ vs. H. Note that hypoxia significantly reduced PINK1 expression in comparison with that of the normoxic control, while activating DOR using UFP-512 significantly attenuated the downregulation of PINK1 protein by hypoxia. **c** PC12 cells were exposed to hypoxia at 1% O_2 for 48 h. C: normoxic control. H: hypoxia. H + U: DOR was activated using UFP-512 in hypoxic conditions. H + U + N: PC12 cells were treated with UFP-512 plus naltrindole at the same time in hypoxic conditions. $N = 3$ for each group. $^{**}p < 0.01$ vs. control. $^{\Delta\Delta}p < 0.01$ vs. H. Note that hypoxia at 1% O_2 for 48 h led to a more severe decrease in PINK1 expression. Activating DOR using UFP-512 increased the expression level of PINK1 more significantly. **d** PC12 cells were exposed to 0.5–2.0 mM MPP$^+$ for 24 h. C: normoxic control. M: MPP$^+$. M + U: DOR was activated using UFP-512 and exposed to MPP$^+$. M + U + N: PC12 cells were treated with UFP-512 plus naltrindole and exposed to MPP$^+$. $N = 3$ in each group. NS: not significant, $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control. $^{\Delta}p < 0.05$ and $^{\Delta\Delta}p < 0.01$ vs. M. Note that the level of PINK1 decreased with the increase in MPP$^+$ concentration, while activating DOR using UFP-512 significantly reversed the reduction of PINK1 expression in MPP$^+$ (1.0 and 2.0 mM) insults

the PINK1 siRNA-treated group after the application of DOR antagonist (naltrindole 1 μM) in both hypoxic and MPP$^+$ conditions.

LDH leakage showed similar results. As shown in Fig. 4b, c, both hypoxia and MPP$^+$ induced an injury to PC12 cells, and the knockdown of PINK1 aggravated this injury by further enhancing the leakage of LDH. Moreover, the DOR activation-mediated cytoprotection was significantly attenuated in the PINK1 siRNA-treated group, which was indicated by an increase in LDH leakage compared to that of the control siRNA-treated group. Under hypoxic conditions, PINK1 knockdown led to a 41.36% increase in LDH leakage after prolonging the exposure time to 48 h ($p < 0.05$ vs. hypoxia plus DOR activation in the control siRNA-treated group, $n = 3$; Fig. 4b, bottom panel). Similarly, the PINK1 siRNA also induced a more serious injury in MPP$^+$ conditions, and DOR activation with UFP-512 induced a minor effect on the LDH leakage (22.91% increase in the

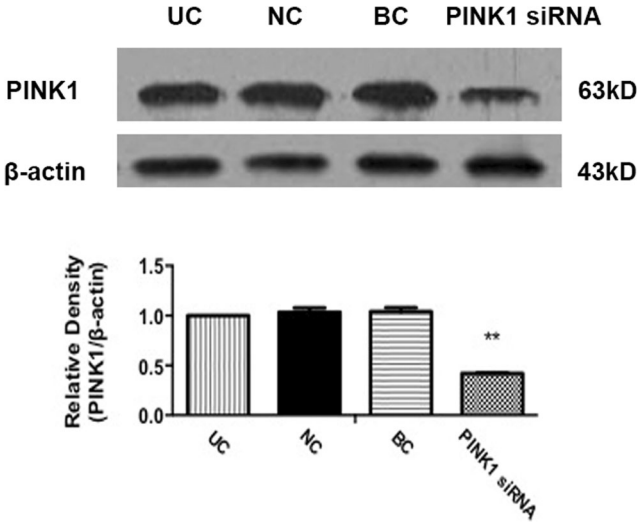


Fig. 3. PINK1 siRNA reduced PINK1 expression in PC12 cells. UC: untransfected PC12 cells. NC: negative control. PC12 cells were transfected with negative control siRNA. BC: blank control. PC12 cells were merely transfected with Lipofectamine 2000. PINK1 siRNA: PC12 cells were transfected with PINK1 siRNA. $N = 3$ for each group. $^{**}p < 0.01$ vs. UC. Note that PINK1 protein expression was significantly reduced by PINK1 siRNA

control siRNA group vs. 28.57% in the PINK1 siRNA group after 24 h of 1.0 mM MPP^+ treatment, $p < 0.05$, $n = 3$; Fig. 4c, bottom panel).

Altogether, the results from cell imaging, MTT, and LDH assays all suggest that PINK1 plays an important role in PC12 cell survival and is directly involved in the DOR-mediated cytoprotection under both hypoxic and MPP^+ insults.

DOR Activation Regulated Akt and Erk Phosphorylation and Caspase-3 Activation

To investigate the signaling integration between DOR and PINK1, we detected the effects of UFP-512 on the molecules involved in cell survival and apoptosis, including Akt/phosphorylated Akt (p-Akt), pro-caspase-3/cleaved caspase-3, Erk/phosphorylated Erk (p-Erk), p38/phosphorylated p38 (p-p38), and JNK/phosphorylated JNK (p-JNK). As shown in Fig. 5, 1%O_2 hypoxia for 48 h or 1.0 mM MPP^+ for 24 h caused a significant reduction in Akt phosphorylation, activated the cleaved caspase-3, and increased the level of phosphorylated p38. The application of the DOR agonist UFP-512 enhanced the phosphorylation of Akt in MPP^+ or hypoxic conditions and suppressed the activation of cleaved caspase-3, but neither p38 nor phosphorylated JNK expression was altered. Furthermore, we found that the treatment of cells with UFP-512 was able to increase the phosphorylation of Erk1/2 under all the conditions including normoxia, MPP^+, and hypoxia.

