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# δ-Opioid Receptor Activation Attenuates the Oligomer Formation Induced by Hypoxia and/or α-Synuclein Overexpression/Mutation Through Dual Signaling Pathways

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## Abstract

We have recently demonstrated that δ-opioid receptor (DOR) activation attenuates α-synuclein expression/aggregation induced by MPP(+) and/or severe hypoxia. Since α-synuclein plays a critical role in the pathogenesis of Parkinson's disease, DOR activation may trigger an antiparkinson pathway(s) against α-synuclein-induced injury. However, the underlying mechanism is unknown yet. In HEK293T and PC12 cells, we investigated the effects of DOR activation on the oligomer formation induced by α-synuclein overexpression and mutation in normoxic and hypoxic conditions and explored the potential signaling pathways for DOR protection. We found that (1) increased expression of both wild-type and A53T-mutant α-synuclein led to the formation of α-synuclein oligomers and cytotoxic injury; (2) DOR activation largely attenuated the formation of toxic α-synuclein oligomers induced by α-synuclein overexpression/mutation and/or hypoxia; (3) DOR activation attenuated α-synuclein-induced cytotoxicity through TORC1/SIK1/CREB, but not the phospho-CREB pathway, while DOR activation reduced hypoxic cell injury through the phospho-CREB mechanism; and (4) the interaction of α-synuclein and the DJ-1 was involved in the mechanisms for DOR-mediated protection against α-synuclein oligomer formation. Our findings suggest that DOR attenuates the formation of toxic α-synuclein oligomers through the phos-CREB pathway under hypoxic conditions, and through TORC1/SIK1/CREB pathways in the conditions of α-synuclein overexpression and mutation. The DJ-1 gene was involved in the DOR protection against parkinsonian injury.

**Keywords** δ-Opioid receptor · α-Synuclein · DJ-1 · CREB · Cytoprotection · Hypoxia · Oligomer formation · TORC1 · Parkinson's disease

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## Introduction

δ-Opioid receptor (DOR) is expressed in abundance in the cortical and striatal regions [1, 2]. DOR has been revealed to pose an especially compelling biological function for new neuroprotective therapies. An early human study suggested that altered opioid transmission is part of the pathophysiology of Parkinson's disease (PD) [3]. Moreover, some studies on rodent and primate PD models showed that systemic administration of a DOR agonist has significant antiparkinson effects [4, 5]. In sharp contrast, other studies showed opposite results [6]. Therefore, the role of DOR in the parkinsonian brain is debatable due to the lack of direct and convincing evidence for the DOR's effect on parkinsonian pathophysiology and methodological limitations in previous opioid research.

Our recent work with current approaches has strongly demonstrated that DOR is a neuroprotector in the brain [7, 8]. Moreover, we found that DOR activation attenuates α-synuclein expression/aggregation and accordingly reduces cytotoxicity in an in vitro PD model exposed to MPP(+) stress [9]. Since α-synuclein plays a critical role in the pathogenesis of PD, our novel finding prompted us to hypothesize that DOR signaling may reduce α-synuclein overexpression and oligomer formation in the parkinsonian brain, suggesting a new therapeutic avenue for the treatment of PD.

Although the precise mechanisms underlying PD are still unclear, several lines of evidence have demonstrated that environmental and genetic factors significantly contribute to PD pathogenesis in an aged brain. MPTP, pesticides (paraquat, organophosphates, and rotenone), hypoxic injury, metals (e.g., manganese, copper, mercury, lead, iron, zinc, and aluminum), diet, head trauma, and infections have been proposed as potential risk factors [10, 11], while caffeine intake and cigarette smoking have been found to reduce the risk of PD [10]. The genes associated with PD are α-synuclein, parkin, ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), PTEN-induced putative kinase 1 (PINK1), DJ-1, and leucine-rich repeat kinase 2 (LRRK2 or dardarin) [11]. Among these key factors, MPP is well recognized as a PD inducer [12]. At molecular level, α-synuclein expression and aggregation are broadly thought as a primary event in the pathophysiology of PD [9, 13]. Therefore, our finding on DOR's attenuation of MPP(+)-induced α-synuclein expression and aggregation is truly significant in both science and medicine. However, the mechanisms for DOR-induced protection againstα-synuclein expression and aggregation are still unknown.

Our previous studies suggest that DOR activation may attenuate MPP(+)- or hypoxia-induced α-synuclein expression/aggregation via a CREB pathway because DOR activation enhances CREB phosphorylation and prevents the collapse of the mitochondrial membrane potential ($\Delta\psi m$) [9]. Several lines of evidence have shown that CREB is regulated through phosphorylationat serine 133 (S133) and starts transcription of the target gene by raising CBP [14]. However, recent studies [15, 16] found that transducer of regulated CREB (TORCs) can be sustained in conjunction with CREB, leading to the transcription of target genes; this transcription is not significantly dependent on phosphorylation at S133. On the other hand, salt-inducible kinase (SIK) was found to be the transcriptional inhibitory kinase of the CREB-TORC complex [16]. One or more isoforms of SIK are the target genes of CREB, which makes it possible to form a complete feedback regulation loop for CREB signaling.

DJ-1 is a small but ubiquitously expressed protein implicated in several pathways associated with PD pathogenesis. Although mutations in the gene encoding DJ-1 lead to familial early-onset PD, the exact mechanisms responsible for its role in PD pathogenesis are still elusive. Recent studies found that DJ-1 interacts directly with α-synuclein monomers and oligomers in vitro, which also occurs in HEK293 cells [17]. Therefore, a direct interaction between DJ-1 and α-synuclein likely constitutes the basis for a neuroprotective mechanism, and familial mutations in DJ-1 may contribute to PD by disrupting these interactions [18].

This study was designed to determine if DOR has an antiparkinson effect on the cell model with α-synuclein overexpression/mutation induced oligomer formation. If this is true, what is the major signaling pathway(s) mediating the DOR effect? More specifically, are CREB, TORC, SIK, and DJ-1 differentially involved in the DOR protection against α-synuclein cytotoxicity? Since DOR activation also attenuates hypoxia-induced α-synuclein aggregation and cell injury [9], we further asked if DOR protects from α-synuclein cytotoxicity through the same pathway(s) in MPP(+) and hypoxic conditions.

## Material and Methods

### Chemicals and Reagents

Deulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) for cell culture medium, protease inhibitors for western blot, and anti-α-synuclein antibody were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Primary antibodies against DOR CREB and CREB/S133 were purchased from Chemicon Inc. (Temecula, CA, USA). Primary antibodies against PARK7/DJ-1, TORC1, and Snf1lk were from Abcam (Cambridge, MA, USA). Cytoscan-LDH cytotoxicity assay kit was purchased from G-Biosciences Inc. (St. Louis, MO, USA). RIPA buffer was from Life Science Research Pierce Biotechnology Inc. (Rockford, IL, USA). Transfer buffer and running buffer for western blotting were purchased from Boston BioProducts Inc. (Ashland, MA, USA). UFP-512 [H-Dmt-Tic-NH-CH (CH2-COOH)-Bid], a

specific and potent DOR agonist [19, 20], was provided by our research team.

## Cell Culture

HEK293T cells and PC12 cells were purchased from ATCC (Manassas, VA, USA). Both cells were cultured in DMEM supplemented with 10% FBS, 4500 mg/L of glucose, 110 mg/L of sodium pyruvate, and L-glutamine in a humidified incubator with 5% $CO_2$ at a temperature of 37 °C. Cells were plated on either 60-mm dishes (for western blotting studies) or 96-well plates (for cell viability, cytotoxicity, and fluorescence microscope studies) at a density of $2 \times 10^5$ cells/mL.

## Cell Transfection

PC12 cells were transfected with either the α-synuclein (wild)-EGFP vector (empty vector control) or the α-synuclein (A53T)-EGFP vector using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were transfected with 16 μg of DNA/106 cells and incubated at 37 °C with 5% $CO_2$ for 24 h, after which they were collected for further experiments. WT/A53T plasmids were available free of charge from Dr. Tomoki Kuwahara of the Graduate School of Pharmaceutical Sciences, University of Tokyo.

## Western Blot Analysis

The harvested HEK293T cells were washed twice with DPBS and then were ultrasonically homogenized in ice-cold RIPA buffer (25-mM Tris-HCl pH 7.6, 150-mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1% SDS) containing a mixture of cocktail proteinase inhibitors (1-mg/mL pepstatin A, 1-mg/mL leupeptin, 1-mg/mL aprotinin, 100-mg/mL phenyl methane sulfonyl fluoride, 100-μg/mL benzamidine, 8-mg/mL calpains I and II; 0.1-mM PMSF). Homogenates were centrifuged (12,000 *g*, 15 min at 4 °C), and the supernatants were used directly for electrophoresis. The concentration of protein in samples was determined by a Bradford protein assay. Samples were separated on a 10% SDS-PAGE under denaturing conditions and transferred onto a polyvinylidenedifluoride membrane (PVDF, Amersham Biosciences, USA). The nonspecific binding was blocked in a blocking buffer (5% non-fat dry milk, 10-mM Tris-HCl, pH 7.5, 150-mM NaCl, 0.1% Tween 20), and different proteins were identified using specific primary antibodies diluted in TBST containing 1% non-fat dry milk incubated overnight at 4 °C. After washing with TBST three times, the membrane was incubated for 1 h at room temperature with goat anti-rabbit IgG (Chemicon Inc. Temecula, CA, USA) for DOR and CREB, or goat anti-mouse IgM (Chemicon Inc. Temecula, CA, USA) for α-synuclein diluted 1:5000 in 1% non-fat dry milk. After thrice washing with TBST, immunoreactive bands were observed under Piercer® ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA). In addition, membranes were stripped by Restore™ Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL, USA) and reprobed with anti β-actin antibody (Chemicon Inc., Temecula, CA, USA) diluted in 1:5000 for quantitative analysis of proteins.