PINK1 Knockdown Largely Reduced Akt Phosphorylation and Upregulated Caspase-3 and p38

Moreover, we transfected the PC12 cells with negative control siRNA or PINK1 siRNA to compare the differences in the expression level of Akt/p-Akt, pro-caspase-3/cleaved caspase-3, Erk/p-Erk, p38/p-p38, and JNK/p-JNK before and after PINK1 knockdown. As delineated in Fig. 6, PINK1 knockdown remarkably reduced the signal density of phosphorylated Akt, promoted the activation of caspase-3, and led to a significant upregulation of phosphorylated p38.

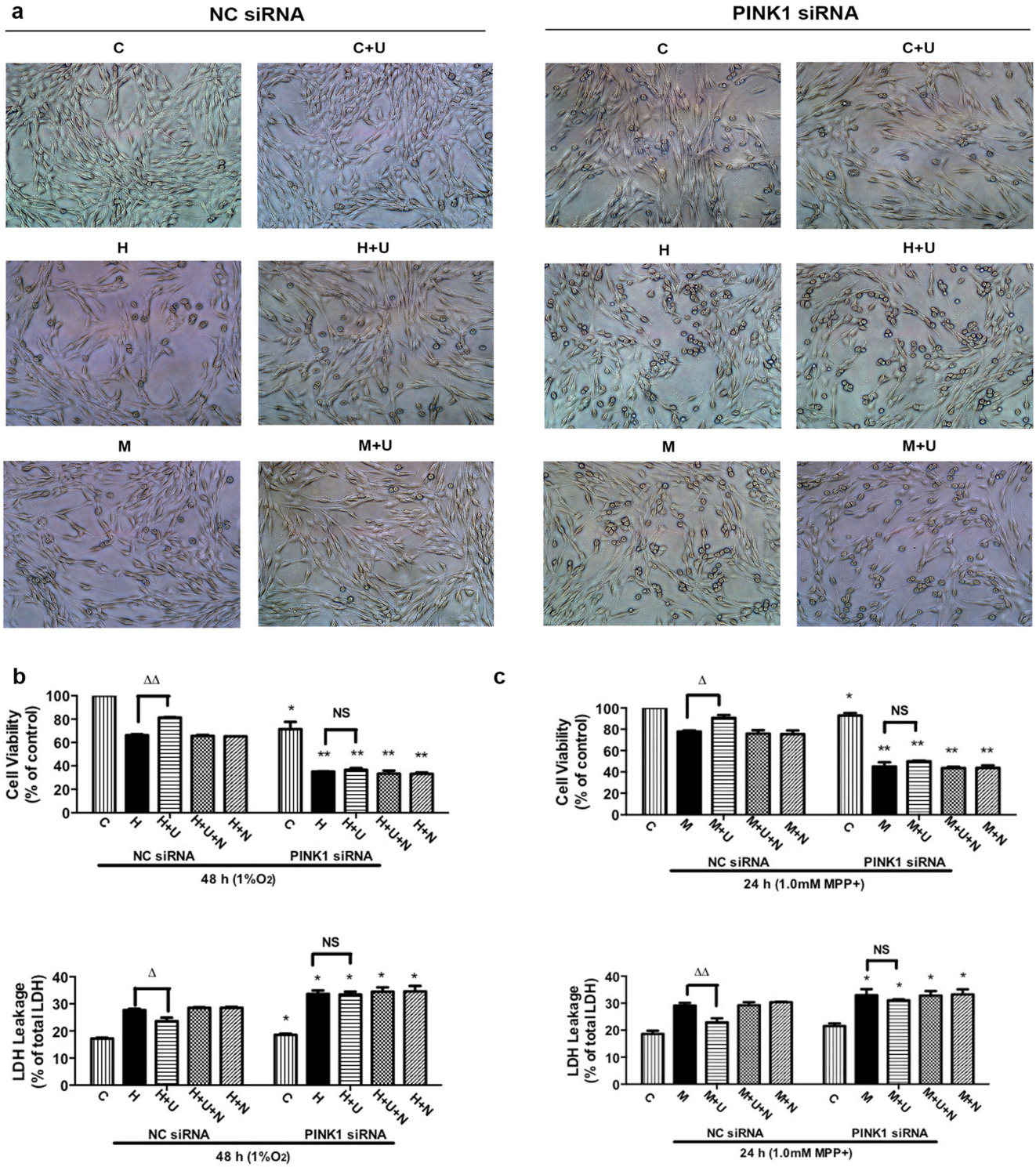
DOR Regulated Akt Phosphorylation Through PINK1 Signaling

Since the above results indicated that both DOR activation and PINK1 knockdown influenced Akt phosphorylation on the conditions of MPP^+ and hypoxia, we further explored the interaction between PINK1 and DOR in the regulation of Akt. PI3K/Akt pathway is an important intracellular signaling pathway that is directly related to cellular proliferation and longevity. Given that DOR protects neurons against hypoxia or MPP^+-induced apoptosis via the PI3K/Akt pathway, we investigated the effects of DOR activation and PINK1 on Akt and Akt phosphorylation. Both control siRNA and PINK1 siRNA-transfected PC12 cells were exposed to hypoxia or 1.0 mM MPP^+ for 24–48 h. As shown in Fig. 7, under hypoxic and MPP^+ insults, both the control siRNA-treated group and the PINK1 siRNA-treated group showed no significant changes in the total Akt expression. However, the expression of phosphorylated Akt was largely reduced by 48-h 1% O_2 hypoxia or 24-h 1.0 mM MPP^+. The application of the DOR agonist UFP-512 effectively increased the phosphorylation of Akt, while the addition of the DOR antagonist naltrindole enhanced the reduction in phosphorylated Akt induced by hypoxic and MPP^+ insults in the control siRNA-treated group (Fig. 7a, b).

Then, we detected the expression of phosphorylated Akt in the PINK1 siRNA-treated group. The basal phosphorylation level of Akt was significantly reduced and was more severe under MPP^+ exposure after cells were transfected with PINK1 siRNA. The knockdown of PINK1 partially blocked the DOR activation-mediated increase in the phosphorylation level of Akt and also attenuated the deprivation in phosphorylated Akt expression induced by naltrindole in both hypoxic and MPP^+ conditions (Fig. 7a, b).

PINK1 Signaling Was Involved in DOR-Mediated Regulation of Caspase-3

Similarly, we investigated whether the DOR-PINK1 axis is involved in the regulation of caspase-3 activation in the DOR-mediated cytoprotection against hypoxic or MPP^+



injury by detecting caspase-3 activation in both the control and PINK1 siRNA-transfected groups. In the control siRNA-treated group, both prolonged MPP^+ and hypoxic insults increased the level of cleaved caspase-3 expression with a decrease in the level of pro-caspase-3 proteins. The activated caspase-3 was significantly attenuated by the application of the DOR agonist with more caspase-3 remaining in a zymogen status (Fig. 8a, b). In contrast, the inhibition of DOR with naltrindole resulted in a marked increase in cleaved caspase-3 levels and a decrease in inactivated caspase-3 levels under both hypoxic and MPP^+ conditions. The cells with PINK1 knockdown showed a higher level of activated caspase-3 and lower levels of pro-caspase-3 proteins. The results are consistent with the changes in Akt phosphorylation.

◀ **Fig. 4.** Knockdown of PINK1 aggravated PC12 cell injury and interfered with DOR-mediated cytoprotection against hypoxia. The PC12 cells transfected with control siRNA or PINK1 siRNA were exposed to hypoxia at 1% O_2 for 48 h or 1.0 mM MPP^+ for 24 h. **a** Morphologic changes, 20× microscope. C: normoxic control. C + U: DOR activation with UFP-512 in normoxic conditions. H: hypoxia. H + U: DOR activation with UFP-512 in hypoxic conditions. M: MPP^+. M + U: DOR activation with UFP-512 under MPP^+ insults. NC siRNA: negative control siRNA-transfected PC12 cells. PINK1 siRNA: PINK1 siRNA-transfected PC12 cells. Note that both hypoxia and MPP^+ induced cell injury and PINK1 knockdown aggravated such injury. Morphologically, it was characterized by a reduction in cell density, and cells became shrinkage and turned round. **b** The cell viability and LDH leakage were measured by MTT and LDH assays under hypoxic conditions. C: normoxic control. H: hypoxia. H + U: DOR activation with UFP-512 in hypoxic condition. H + U + N: PC12 cells were treated with UFP-512 plus naltrindole at the same time in hypoxic conditions. H + N: PC12 cells were treated with naltrindole in hypoxic conditions. $N = 3$ in each group. NS: not significant. $^*p < 0.05$ and $^{**}p < 0.01$, the subgroup of the control siRNA group vs. the corresponding subgroup of the PINK1 siRNA group. $^{\Delta}p < 0.05$ and $^{\Delta\Delta}p < 0.01$ vs. H within the same group. Note that the cell viability baseline was significantly reduced by PINK1 knockdown in hypoxic conditions, consistent with the increase in LDH leakage. Knockdown of PINK1 attenuated DOR-mediated cytoprotection against hypoxia. **c** The PC12 cells transfected with control siRNA or PINK1 siRNA were exposed to 1.0 mM MPP^+ for 24 h, and then the cell viability and LDH leakage were measured using MTT and LDH assays. C: normoxic control. M: MPP^+. M + U: DOR was activated using UFP-512 and then exposed to MPP^+. M + U + N: PC12 cells were treated with UFP-512 plus naltrindole at the same time and then exposed to MPP^+. M + N: DOR was inhibited using naltrindole and then exposed to MPP^+. $N = 3$ in each group. NS: not significant, $^*p < 0.05$ and $^{**}p < 0.01$, the subgroup of the control siRNA group vs. the corresponding subgroup of the PINK1 siRNA group. $^{\Delta}p < 0.05$ and $^{\Delta\Delta}p < 0.01$ vs. M within the same group. Note that the cell viability was significantly decreased by the PINK1 knockdown under MPP^+ conditions, which was consistent with the increase in LDH leakage. Knockdown of PINK1 also attenuated DOR-mediated cytoprotection against MPP^+

The effects of DOR activation on both PI3K/Akt and caspases were inhibited by the knockdown of PINK1.

Discussion

The present study demonstrated that (i) DOR activation enhanced PINK1 expression in hypoxia and/or MPP^+ stress, (ii) PINK1 knockdown attenuated DOR-mediated cytoprotection against hypoxic and MPP^+ stress, and (iii) the DOR-mediated regulation of Akt and caspase-3 signaling relied, at least partly, on the function of PINK1. These novel findings suggest a link between DOR, PINK1, and two important signaling molecules involved in the regulation of cell proliferation and apoptosis. Our data indicates the presence of a new mechanism underlying the DOR-mediated neuroprotection against hypoxic and MPP^+ insults.

We used the highly differentiated PC12 cell line in this work for several reasons. Firstly, PC12 cells are electrically excitable and express membrane-bound receptors and cytosolic macromolecules similar to those expressed in the neurons of a PD brain [30–33]. They also release various neurotransmitters [31, 33] and express a significant amount of DOR [20, 21], which makes them suitable for us to conduct DOR research on. Therefore, this cell line is an optimal choice for achieving our research goal in this work. In addition, we have used this cell model in our previous studies [21].