## Immunofluorescence Assay

PC12 cells ($0.5 \times 10^4$ cells/well) were seeded into 96-well plates with sterile aseptic cover glasses and cultured. After the treatment, the cells were washed and fixed with 4% paraformaldehyde for 20 min at room temperature, then washed and blocked with blocking buffer (5% non-fat dry milk in PBS) for 2 h at room temperature. The cover glass with PC12 cells was incubated with mouse anti-human α-synuclein antibody (1:50) (Sigma Chemical Co. St. Louis, MO, USA) primary antibody for 1 h at room temperature. Cells were washed and incubated with FITC-labeled goat anti-mouse IgG as secondary antibodies (1:200) for 30 min at room temperature; slides were protected from light, starting from this step to the end, by covering slides with aluminum foil. Then, they were washed in PBS-Tween 20 three times and mounted with 50% glycerol. Images of stained cells were captured using the Leica DMIL® fluorescent microscope.

## Cell Injury Assessment

Cytotoxicity was quantitatively evaluated by measuring the LDH activity in the medium with the Cytoscan-LDH cytotoxicity assay kit (G-Biosciences Inc., St Louis, MO, USA). Cells were treated with indicated compound(s) and incubated for the desired period (include no-treatment controls). Fifty microliters of the supernatant was transferred to each well of a flat-bottom 96-well enzymatic assay plate and mixed with 50 μL of the reconstituted Substrate Mix; these mixtures were then incubated at room temperature and protected from light for 30 min. Then, 50 μL of the Stop Solution was added to terminate the reaction. The solutions were then used for LDH assay by a microplate reader (Tecan Sunrise, Switzerland) with the absorbance wavelength at 490 nm.

## Statistical Analysis

All values are expressed as mean ± S.E. One-way analysis of variance (ANOVA) followed by Bonferroni test was used for multiple pairwise tests to determine the significance. *P* values less than 0.05 were considered statistically significant.

## Availability of Data and Materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

## Results

### Effects of Hypoxia and α-Synuclein Expression on Cell Viability

The leakage of LDH was measured to determine cell viability, since it is a reliable index of cell injury [21]. First, we examined the effects of 24-h hypoxia at 0.5% $O_2$ and transfection of α-synuclein (wild-type and A53T mutant type) on cellular cytotoxicity in the HEK293T cell. As shown in Fig. 1a that summarizes the results of three independent experiments, hypoxic exposure increased LDH leakage (35.68% over the control, $P < 0.05$).

The transfection of wild-type α-synuclein or A53T-mutant α-synuclein caused more LDH leakage (56.24% over the control with wild-type α-synuclein and 56.60% over the control with A53T-mutant α-synuclein, $P < 0.05$) under normoxic conditions. Injury after transfection of wild-type α-synuclein or A53T-mutant α-synuclein was severer than injury after hypoxia (56.24 and 56.60 vs 35.68%, $P < 0.05$).

Under hypoxic conditions, the same transfection of wild-type or A53T-mutant α-synuclein further increased LDH leakage (70.71% over the control with wild-type α-synuclein and 67.93% over the control with A53T-mutant α-synuclein, $P < 0.05$). There was, however, no significant difference between the transfection of wild-type α-synuclein and that of A53T-mutant α-synuclein in terms of cytotoxic injury.

These data suggest that both severe hypoxia and α-synuclein over-expression (either wild-type or A53T-mutant) lead to significant cytotoxicity, while the combination of both stresses causes even more injury.

### DOR Protection Against Cell Injury Induced by Hypoxia and/or α-Synuclein Overexpression

To examine the effects of DOR activation on cell injury induced by hypoxia and α-synuclein overexpression and DOR activation with UFP-512, a potent and specific DOR agonist (5 μM) [9, 19, 20] was applied to HEK293T cells with and without the transfection of wild-type/A53T-mutant α-synuclein under hypoxia. The cell injury was then determined by an LDH release assay [9].

DOR activation significantly attenuated cytotoxicity induced by α-synuclein overexpression. UFP-512 administration decreased LDH leakage from 53.36 to 21.86% ($P < 0.05$) of the control in the group of wild-type α-

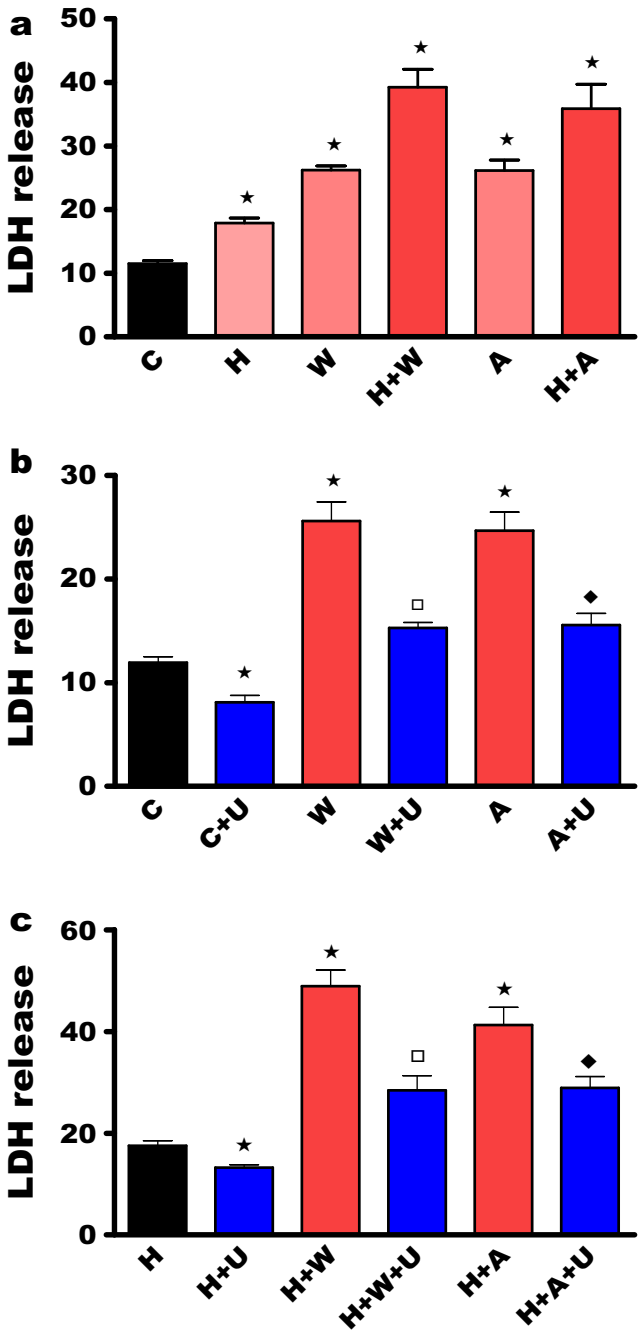


**Fig. 1** Effect of DOR activation on cell injury of HEK293T induced by the transfection of wild-type or A53T-mutant α-synuclein under normoxic or hypoxic conditions. LDH leakage was measured as an index of cellular injury. **a** Overexpression or mutation of α-synuclein with or without hypoxia. **b** DOR agonist UFP512 attenuated the cellular injury induced by overexpression or mutation of α-synuclein. **c** DOR agonist UFP512 attenuated the cellular injury induced by overexpression or mutation of α-synuclein under hypoxia. C, normoxic control; H, hypoxia; W, transfection of wild-type α-synuclein plasmid; A, transfection of A53T-mutant α-synuclein plasmid; U, UFP-512. $N = 3$. * $P < 0.05$ vs. the control. □ $P < 0.01$ vs. wild-type α-synuclein (**b**) or wild-type α-synuclein under hypoxia (**c**). ♦ $P < 0.01$ vs. A53T-mutant α-synuclein (**b**) or A53T-mutant α-synuclein under hypoxia (**c**). Note that DOR activation is cytoprotective against cellular injury induced by the overexpression or mutation of α-synuclein in both normoxic and hypoxic conditions

synuclein and from 51.58 to 23.26% ($P < 0.05$) of the control in the group of A53T-mutant α-synuclein ($P < 0.05$; Fig. 1b). The same treatment with UFP-512 also reduced LDH leakage by 32.58% in hypoxia group ($P < 0.05$ vs. the control; Fig. 1c).

Under hypoxic conditions, DOR activation still induced a significant reduction in cell injury after transfection of wild-type or A53T-mutant α-synuclein. As shown in Fig. 1c, LDH leakage decreased from 64.12 to 38.32% ($P < 0.05$) of the control after 24 h of hypoxia and from 57.48 to 39.33% of the control ($P < 0.05$) in the group of A53T-mutant α-synuclein after 24 h hypoxia at 0.5% $O_2$.

These results suggest that DOR activation is cytoprotective against hypoxia and/or α-synuclein overexpression induced cytotoxicity in the HEK293T cell.

## Effect of DOR Activation on DOR Protein Expression Under the Conditions of Hypoxia and α-Synuclein Overexpression

We have previously found that DOR is sensitive to hypoxic stress with its protein levels decreasing in response to severe stress [7, 22]. In this work, we observed that the level of DOR protein in HEK293T cell significantly decreased after the overexpression of wild-type or A53T-mutant α-synuclein, while UFP-512 application (5 μM) significantly increased the level of DOR (Fig. 2a). Interestingly, DOR activation induced a slight overshoot of DOR expression in both naive cells (13.15%, $P < 0.05$ vs. the control; Fig. 2a) and in those with the overexpression of wild-type α-synuclein (12.72%, $P < 0.05$ vs. the control; Fig. 2a), while it could only restore the expression of DOR protein to the control level in the cells with the overexpression of A53T-mutant α-synuclein ($P > 0.05$ vs. the control; Fig. 2a). In the hypoxic condition, the DOR protein greatly decreased in all three groups: hypoxia alone (H) (53.42%, $P < 0.05$ vs. the control; Fig. 2b), hypoxia plus the overexpression of wild-type α-synuclein (H + W) (58.48%, $P < 0.05$ vs. the control; Fig. 2b), and hypoxia plus the overexpression of A53T-mutant α-synuclein (H + A) (76.14%, $P < 0.05$ vs. the control; Fig. 2b), especially in the group of H + A. However, DOR activation almost doubled the level of DOR proteins, especially in the groups of H and H + W (54.14 and 52.73%, $P < 0.05$ vs. H; Fig. 2b).

These results suggest that DOR activation can reverse the decrease in DOR expression resulting from severe hypoxia or α-synuclein insults.

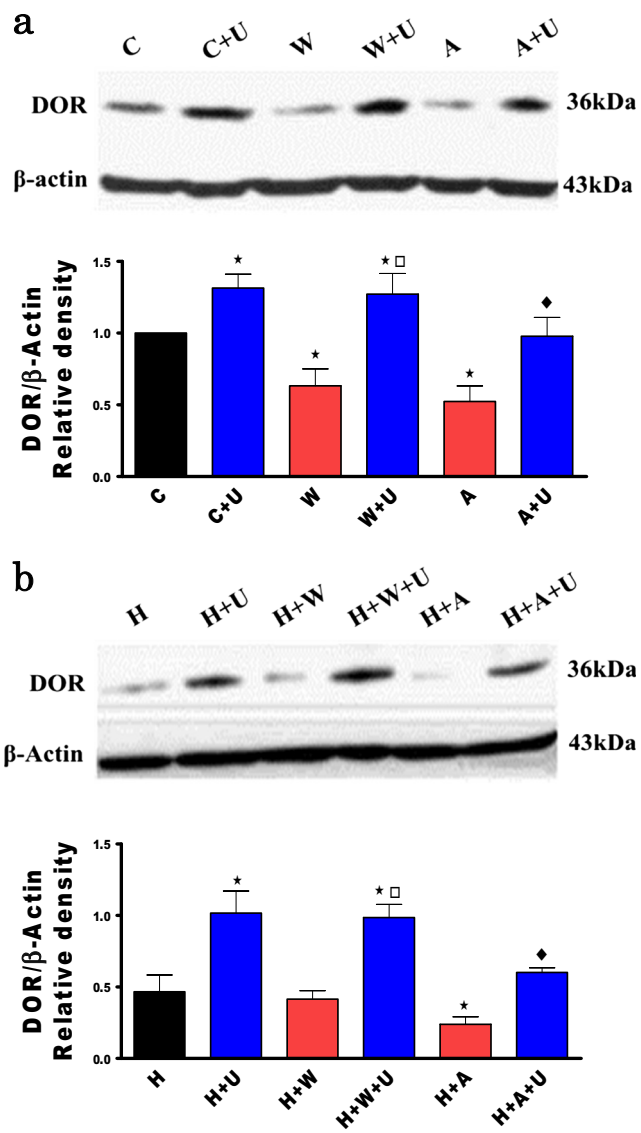


**Fig. 2** Effects of UFP-512 on DOR protein expression in HEK293T cells with the transfection of wild-type or A53T-mutant α-synuclein under normoxic or hypoxic conditions. **a** Representative blots of western blot analysis of DOR expression after transfection of wild-type α-synuclein or A53T-mutant α-synuclein plasmids under normoxic conditions. **b** Representative blots of western blot analysis of DOR expression after transfection of wild-type α-synuclein or A53T-mutant α-synuclein plasmids under hypoxic conditions. C, normoxic control; H, hypoxia; W, transfection of wild-type α-synuclein plasmid; A, transfection of A53T-mutant α-synuclein plasmid; U, UFP-512. $N = 3$. * $P < 0.05$ vs. the control. □ $P < 0.01$ vs. wild-type α-synuclein (**a**) or Wild-type α-synuclein under hypoxia (**b**). ◆ $P < 0.01$ vs. A53T-mutant α-synuclein (**a**) or A53T-mutant α-synuclein under hypoxia (**b**). Note that DOR activation increases DOR protein level in the cells with α-synuclein overexpression or mutation under normoxic or hypoxic condition

## DOR Inhibition of α-Synuclein Overexpression and Oligomer Formation

We then determined whether DOR activation directly inhibits hypoxia-induced α-synuclein expression and the overexpression of wild-type and A53T-mutant α-synuclein in HEK293T cells. We found that in normoxic conditions, DOR activation had no appreciable effect on the level of α-synuclein (Fig. 3a), while in hypoxic conditions, DOR activation significantly reduced the level of α-synuclein by 22.2% ($P < 0.05$ vs. the control; Fig. 3b).

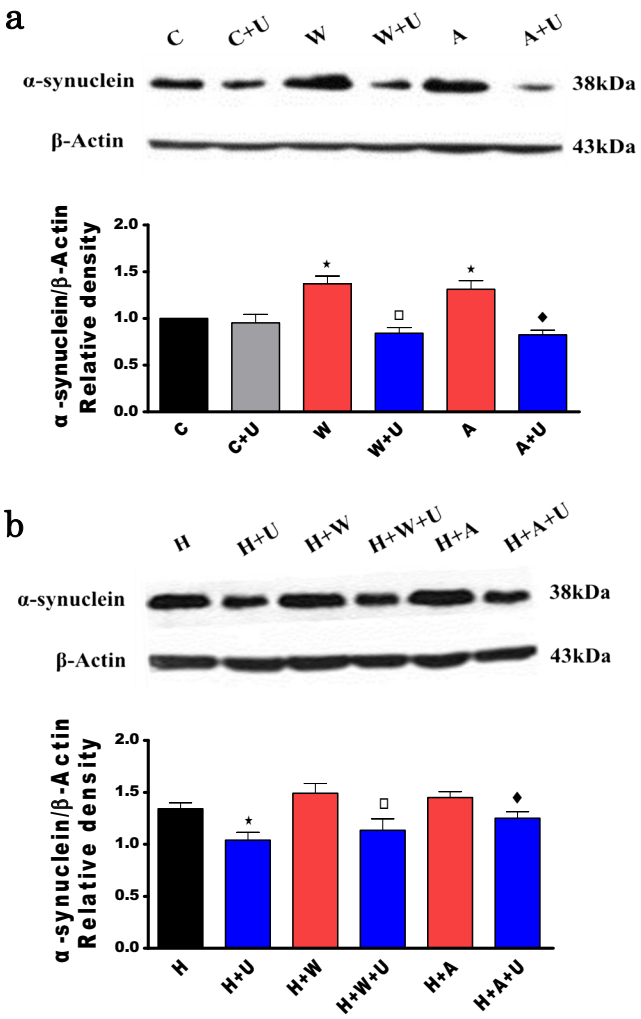


**Fig. 3** Effects of UFP-512 on the α-synuclein protein expression in HEK293T cells with the transfection of wild-type or A53T-mutant α-synuclein under normoxic or hypoxic conditions. **a** Representative blots of western blot analysis of α-synuclein expression after transfection of wild-type α-synuclein or A53T-mutant α-synuclein plasmids under normoxic conditions. **b** Representative blots of western blot analysis of α-synuclein expression after transfection of wild-type α-synuclein or A53T-mutant α-synuclein plasmid under hypoxic conditions. C, normoxic control; H, hypoxia; W, transfection of wild-type α-synuclein plasmid; A, transfection of A53T-mutant α-synuclein plasmid; U, UFP-512. $N = 3$. * $P < 0.05$ vs. the control. □ $P < 0.01$ vs. wild-type α-synuclein (**a**) or wild-type α-synuclein under hypoxia (**b**). ◆ $P < 0.01$ vs. A53T-mutant α-synuclein (**a**) or A53T-mutant α-synuclein under hypoxia (**b**). Note that α-synuclein protein expression increases after transfection of wild-type α-synuclein or A53T-mutant α-synuclein plasmids under normoxic or hypoxic conditions, while DOR activation lowered the level of α-synuclein protein in the cells with transfection of wild-type α-synuclein or A53T-mutant α-synuclein plasmids in both normoxic and hypoxic conditions

In the cells with an overexpression of wild-type or A53T-mutant α-synuclein, DOR activation also significantly reduced the levels of both wild-type and A53T-mutant α-synuclein. As shown Fig. 3a, the level of wild-type α-synuclein (W) was reduced by 38.63% ($P < 0.05$ vs. W), and those of A53T-mutant α-synuclein (A) by37.18% ($P < 0.05$ vs. A) after DOR activation. These findings strongly suggest that DOR activation has an inhibitory effect on α-synuclein overexpression.