Mitochondrial dysfunction, oxidative stress, and impaired ubiquitin-proteasome system (UPS) functioning are three common denominators of neurological disorders, including PD [34–38]. PINK1 plays an important role in promoting mitochondrial homeostasis [11–17]. The identification of the association between the PINK1 gene and autosomal recessive PD has led to a significant insight into the correlation between the pathogenesis of PD and the impaired mitochondrial function and dynamics. On the other hand, MPP^+ or hypoxic insults are direct causes for mitochondrial dysfunction. MPP^+ was found to bring about parkinsonism and dopamine (DA) neurodegeneration by selectively entering DA neurons, causing an inhibition of I, a mitochondrial respiratory chain component of the oxidative phosphorylation machinery [39, 40]. Once the mitochondrial membrane is depolarized when exposed to MPP^+ insults, a number of molecules, including caspases and cytochrome c, can freely diffuse from the mitochondria to the cytoplasm and initiate cell apoptosis [41]. In contrast to MPP^+ insult, hypoxic injury resulting from insufficient supply of oxygen and/or blood flow increases with age and makes neurons more vulnerable to neurotoxicity. By inducing oxidative stress and upregulating intracellular calcium, hypoxia causes the depolarization of the mitochondrial membrane potential indirectly [42–44]. Our recent work in a cellular model in vitro showed that prolonged hypoxia, similar to MPP^+, enhanced α-synuclein expression and promoted its oligomer formation [20], further confirming the close linkage between PD pathogenesis and hypoxic injury. Since both MPP^+ and hypoxic stress have been demonstrated as critical pathogenic factors that contribute to the development of PD [39–44] and negatively affect mitochondrial maintenance, the inextricable links between mitochondrial integrity and PD stimulated our interests in exploring a new way for PD treatment by targeting mitochondrial dysfunction.

Our previous studies have proven the important role of DOR in protecting PC12 cells against MPP^+ and hypoxic insults [21]. In the present work, we found that both hypoxia and MPP^+ induced a reduction in the expression of PINK1, whereas DOR activation attenuated this reduction in PINK1 protein. Since both MPP^+ and hypoxia have a negative impact on mitochondria, it is not surprising that PINK1, as a mitochondria protector, was downregulated by these two insults. What is strikingly noticeable is that DOR activation significantly increased the level of PINK1 protein under hypoxic and/or MPP^+ conditions, while DOR antagonism completely blocked this effect. Several lines of evidence support the fact

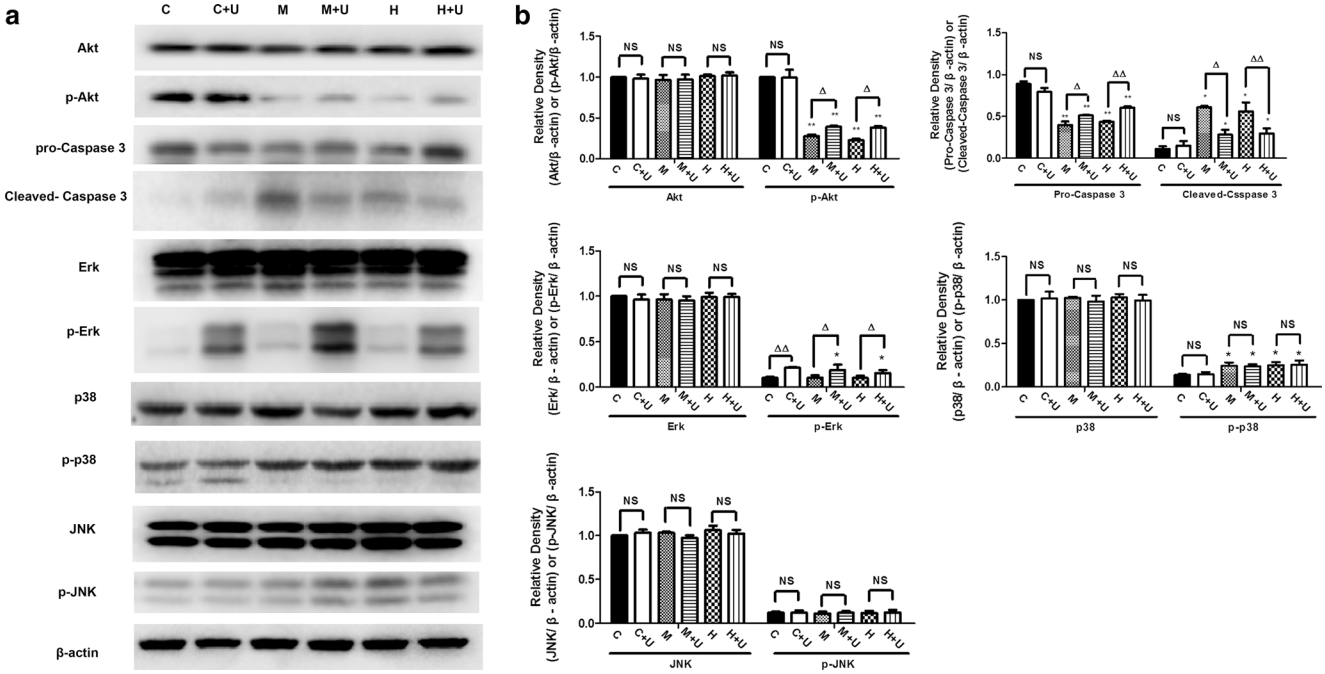


Fig. 5. **a**, **b** Effects of DOR activation on Akt, caspase-3, MAPK under normoxia, hypoxia, or MPP^+ stress. PC12 cells were treated with 5 μM of UFP-512 to activate DOR under normoxic, hypoxic, or MPP^+ condition. The levels of Akt/p-Akt, pro-caspase-3/cleaved caspase-3, Erk/p-Erk, p38/p-p38, and JNK/p-JNK were examined by Western blot. Quantitative data were subjected to statistical analysis. C: normoxic control. C + U: DOR activation with UFP-512 in normoxic conditions. M: MPP^+. M + U: DOR activation with UFP-512 under MPP^+ insults. H: hypoxia. H + U: DOR activation with UFP-512 in hypoxic conditions. $N = 3$ in each group. NS: not significant. $^*p < 0.05$ and $^{**}p < 0.01$, vs. the control within the subgroup. $^{\Delta}p < 0.05$ and $^{\Delta\Delta}p < 0.01$ vs. C or M or H within the same group. Note that MPP^+ and hypoxia led to a decrease in p-Akt, the activation of cleaved caspase-3, and an increase in p-p38 expression. DOR activation attenuated the MPP^+ and/or hypoxia-induced dephosphorylation of Akt and suppressed the activation of cleaved caspase-3. The application of UFP-512 also upregulated p-Erk in normoxic, MPP^+, and hypoxic conditions

that an overexpression of wild-type PINK1 can stabilize mitochondrial networks by maintaining mitochondrial membrane potential, reducing basal and neurotoxin-induced ROS, suppressing cytochrome c release, reversing toxin-induced fission [6, 45–47], and regulating calcium homeostasis [48]. Furthermore, there is evidence to show that the loss of PINK1 leads to severe mitochondrial dysfunction including aberrations in mitochondrial dynamics, calcium homeostasis, biosynthetic pathways, and mitophagy [39, 49]. Apparently, an upregulation of PINK1 is of great benefit to normal mitochondrial function. In fact, our data has well demonstrated that under hypoxia or MPP^+ stress, DOR attenuated the downregulation of PINK1, increased cell viability (MTT assay), and decreased cell injury (LDH assay), while these changes could be mostly reversed by DOR antagonism. This notion is further supported by the fact that the knockdown of PINK1 with PINK1 siRNA significantly reduced PC12 cell viability, aggravated cell injury, and attenuated DOR-mediated cytoprotection against hypoxia and MPP^+ injury.

While exploring the link between the DOR-PINK1 axis and cell survival/apoptosis, our first finding was that Akt is an important molecule of DOR-PINK1 action in both the conditions of hypoxic and MPP^+ insults. A large number of in vitro studies have shown that Akt is critical in maintaining neuronal survival by promoting activities of a wide range of neurotrophic factors. PC12 and HeLa cell lines have been reported to induce the activation of the PI3K/Akt survival pathway in response to hypoxia [50, 51]. Several genes involved in the genesis of PD have been shown to be related to the instability of Akt, and some toxins, including MPTP, 6-OHDA, rotenone, and C2 ceramide, are also referred to as negative regulators of Akt activity [52–54]. Furthermore, Murata et al. reported that PINK1 regulated the activity of Akt by activation of MTORC2 multiprotein complex [55]. Overexpression of wild-type PINK1 can protect neurons against various injuries by storing the activation of the PI3K/Akt pathway [54]. In the present work, we found that both hypoxia and MPP^+ reduced Akt phosphorylation, and this reduction was greatly reversed by DOR activation, suggesting a positive effect of DOR activation on Akt phosphorylation. Furthermore, the downregulation of PINK1 expression led to a significant decrease in the basal level of phosphorylated Akt, and this decrease could not be changed even after DOR activation in both hypoxic and MPP^+ models. Accordingly, we confirmed that Akt is actively involved in the cellular activities in both hypoxic and PD models, which is regulated by DOR-PINK1 signaling. An interference with PINK1 expression and/or function may abolish the capacity of DOR