To elucidate the mechanisms behind DOR-mediated cytoprotection, we examined the formation of toxic α-synuclein oligomers in PC12 cells with the overexpression of wild-type and A53T-mutant α-synuclein using an immunofluorescence assay with monoclonal anti-SNCA antibody (clone 2E4, Cat # WH0006622M1). As shown in Fig. 4, the samples harvested from cell cultures with the overexpression of wild-type and A53T-mutant α-synuclein were all strongly labeled by the α-synuclein antibody (red) and showed typical aggregation of oligomers and fibrils. Meanwhile, DOR activation with UFP-512 greatly inhibited such aggregation (Fig. 4b). These observations suggest that DOR activation is inhibitory to toxic aggregation of α-synuclein.

## DOR-Induced Changes in Ser-133/CREB in Hypoxia and α-Synuclein Injury

To determine whether DOR effects involve the transcription factor CREB, we examined the effect of DOR activation on the phosphorylation of CREB in HEK293T cells under both normoxic and hypoxic conditions. The cells were incubated with UFP-512 at 5 μM for 24 h under either normoxia or hypoxia. CREB protein expression was detected by western blot analysis with anti-Ser 133 phosphorylated CREB and anti-CREB antibodies. The results revealed that DOR activation had upregulated Ser-133 CREB phosphorylation (Fig. 5a). Hypoxia slightly decreased the levels of Ser-133 CREB phosphorylation, whereas DOR activation greatly increased the level of Ser-133 CREB phosphorylation ($P < 0.05$ vs. the control; Fig. 5a). No significant changes in the total amount of CREB levels were found in these conditions (Fig. 5a). Unexpectedly, we found that the overexpression of wild-type and A53T-mutant α-synuclein, unlike that under hypoxic stress, did not induce any significant change in Ser-133 CREB phosphorylation. Also, DOR activation had no appreciable effect on Ser-133 CREB phosphorylation in the cells with α-synuclein overexpression (Fig. 5b). These data suggest that hypoxia and α-synuclein insults differentially affect Ser-133 CREB phosphorylation.

## DOR-Induced Changes in SIK1 and TORC1 Under α-Synuclein Injury

We further explored the mechanisms underlying the DOR-mediated cytoprotection against α-synuclein injury by investigating the effect of DOR activation on SIK1,

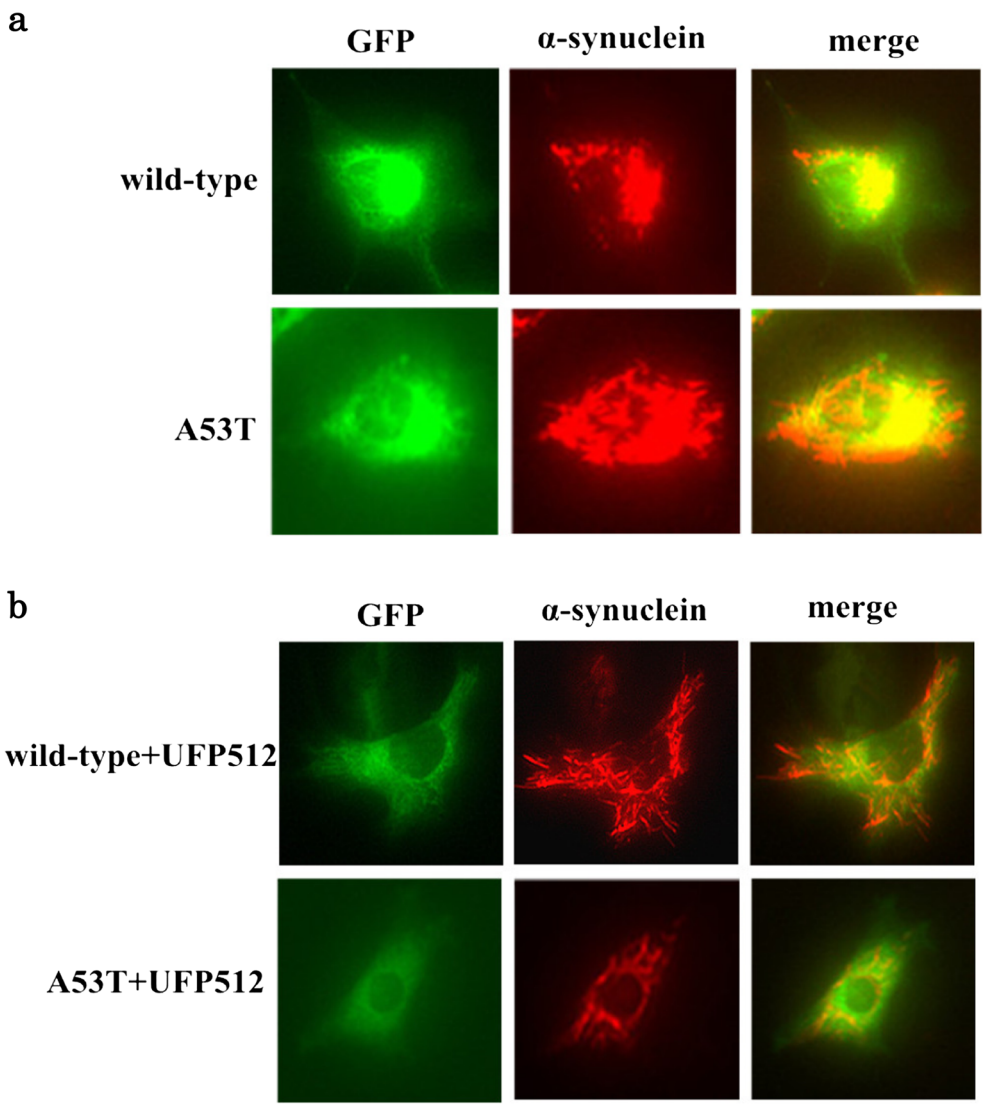


**Fig. 4** Immunofluorescence of α-synuclein in PC12 cells. **a** The abnormal protein aggregation in the cells with the transfection of wild-type α-synuclein or A53T-mutant α-synuclein. **b** DOR activation induced reduction of abnormal protein aggregation in the cells with the transfection of wild-type or A53T-mutant α-synuclein. GFP (green), PC12 cells stably expressing GFP-α-synuclein protein (green fluorescence) were transfected with pEGFP-synuclein (wt/mut) plasmid, and then stained with anti-α-synuclein and FITC-labeled goat anti-mouse IgG as secondary antibodies (red fluorescence). Merg, Merg GFP + α-synuclein. Note that pre-treatment with UFP-512 remarkably reduces abnormal protein aggregation in the cells with transfection of wild-type or A53T-mutant α-synuclein. Representative cells are shown from one experiment out of three total experiments

TORC1, and CREB in HEK293T cells. In the western blot analysis, SIK1 protein expression was detected by the anti-Snf1lk antibody, and TORC1 protein was detected by anti-Ser 151 phosphorylated TORC1 and anti-TORC. The results showed that the levels of SIK1 and Ser 151 TORC1 phosphorylation increased in the cells transfected wild-type or A53T-mutant α-synuclein, whereas DOR agonist UFP-512 reversed the overexpression of α-synuclein (wild/A53T)-induced increase in the level of Ser 151 TORC1 phosphorylation and SIK1 (Fig. 6). No significant changes in the expression of SIK1 and TORC1 protein were found under hypoxic conditions (Fig. 6). Such findings raise the possibility that in these cells transfected wild-type or A53T-mutant α-synuclein, the primary pathway of DOR activation attenuated α-synuclein-induced cytotoxicity through TORC1/SIK1/CREB, but not the phospho-CREB pathway, while DOR reduced hypoxic cell injury depended on the phospho-CREB mechanism.