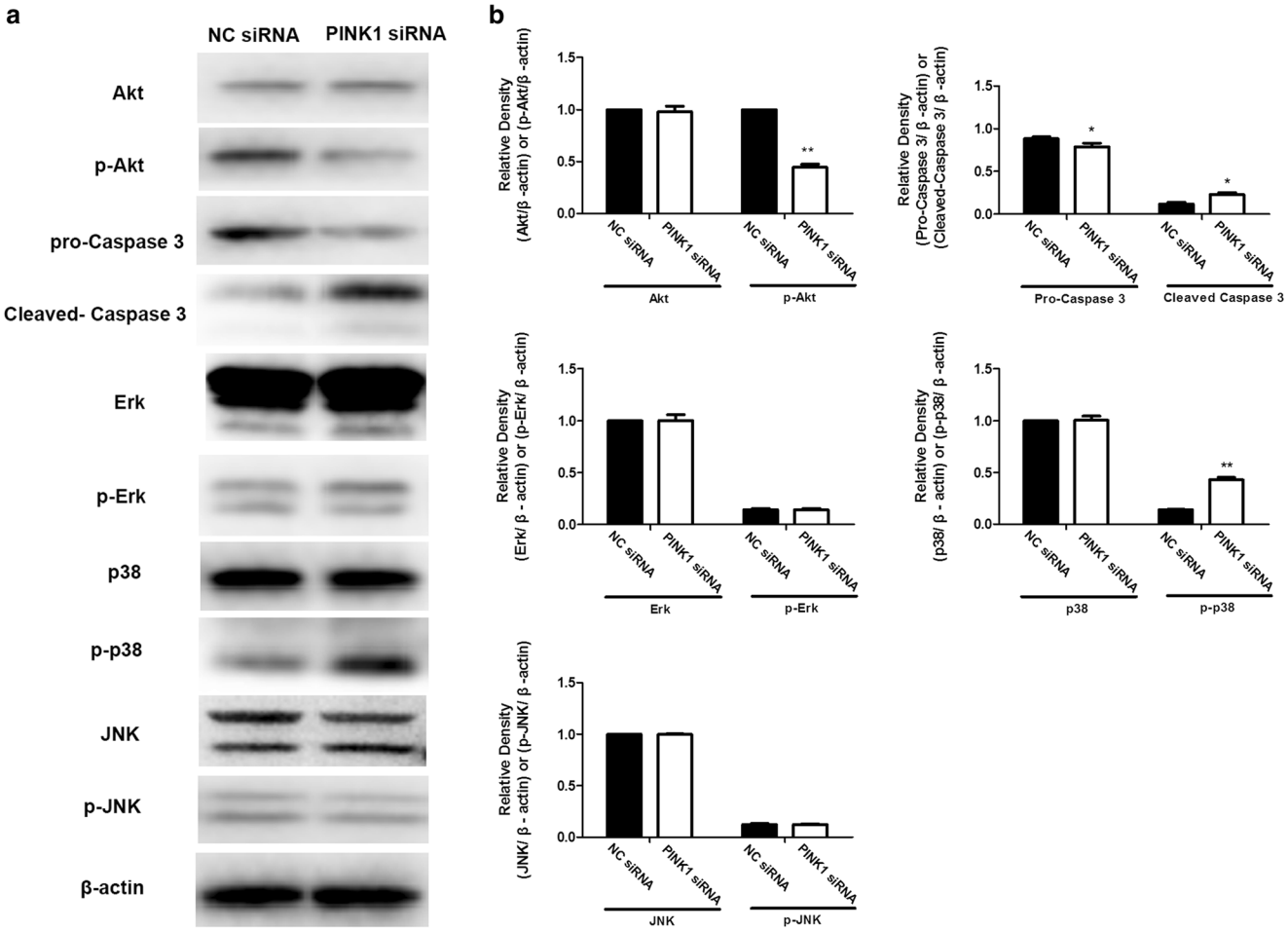


Fig. 6. **a**, **b** Differential regulation of Akt, caspase-3, and MAPK before and after PINK1 knockdown. PC12 cells were transfected with negative control siRNA or PINK1 siRNA in normoxic, MPP^+, and hypoxic conditions. Akt/p-Akt, pro-caspase-3/cleaved caspase-3, Erk/p-Erk, p38/p-p38, and JNK/p-JNK protein expression levels were determined by Western blot. C: normoxic control. C + U: DOR activation with UFP-512 in normoxic conditions. M: MPP^+. M + U: DOR activation with UFP-512 under MPP^+ insults. H: hypoxia. H + U: DOR activation with UFP-512 in hypoxic conditions. $n = 3$ in each group. NS: not significant. $^*p < 0.05$ and $^{**}p < 0.01$, vs. NC siRNA within the same group. Note that PINK1 knockdown led to a reduction in p-Akt expression with increased caspase-3 and p-p38

activation to upregulate Akt signaling and protect cells from hypoxic and MPP^+ insults.

Caspases are a well-known family in the regulation of cell apoptosis. Although the signaling process of cell survival and death is different among various models in different conditions, caspase-3 activation has been seen as a common change in different neurons in both hypoxic and MPP^+ conditions [56, 57]. In most cases, apoptosis is caspase-dependent [58]. Once initiator caspases are activated, they produce a chain reaction, activating several other executioner caspases, bringing about the irreversible changes in the cell death process [59, 60]. Since our previous studies [21] have demonstrated that the caspase signaling pathway is targeted by DOR, we further investigated the role of the DOR-PINK1 bond in the regulation of caspase-3. We found that PINK1 knockdown increased caspase-3 activation and greatly attenuated DOR activation-induced reduction of cleaved caspase-3 and increase in pro-caspase-3. Therefore, it is very likely that DOR-PINK1-mediated cytoprotection is, at least partially, mediated by the regulation of caspases.

In addition, in the way of screening the potential molecules related to DOR and PINK1, we also found that DOR activation was able to activate the Erk1/2 pathway in normoxic, hypoxic, and MPP^+ conditions as shown in this work, which is consistent with our previous observation on primary cultured cortical neurons [28, 29, 61]. Since Erk1/2 is known to mediate neuronal survival [19, 28], it is likely an important mediator in the DOR-mediated cytoprotection. Moreover, we observed that PINK1 knockdown led to an increase in p38 phosphorylation, suggesting an inhibitory role of PINK1 in the regulation of p38 signaling. Several lines of evidence indicate that the p38 MAPK pathway is involved in neuronal injury [28] and some brain diseases such as Alzheimer's disease, PD, and multiple sclerosis [62]. As stress-induced activation of p38 MAPK can facilitate neuronal apoptosis in neurodegenerative diseases [62, 63], the PINK1-mediated