## DOR-Induced Changes in DJ-1 Under α-Synuclein Injury

Since DJ-1 interacts with α-synuclein and inhibits α-synuclein aggregation [17], we further asked if DOR prevents α-synuclein injury by increasing DJ-1 expression. We found that both wild-type and A53T-mutant α-synuclein expression led to a significant decrease in DJ-1 levels. However, DOR activation greatly increased the levels of DJ-1 in HEK293T cells with wild-type or A53T-mutant α-synuclein overexpression (Fig. 7). These results suggest that DJ-1 may play an important role in the DOR-mediated inhibition of α-synuclein injury. It is a remarkable fact that the expression of α-synuclein protein increased under hypoxic conditions can be attenuated by DOR activation, which is consistent with our previous studies [21]. At the same time, DOR activation induced the expression of DJ-1 protein corresponding increase (Fig. 7), suggesting that the interaction of α-synuclein and the

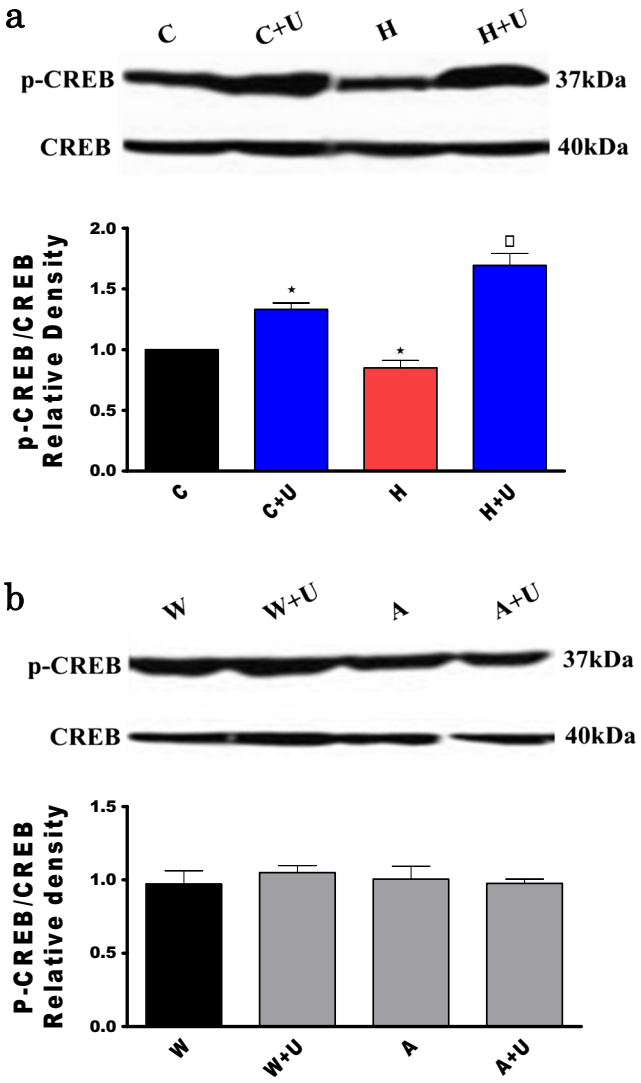


**Fig. 5** Effect of DOR activation on CREB phosphorylation in HEK293T cells with the transfection of wild-type or A53T-mutant α-synuclein under normoxic or under hypoxic conditions. **a** Pre-treatment with UFP-512 in the cells under normoxic and hypoxic conditions. **b** Pre-treatment with UFP-512 in the cells with the transfection of wild-type α-synuclein or A53T-mutant α-synuclein plasmids. C, normoxic control; H, hypoxia; W, transfection of wild-type α-synuclein plasmid; A, transfection of A53T-mutant α-synuclein plasmid; U, UFP-512. $N = 3$. * $P < 0.05$ vs. the control. □ $P < 0.05$ vs. hypoxia. Note that DOR activation increases p-CREB in hypoxia or normoxia, but had no appreciable effect on p-CREB in the cells with the transfection of wild-type α-synuclein or A53T-mutant α-synuclein plasmids

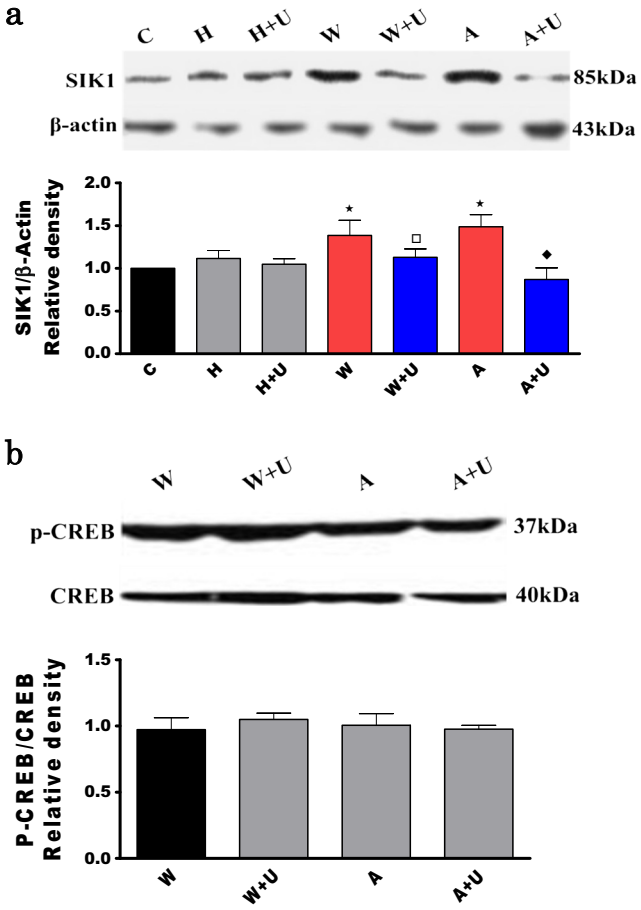


**Fig. 6** Effect of DOR activation on the SIK1 and p-TORC1 protein expression in HEK293T cells with the transfection of wild-type or A53T-mutant α-synuclein. **a** Pre-treatment with UFP-512. The representative blots of western blot analysis show SIK1 expression in the cells with the transfection of wild-type or A53T-mutant α-synuclein plasmids. **b** Pre-treatment with UFP-512. The representative blots of western blot analysis show p-TORC1 protein expression in the cells with the transfection of wild-type or A53T-mutant α-synuclein plasmid. W, wild-type α-synuclein plasmid; A, A53T-mutant α-synuclein plasmid; U, UFP-512. $N = 3$. * $P < 0.05$ vs. the control. □ $P < 0.05$ vs. wild-type α-synuclein. ◆ $P < 0.05$ vs. A53T-mutant α-synuclein. Note that DOR activation decreases the expression of p-TORC1 and SIK1 proteins in the cells with α-synuclein overexpression or mutation

DJ-1 was involved in the mechanisms for DOR-mediated protection against α-synuclein oligomer formation.

## Discussion

We have made several interesting findings in this work. First, DOR activation largely attenuated the formation of toxic α-synuclein oligomers and cell injury in the cells with α-synuclein overexpression/mutation and/or the cells exposed to prolonged hypoxia. Secondly, the DOR protection against α-synuclein overexpression and mutation was associated with TORC1 and SIK1 downregulation without any significant changes in CREB phosphorylation. In contrast, the DOR protection against α-synuclein dysregulation under hypoxic conditions was associated with an upregulated CREB phosphorylation without any appreciable change in TORC1 and SIK1. Finally, DOR protections upregulated DJ-1 expression in either conditions of α-synuclein overexpression/mutation and hypoxia. Our novel finding suggests that DOR upregulates DJ-1 expression and suppresses α-synuclein dysregulation through dual pathways.

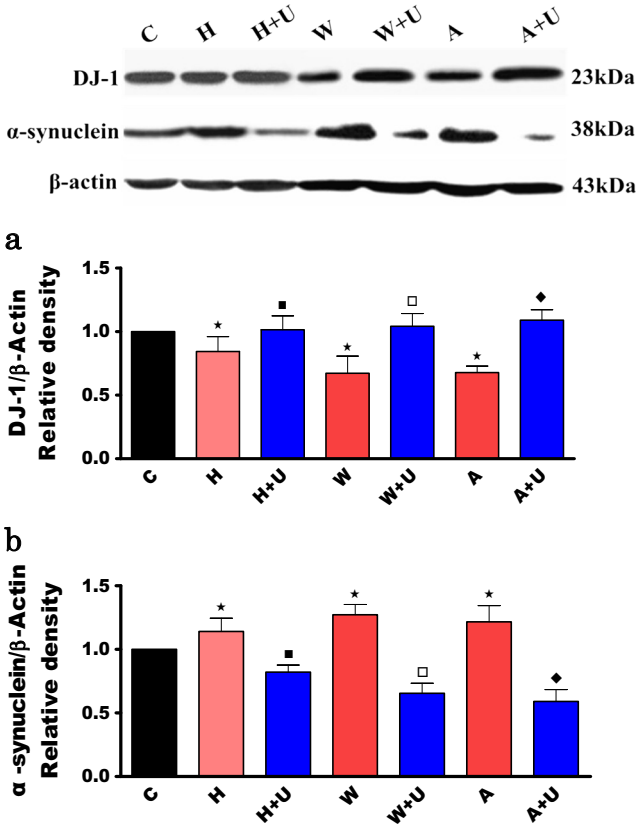


**Fig. 7** Effect of DOR activation on the expression of the DJ-1 protein in HEK293T cells with the transfection of wild-type or A53T-mutant α-synuclein. **a** Pre-treatment with UFP-512. The representative blots of western blot analysis show DJ-1 expression in the cells with the transfection of wild-type or A53T-mutant α-synuclein plasmids. **b** Pre-treatment with UFP-512. The representative blots of western blot analysis show α-synuclein protein expression in the cells with the transfection of wild-type or A53T-mutant α-synuclein plasmid. C, normoxic control; H, hypoxia; W, transfection of wild-type α-synuclein plasmid; A, transfection of A53T-mutant α-synuclein plasmid; U, UFP-512. $N = 3$. * $P < 0.05$ vs. control. ■ $P < 0.05$ vs. hypoxia. □ $P < 0.05$ vs. wild-type α-synuclein. ◆ $P < 0.05$ vs. A53T-mutant α-synuclein. Note that DOR activation decreases α-synuclein protein expression in the cells with α-synuclein overexpression or mutation by increasing DJ-1 protein expression

The cause of Parkinson's disease is generally unclear, but believed to involve both genetic and environmental factors. Since the discovery of missense mutations (A53T and A30P) of α-synuclein in familial cases of PD (PARK1), numerous histological studies have shown that α-synuclein dysregulation is a major component in Lewy bodies [23, 24]. There is also an increased risk in people exposed to certain pesticides, hypoxic/ischemic injury, and prior head injuries, while there is a reduced risk in tobacco smokers and those who drink coffee or tea [25, 26]. The motor symptoms of the disease result from the death of cells in the substantia nigra, a region of the midbrain. This results in not enough dopamine in these areas. The reason for this cell death is poorly understood, but involves the build-up of α-synuclein proteins into Lewy bodies in the neurons [24, 27].