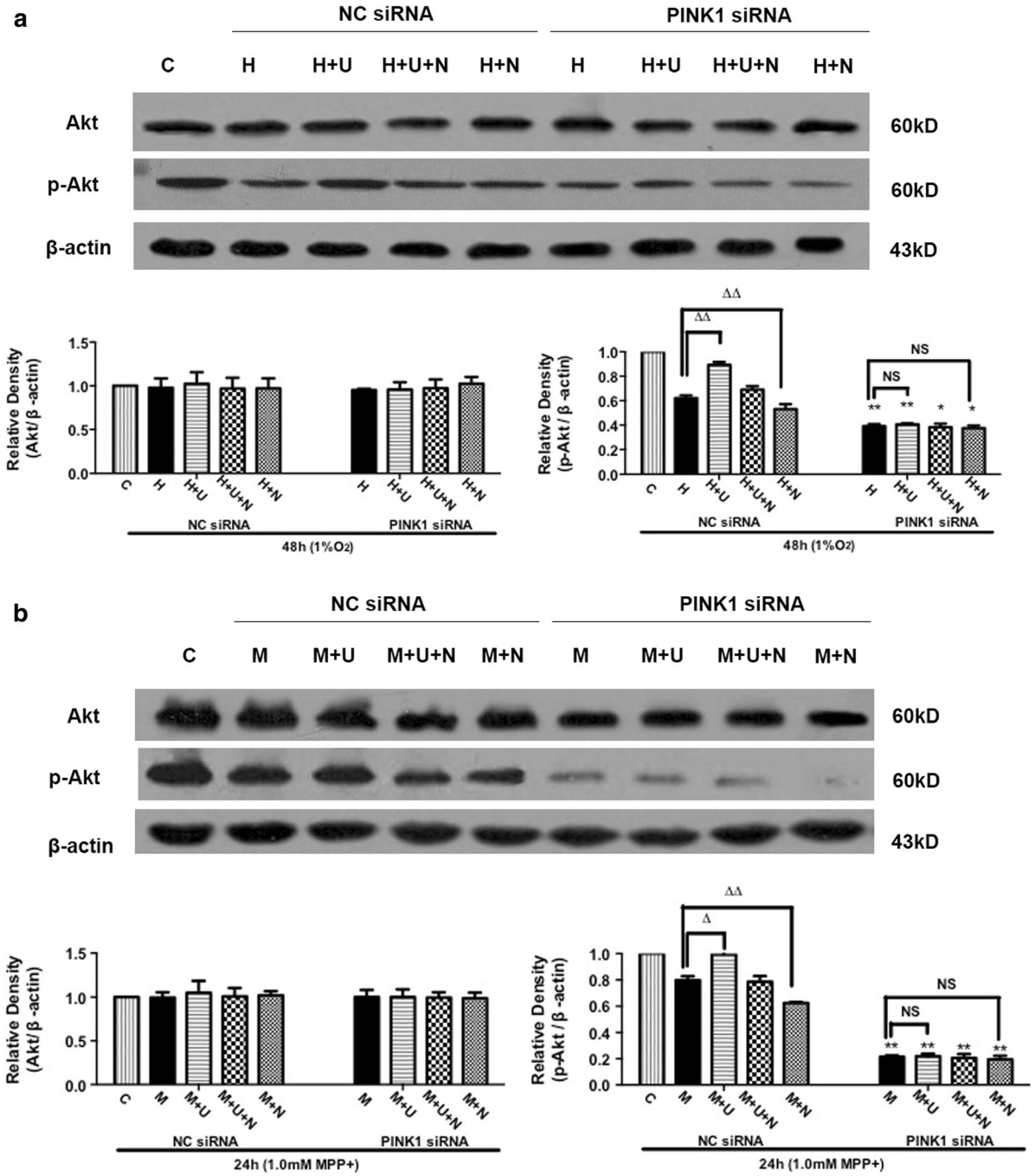


Fig. 7. DOR regulated Akt phosphorylation through PINK1 signaling in PC12 cells under both hypoxic and MPP^+ stress. **a** The experiments were conducted at 1% O_2 for 48 h. Akt/p-Akt was detected using Western blot. C: normoxic control. H: hypoxia. H + U: DOR was activated using UFP-512 in hypoxic conditions. H + U + N: DOR was treated with UFP-512 plus naltrindole at the same time in hypoxic conditions. H + N: DOR was inhibited using naltrindole in hypoxic conditions. $N = 3$ for each group. NS: not significant, $^*p < 0.05$, $^{**}p < 0.01$, the subgroup of the control siRNA group vs. the corresponding subgroup of the PINK1 siRNA group. $^{\Delta}p < 0.05$ and $^{\Delta\Delta}p < 0.01$ vs. H within the same group. Note that the hypoxia-induced decrease in Akt phosphorylation was significantly reversed by DOR activation. PINK1 knockdown led to a decrease in the basal phosphorylation level of Akt and attenuated DOR-mediated phosphorylation of Akt expression. **b** The experiments were conducted in the conditions of 1.0 mM MPP^+ for 24 h, and Akt/p-Akt was detected using Western blot. M: MPP^+. M + U: DOR was activated using UFP-512 and then exposed to MPP^+. M + U + N: DOR was treated with UFP-512 plus naltrindole at the same time under MPP^+ insult. M + N: DOR was inhibited using naltrindole and then exposed to MPP^+. $N = 3$ for each group. NS: not significant. $^{**}p < 0.01$, the subgroup of the control siRNA group vs. the corresponding subgroup of the PINK1 siRNA group. $^{\Delta}p < 0.05$ and $^{\Delta\Delta}p < 0.01$ vs. M within the same group. Note that in accordance with the results of hypoxia, MPP^+ also induced a decrease in Akt phosphorylation, which was significantly promoted by DOR activation. Also note that PINK1 knockdown led to a more severe decrease in the basal level of phosphorylated Akt and catastrophically attenuated DOR-mediated Akt phosphorylation