A collection of studies from our laboratory and those of others have well demonstrated the role of DOR in neuroprotection [3–5, 7, 9, 20, 22, 28–33]. Specific agonists of delta-opioid receptors, such as UFP-512 and DADLE [(D-Ala2, D-Leu5) encephalin], have displayed the ability to promote neuronal survival and mitigate apoptotic pathways [22, 28, 34]. Delayed neuronal death is the primary target of neuroprotective strategies [35, 36]. Studies have shown that exposure of neurons to opioids immediately before ischemia induces ischemia tolerance and delays neuronal death. This phenomenon is called acute opioid preconditioning and this neuroprotection may be delta1-opioid receptor dependent and may involve mitochondrial adenosine triphosphate-sensitive potassium channel activation, free radical production [37, 38]. Indeed, the induction of nuclear and mitochondrial gene expression and the maintenance of protein phosphatases levels may be involved the neuroprotective mechanism, in a manner that likely involves modulation of the phosphorylation state of signaling kinases and mitochondrial pro- and anti-apoptotic proteins [39, 40].

However, it is debatable if DOR plays a protective role against parkinsonism or not [6, 41, 42]. Recently, our evidence suggests that DOR downregulation is potentially involved in the genesis of parkinsonian pathology [21, 43–45]. In an in vitro cell model, for example, we found that MPTP treatment led to a marked reduction of DOR expression with α-synuclein overproduction/aggregation and cell injury [9]. The present study further demonstrated that DOR activation indeed attenuated α-synuclein dysregulation in both genetic and environmental abnormalities. It is well-recognized that aggregation of α-synuclein contributes to the formation of Lewy bodies, which then leads to neurodegeneration in PD [24, 46–48]. Since our studies repeatedly demonstrated that DOR activation is inhibitory to α-synuclein dysregulation, it is very likely that DOR signaling may be antiparkinsonian by targeting the α-synuclein molecule.

There is no information available regarding the signaling mechanisms underlying the DOR-mediated protection against α-synuclein dysregulation. We made the first exploration in this work. CREB, a cellular transcription factor, binds to certain DNA sequences called cAMP response elements (CRE), thereby regulating the transcription of the downstream genes [49]. The CREB-dependent gene expression is widely involved in synaptic transmission, signal transduction, gene transcription, and metabolic and other physiological functions [50, 51]. In pathophysiological conditions, CREB downregulation is implicated in the pathology of some neurodegenerative diseases such as Alzheimer's disease, while an increase in CREB expression is considered a possible therapeutic target [52]. Indeed, we found a significant increase in phospho-

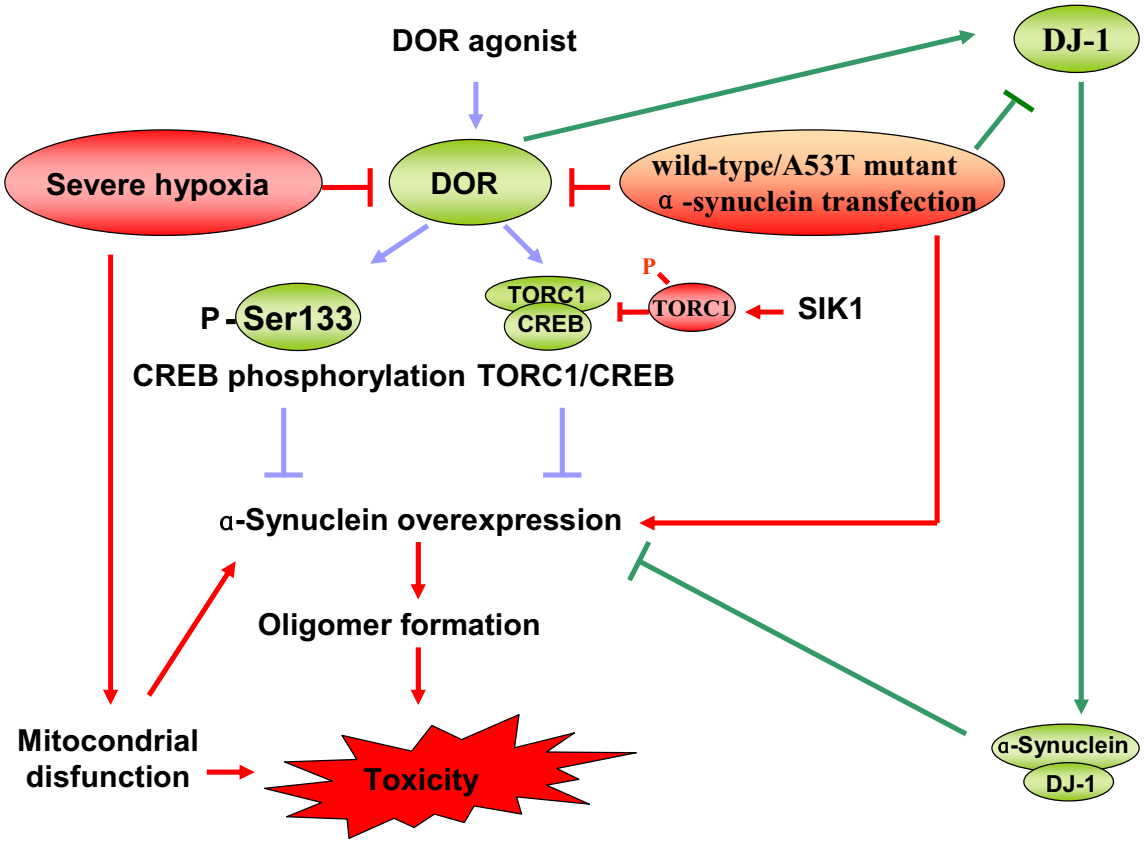


**Fig. 8** Schematical illustration of potential mechanisms behind DOR protection against α-synuclein aggregation

CREB expression after DOR activation under hypoxic conditions, which is in parallel with the attenuation of α-synuclein dysregulation. In sharp contrast, the upregulation of phospho-CREB expression was not seen after DOR activation in the cells with the transfection of wild-type or A53T-mutant α-synuclein. These results suggest that CREB is differentially involved in the DOR protection against α-synuclein dysregulation in genetic versus environmental abnormalities.

A conserved family of coactivators, called transducers of regulated CREB (TORCs) [53–57] has been found to increase the expression of cAMP responsive genes. Under basal conditions, TORC1s are sequestered in the cytoplasm through phosphorylation by the salt inducible kinase 1(SIK1) and other members of the AMPK family of Ser/Thr kinases [16, 58]. Following exposure to cAMP, TORC1 is dephosphorylated and translocated to the nucleus, where it associates with CREB and target gene expression. Our data in this work clearly show that SIK1 protein expression and TORC1 phosphorylation are significantly increased by the transfection of wild-type or A53T-mutant α-synuclein, while such an increase was completely reversed by DOR activation. The DOR inhibited SIK1 expression, might have caused TORC1 to be "translocated" from the cytoplasm to the nucleus by dephosphorylation [53], where it interacted with the bZIP domain of CREB [53–55], and thus reduced α-synuclein protein expression. Therefore, DOR signaling may antagonize α-synuclein dysregulation induced by α-synuclein overexpression and mutation by attenuating the upregulation of SIK1 and TORC1. This mechanism is different from that of DOR activation in hypoxic conditions.

DJ-1 is a small ubiquitously expressed protein implicated in several pathways associated with PD pathogenesis [59–61]. Interestingly, in both genetic (transfection of wild-type or A53T-mutant α-synuclein) and environmental (hypoxic) conditions as shown in this work, DJ-1 protein levels were largely decreased, whereas DOR activation could restore it to normal levels, or even higher. Several lines of evidence show that direct interactions between α-synuclein and DJ-1, two critical proteins in the control of PD pathogenesis, constitute the basis for a neuroprotective mechanism [17, 62, 63]. Our results strongly support that DJ-1 and α-synuclein change oppositely in response to genetic and environmental stress, therefore supporting the view that α-synucleinand DJ-1 interact with each other functionally. DOR signaling may attenuate α-synuclein dysregulation via DJ-1 upregulation in both genetic (transfection of wild-type or A53T-mutant α-synuclein) and environmental (hypoxic) conditions, although CREB and SIK1/TORC1 pathways are

differentially involved in DOR action in different conditions. In fact, DJ-1 may be an important redox-reactive neuroprotective protein implicated in the regulation of oxidative stress after ischemia. DJ-1 was detected immediately after a stroke, and efficiently translocated into the mitochondria [64], serving a neuroprotective role [65].