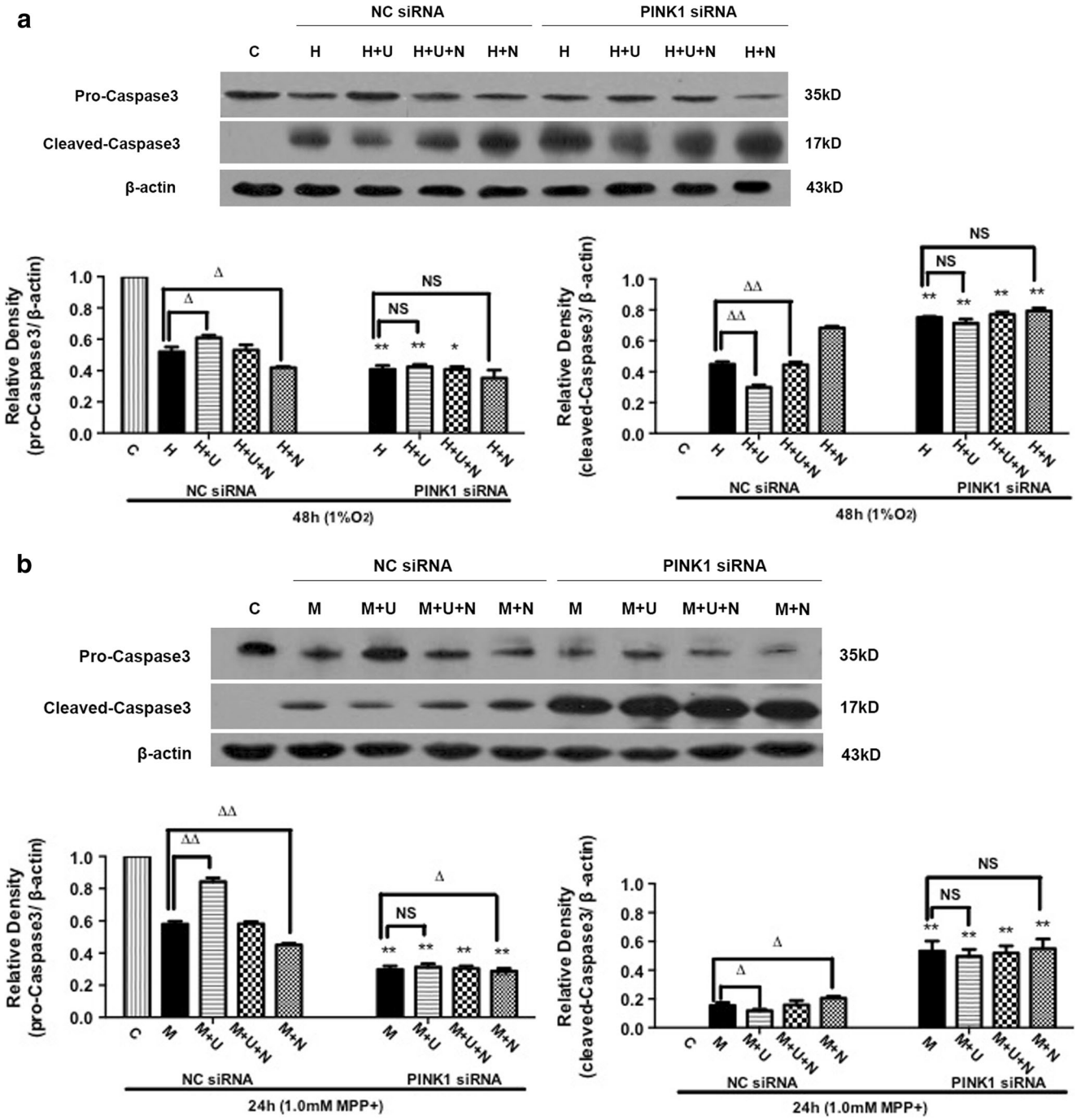


Fig. 8. DOR mediated the regulation of caspase-3 through PINK1 signaling. **a** The experiments were conducted at 1% O_2 for 48 h, and pro-caspase-3/cleaved caspase-3 levels were detected using Western blot assay. C: normoxic control. H: hypoxia. H + U: DOR was activated using UFP-512 in hypoxic conditions. H + U + N: DOR was treated with UFP-512 plus naltrindole simultaneously in hypoxic condition. H + N: DOR was inhibited using naltrindole in hypoxic conditions. $N = 3$ for each group. NS: not significant. $^{*}p < 0.05$ and $^{**}p < 0.01$, the subgroup of the control siRNA group vs. the corresponding subgroup of the PINK1 siRNA group. $^{\Delta}p < 0.05$ and $^{\Delta\Delta}p < 0.01$ vs. H within the same group. Note that the increase in cleaved caspase-3 and the parallel decrease in pro-caspase-3 after exposure to 1% O_2 for 48 h. The alternations in pro-caspase-3/cleaved caspase-3 levels became more severe after the transfection with PINK1 siRNA, while the DOR effects on pro-caspase-3/cleaved caspase-3 levels were significantly reduced by PINK1 knockdown. **b** The experiments were conducted in the PC12 cells exposed to 1.0 mM MPP^+ for 24 h. Pro-caspase-3/cleaved caspase-3 was detected using Western blot assay. M: MPP^+. M + U: DOR was activated using UFP-512 and then exposed to MPP^+. M + U + N: DOR was treated with UFP-512 plus naltrindole simultaneously under MPP^+ insult. M + N: DOR was inhibited using naltrindole and then exposed to MPP^+. $N = 3$ for each group. NS: not significant. $^{**}p < 0.01$, the subgroup of the control siRNA group vs. the corresponding subgroup of the PINK1 siRNA group. $^{\Delta}p < 0.05$ and $^{\Delta\Delta}p < 0.01$ vs. M within the same group. Note that MPP^+ insults increased the level of cleaved caspase-3 and decreased the level of pro-caspase-3. The alternations in pro-caspase-3/cleaved caspase-3 levels became more severe in PINK1 siRNA-transfected PC12 cells, and DOR-mediated regulation of caspase-3 was significantly attenuated

inhibition of the p38 signaling may play an important role in neuroprotection against neurodegenerative diseases, especially in PD. Altogether, the present study suggests that the interaction between DOR and PINK1 with differential regulation of Erk1/2 and p38 contributes to neuroprotection against parkinsonian injury.

In summary, we have established a DOR-PINK1 axis as a critical component of neuroprotection against MPP^+ and hypoxic injury. Our data provides compelling evidence that the DOR-mediated cytoprotection against hypoxic/MPP^+ injury greatly depends on PINK1 signaling. The interaction between DOR and PINK1 may protect the cells against parkinsonian injury resulting from hypoxia and/or MPP^+ through a positive regulation of Akt signaling and negative modulation of caspase-3 activity. Our findings raise a possibility for mitochondria protection through DOR-PINK1 signaling. It is our belief that more mechanistic and in-depth investigations may provide a novel clue for new therapeutic strategies for PD treatment as well as other neurodegenerative disorders.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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