We used HEK293T and PC12 cells in the present study to explore DOR's effect on the oligomer formation induced by hypoxia and/or α-synuclein overexpression/mutation. HEK 293T cells have been widely used as a cell model for many years because of their reliable growth and propensity for transfection, achieving efficiencies approaching 100% [66, 67]. Moreover, we found that these cells express DOR and are suitable for DOR-relevant research [9]. The PC12 cell line has been used to obtain cellular and molecular information about diseases of the brain because of its extreme versatility for pharmacological manipulation, ease of culture, and the large amount of information on their proliferation and differentiation [68]. PC12 cells have smaller vesicles holding an average $1.9 \times 10^{-19}$ mol of neurotransmitter to be released [68]. The vesicles hold catecholamines, not only dopamine but also limited amounts of norepinephrine. PC12 cell line use has given much information to the function of proteins underlying vesicle fusion. This cell line has been used to understand the role of synaptotagmin in vesicle-cell membrane fusion [69]. Therefore, a combined use of these two cell models is helpful for understanding the regulation of cellular and molecular mechanisms of PD.

In summary, our novel data suggest that DOR activation can largely attenuate α-synuclein dysregulation via DJ-1 upregulation in both genetic (transfection of wild-type or A53T-mutant α-synuclein) and environmental (hypoxic) conditions; the DOR action involves TORC1/SIK1 downregulation in the former condition and CREB phosphorylation in the latter condition. Figure 8 schematically shows the potential mechanisms of the DOR-mediated protection against α-synuclein dysregulation. It is our belief that DOR signaling is an anti-parkinsonian factor through the mechanism of DJ-1 upregulation versus α-synuclein downregulation, while the regulation of DJ-1 and α-synuclein differentially involve CREB and TORC1/SIK1 signaling, depending on the condition.

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**Author Contributions** YX conceived the project. TC designed and performed the experiments, analyzed the data, and drafted the manuscript. QW and DC performed experiments and analyzed data. TCX, SYS, ZRL, JNZ, GQW, and GHD aided with data interpretation and manuscript revision. YX provided reagents/materials/analysis tools, interpreted data, revised, and finalized the manuscript for publication. All authors read and approved the final manuscript.

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## Compliance with Ethical Standards

**Conflict of Interests** The authors declare that they have no competing interests.

**Consent for Publication** Yes.

**Ethics Approval** Not applicable.

**Abbreviations** ANOVA, one-way analysis of variance; CREB, cAMP-response element binding protein; CBP, CREB-binding protein; DMEM, Deulbecco's Modified Eagle Medium; DOR, delta opioid receptor; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; H, hypoxia; H + W, hypoxia plus the overexpression of wild-type α-synuclein; H + A, hypoxia plus the overexpression of A53T-mutant α-synuclein l; LDH, Lactic Dehydrogenase; MPTP, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson disease; PVDF, polyvinylidenedifluoride membrane; SIK, salt-inducible kinase; TORC, transducer of regulated CREB

## References

1. Xia Y, Haddad GG (1991) Ontogeny and distribution of opioid receptors in the rat brainstem. Brain Res 549(2):181–193
2. Xia Y, Haddad GG (2001) Major difference in the expression of delta- and mu-opioid receptors between turtle and rat brain. J Comp Neurol 436(2):202–210
3. Piccini P, Weeks RA, Brooks DJ (1997) Alterations in opioid receptor binding in Parkinson's disease patients with levodopa-induced dyskinesias. Ann Neurol 42(5):720–726
4. Hille CJ, Fox SH, Maneuf YP, Crossman AR, Brotchie JM (2001) Antiparkinsonian action of a delta opioid agonist in rodent and primate models of Parkinson's disease. Exp Neurol 172(1):189–198
5. Hill MP, Hille CJ, Brotchie JM (2000) Delta-opioid receptor agonists as a therapeutic approach in Parkinson's disease. Drug News Perspect 13(5):261–268
6. Fox S, Silverdale M, Kellett M, Davies R, Steiger M, Fletcher N, Crossman A, Brotchie J (2004) Non-subtype-selective opioid receptor antagonism in treatment of levodopa-induced motor complications in Parkinson's disease. Mov Disord 19(5):554–560
7. Chao D, Xia Y (2010) Ionic storm in hypoxic/ischemic stress: can opioid receptors subside it. Prog Neurobiol 90(4):439–470
8. Crowley MG, Liska MG, Lippert T, Corey S, Borlongan CV (2017) Utilizing delta opioid receptors and peptides for cytoprotection: implications in stroke and other neurological disorders. CNS & Neurological Disorders Drug Targets 16(4):414–424
9. Chen T, Li J, Chao D, Sandhu HK, Liao X, Zhao J, Wen G, Xia Y (2014) δ-Opioid receptor activation reduces α-synuclein overexpression and oligomer formation induced byMPP(+) and/or hypoxia. Exp Neurol 255:127–136
10. James WH (2003) Coffee drinking, cigarette smoking, and Parkinson's disease. Ann Neurol 53(4):546

11. Dorszewska J (2013) Genetic factors in Parkinson's disease. Current Genomics 14(8):485
12. Tang W, Bansal S, Chowdhury G, Cheng Q, Fang JK, Martin MV, Guengerich FP, Avadhani NG (2013) Metabolism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine by mitochondrion-targeted cytochrome P450 2D6: implications in Parkinson disease. J Biol Chem 288(6):4436–4451
13. Kalia LV, Kalia SK, McLean PJ, Lozano AM, Lang AE (2013) α-Synuclein oligomers and clinical implications for Parkinson disease. Ann Neurol 73(2):155–169
14. Rogge GA, Shen LL, Kuhar MJ (2010) Chromatin immunoprecipitation assays revealed CREB and serine 133 phospho-CREB binding to the CART gene proximal promoter. Brain Res 1344:1–12
15. Tiwari SK, Mishra D, Agarwal S, Kim YS, Beal MF (2012) Transducer of regulated CREB-binding proteins (TORCs) transcription and function is impaired in Huntington's disease. Hum Mol Genet 21(15):3474–3488
16. Liu Y, Li J, Jin DQ, Tang XD (2009) Regulation of TORC-CREB complex by salt-inducible kinases: implications in hypertension and diabetes mellitus. Prog Biochem Biophys 36(3):274–279
17. Zondler L, Miller-Fleming L, Repici M, Gonçalves S, Tenreiro S, Rosado-Ramos R, Betzer C, Straatman KR et al (2014) DJ-1 interactions with α-ynuclein attenuate aggregation and cellular toxicity inmodels of Parkinson's disease. Cell Death Dis 5:e1350
18. Zondler L, Miller-Fleming L, Repici M, Gonçalves S, Tenreiro S, Rosado-Ramos R, Betzer C, Straatman KR et al (2014) DJ-1 interactions with α-synuclein attenuate aggregation and cellular toxicity in models of Parkinson's disease. Cell Death Dis 5:e1350
19. Mabrouk OS, Marti M, Salvadori S, Morari M (2009) The novel delta opioid receptor agonist UFP-512 dually modulates motor activity in hemiparkinsonian rats via control of the nigro-thalamic pathway. Neuroscience 164(2):360–369
20. Cao S, Chao D, Zhou H, Balboni G, Xia Y (2015) A novel mechanism for cytoprotection against hypoxic injury: δ-opioid receptor-mediated increase in Nrf2 translocation. Br J Pharmacol 172(7): 1869–1881
21. Tanner CM, Langston JW (1990) Do environmental toxins cause Parkinson's disease? A critical review. Neurology 40(10 Suppl 3): suppl 17–30
22. Ma MC, Qian H, Ghassemi F, Zhao P, Xia Y (2005) Oxygen-sensitive {delta}-opioid receptor-regulated survival and death signals: novel insights into neuronal preconditioning and protection. J Biol Chem 280(16):16208–16218
23. Hashimoto M, Masliah E (1999) Alpha-synuclein in Lewy body disease and Alzheimer's disease. Brain Pathol 9:707–720
24. Araki K, Yagi N, Ikemoto Y, Yagi H, Choong CJ, Hayakawa H, Beck G, Sumi H et al (2015) Synchrotron FTIR micro-spectroscopy for structural analysis of Lewy bodies in the brain of Parkinson's disease patients. Scientific Reports 5:17625
25. Barranco Quintana JL, Allam MF, Serrano Del Castillo A, Fernandez-Crehuet NR (2009) Parkinson's disease and tea: a quantitative review. J Am Coll Nutr 28(1):1–6
26. Kalia LV, Lang AE. Parkinson's disease. Lancet (London, England) 2015.
27. Shulman JM, De Jager PL, Feany MB (2011) Parkinson's disease: genetics and pathogenesis. Annu Rev Pathol 6:193–222
28. He X, Sandhu HK, Yang Y, Hua F, Belser N, Kim DH, Xia Y (2013) Neuroprotection against hypoxia/ischemia: δ-opioid receptor-mediated cellular/molecular events. Cell Mol Life Sci 70(13): 2291–2303
29. Staples M, Acosta S, Tajiri N, Pabon M, Kaneko Y, Borlongan C (2013) Delta opioid receptor and its peptide: a receptor-ligand neuroprotection. Int J Mol Sci 14(9):17410–17419
30. Sen D, Huchital M, Chen Y (2013) Crosstalk between delta opioid receptor and nerve growth factor signaling modulates neuroprotection and differentiation in rodent cell models. Int J Mol Sci 14(10):21114–21139
31. Wang SY, Duan YL, Zhao B, Wang XR, Zhao Z, Zhang GM (2016) Effect of delta opioid receptor activation on spatial cognition and neurogenesis in cerebral ischemic rats. Neurosci Lett 620:20–26
32. Borlongan CV, Su TP, Wang Y (2001) Delta opioid peptide augments functional effects and intrastriatal graft survival of rat fetal ventral mesencephalic cells. Cell Transplant 10:53–58
33. Borlongan CV, Su TP, Wang Y (2000) Treatment with delta opioid peptide enhances in vitro and in vivo survival of rat dopaminergic neurons. Neuroreport 11:923–926
34. Kermer P, Klocker N, Bahr M (1999) Neuronal death after brain injury. Models, mechanisms, and therapeutic strategies in vivo. Cell Tissue Res 298(3):383–395
35. Zhang Z, Yang X, Zhang S, Ma X, Kong J (2007) BNIP3 upregulation and EndoG translocation in delayed neuronal death in stroke and in hypoxia. Stroke 38(5):1606–1613
36. Zhao P, Huang Y, Zuo Z (2006) Opioid preconditioning induces opioid receptor-dependent delayed neuroprotection against ischemia in rats. J Neuropathol Exp Neurol 65(10):945–952
37. Lim YJ, Zheng S, Zuo Z (2004) Morphine preconditions Purkinje cells against cell death under in vitro simulated ischemia-reperfusion conditions. Anesthesiology 100(3):562–568
38. Simpkins JW, Yi KD, Yang SH (2009) Role of protein phosphatases and mitochondria in the neuroprotective effects of estrogens. Front Neuroendocrinol 30(2):93–105
39. Liu R, Wang X, Liu Q, Yang SH, Simpkins JW (2007) Dose dependence and therapeutic window for the neuroprotective effects of 17beta-estradiol when administered after cerebral ischemia. Neurosci Lett 415(3):237–241
40. Silkis IG (2007) The role of opioid receptor agonists and antagonists in the treatment of Parkinson's disease. Neurochem 1:281–287
41. Chao D, Xia Y (2015) The delta-opioid system in the brain: a neglected element in Parkinson's disease? In Xia Y (ed). Neural functions of the delta-opioid receptor. Springer (New York, Heidelberg, Dordrecht, London) pp 461–520
42. Xu Y, Zhi F, Shao N, Wang R, Yang Y, Xia Y (2016) Cytoprotection against hypoxic and/or $MPP^+$ injury: effect of δ-opioid receptor activation on caspase 3. Int J Mol Sci 17(8):1179
43. Xu Y, Zhi F, Peng Y, Shao NY, Khiati D, Balboni G, Yang YL, Xia Y (2018) δ -Opioid receptor activation attenuates hypoxia/MPP+-induced downregulation of PINK1: a novel mechanism of neuroprotection against parkinsonism injury. Mol Neurobiol:1–15
44. Jiang B, Chen T, Chao D, Wen G, Xia Y (2015) The δ-opioid receptor and α-synuclein in parkinsonism conditions. The proceedings of 2nd Zing Neurodegeneration Conference:102
45. Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M (1998) Alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. Proc Natl Acad Sci U S A 95:6469–6473
46. Wilson RS, Yu L, Schneider JA (2011) Lewy bodies and olfactory dysfunction in old age. Chem Senses 36(4):367–373
47. Putcha P, Danzer KM, Kranich L (2010) Brain-permeable small-molecule inhibitors of Hsp90 prevent alpha-synuclein oligomer formation and rescue alpha-synuclein-induced toxicity. J Pharmacol Exp Ther 332(3):849–857
48. Shaywitz AJ, Greenberg ME (1999) CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annual Review of Annu Rev Biochem 68:821–861
49. Pardo L, Valor LM, Eraso-Pichot A, Barco A, Golbano A, Hardingham GE, Masgrau R, Galea E (2017) CREB regulates distinct adaptive transcriptional programs in astrocytes and neurons. Sci Rep 7(1):6390
50. Chun H, Hao W, Honghai Z, Ning L, Yasong W, Chen D (2009) CCL3L1 prevents gp120-induced neuron death via the CREB cell signaling pathway. Brain Res 1257:75–88

51. Carlezon WA, Duman RS, Nestler E (2005) The many faces of CREB. Trends Neurosci 28(8):436–445
52. Conkright MD, Canettieri G, Screaton R, Guzman E, Miraglia L, Hogenesch JB, Montminy M (2003) TORCs: transducers of regulated CREB activity. Mol Cell 12(2):413–423
53. Bittinger MA, McWhinnie E, Meltzer J, Iourgenko V, Latario B, Liu X, Chen CH, Song C et al (2004) Activation of cAMP response element- mediated gene expression by regulated nucleartransport of TORC proteins. Curr Biol 14(23):2156–2161
54. Screaton RA, Conkright MD, Katoh Y, Best JL, Canettieri G, Jeffries S, Guzman E, Niessen S et al (2004) The CREB coactivator TORC2 functions as a calcium- and cAMP-sensitive coincidence detector. Cell 119(1):61–74
55. Liu L, Zhang X, Wang L, Yang R, Cui L, Li M, Du W, Wang S (2010) The neuroprotective effects of Tanshinone IIA are associated with induced nuclear translocation of TORC1 and upregulated expression of TORC1, pCREB and BDNF in the acute stage of ischemic stroke. Brain Res Bull 82(3/4):228–233
56. Sekeres MJ, Mercaldo V, Richards B, Sargin D, Mahadevan V, Woodin MA, Frankland PW, Josselyn SA (2012) Increasing CRTC1 function in the dentate gyrus during memory formation or reactivation increases memory strength without compromising memory quality. J Neurosci Off J Soc Neurosci 32(49):17857–17868
57. Koo SH, Flechner L, Qi L, Zhang X, Screaton RA, Jeffries S, Hedrick S, Xu W et al (2005) The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. Nature 437(7062): 1109–1111
58. Bonifati V, Rizzu P, Squitieri F, Krieger E, Vanacore N, van Swieten JC, Brice A, van Duijn CM et al (2003) DJ-1(PARK7), a novel gene for autosomal recessive, early onset parkinsonism. Neurol Sci 24(3):159–160
59. Raquel RA, Irene LF, Daniel JB, Emilio F, Angeles A, Bolaños JP (2015) DJ1 represses glycolysis and cell proliferation by transcriptionally up-regulating Pink1. The Biochemical journal 467(2):303–310
60. Bandmann O (2004) DJ-1: the second gene for early onset Parkinson disease. Neurology 62(3):357–358
61. Jin J, Li GJ, Davis J, Zhu, Wang, Pan, Zhang J (2006) Identification of novel proteins associated with both α-synuclein and DJ-1. Mol Cell Proteomics 6(5):845–859
62. Kthiri F, Le HT, Gautie V, Caldas T, Malki A, Landoulsi A, Bohn C, Bouloc P et al (2010) Protein aggregation in a mutant deficient in yajL, the bacterial homolog of the parkinsonism-associated protein DJ-1. J Biol Chem 285(14):10328–10336
63. Kaneko Y, Shojo H, Burns J, Staples M, Tajiri N, Borlongal CV (2014) DJ-1 ameliorates ischemic cell death in vitro possibly via mitochondrial pathway. Neurobiol Dis 62:56–61
64. De Miranda BR, Rocha EM, Bai Q, El Ayadi A, Hinkle D, Burton EA, Greenamyre JT (2018) Astrocyte-specific DJ-1 overexpression protects against rotenone-induced neurotoxicity in a rat model of Parkinson's disease. Neurobiol Dis 115:101–114
65. Liu W, Guo Q, Hu X, Peng L, Zhou B (2015) Induction of DJ-1 protects neuronal cells from isoflurane induced neurotoxicity. Metab Brain Dis 30(3):703–709
66. Storch A, Ludolph AC, Schwarz J (1999) HEK-293 cells expressing the human dopamine transporter are susceptible to low concentrations of 1-methyl-4-phenylpyridine (MPP+) via impairment of energy metabolism. Neurochem Int 35(5):393–403
67. Chen T, Liao X, Wen G, Deng Y, Guo M, Long Z, Ouyang F (2012) Influence of RNA interference on the mitochondrial subcellular localization of alpha-synuclein and on the formation of Lewy body-like inclusions in the cytoplasm of human embryonic kidney 293 cells induced by the overexpression of alpha-synuclein. Neural Regen Res 7(2):1–7
68. Westerink RH, Ewing AG (2008) The PC12 cell as model for neurosecretion. Acta Physiol (Oxford, England) 192(2):273–285
69. Gustavsson N, Han W (2009) Calcium-sensing beyond neurotransmitters: functions of synaptotagmins in neuroendocrine and endocrine secretion. Biosci Rep 29(4):245–259