NEURAL REGENERATION RESEARCH
August 2015, Volume 10, Issue 8
www.nrronline.org

RESEARCH ARTICLE

The E3 ubiquitin ligase seven in absentia homolog 1 may be a potential new therapeutic target for Parkinson's disease

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doi:*10.4103/1673-5374.162763*
http:*//www.nrronline.org/*

Accepted: *2015-06-09*

Abstract

In this study, we investigated the effect of an antibody against E3 ubiquitin ligase seven in absentia homolog 1 (SIAH-1) in PC12 cells. 1-Methyl-4-phenylpyridinium (MPP^+) treatment increased α-synuclein, E1 and SIAH-1 protein levels in PC12 cells, and it reduced cell viability; however, there was no significant change in light chain 3 expression. Treatment with an SIAH-1 antibody decreased mRNA expression levels of α-synuclein, light chain 3 and SIAH-1, but increased E1 mRNA expression. It also increased cell viability. Combined treatment with MPP^+ and rapamycin reduced SIAH-1 and α-synuclein levels. Treatment with SIAH-1 antibody alone diminished α-synuclein immunoreactivity in PC12 cells, and reduced the colocalization of α-synuclein and light chain 3. These findings suggest that the SIAH-1 antibody reduces the monoubiquitination and aggregation of α-synuclein, promoting its degradation by the ubiquitin-proteasome pathway. Consequently, SIAH-1 may be a potential new therapeutic target for Parkinson's disease.

Key Words: *nerve regeneration; neurodegeneration; Parkinson's disease; ubiquitin-proteasome system; autophagy; E3 ubiquitin ligase seven in absentia homolog 1; 1-methyl-4-phenylpyridinium; rapamycin; neural regeneration*

Funding: *This study was supported by the China Postdoctoral Science Foundation, No. 1630; the Natural Science Foundation of Jiangsu Province in China, No. BK2011402; the Jiangsu Province Postdoctoral Research Foundation in China, No. 1301174C; the Jiangsu Province Health Department Foundation in China, No. H201361.*

Cai ZL, Xu J, Xue SR, Liu YY, Zhang YJ, Zhang XZ, Wang X, Wu FP, Li XM (2015) The E3 ubiquitin ligase seven in absentia homolog 1 may be a potential new therapeutic target for Parkinson's disease. Neural Regen Res 10(8):1286-1291.

Introduction

The presence of α-synuclein aggregates is a characteristic of many neurodegenerative diseases, including Parkinson's disease (PD) and Lewy body dementia (Kalia et al., 2013; Tanik et al., 2013). Accumulating evidence shows that α-synuclein misfolding, aggregation and abnormal degradation cause dopaminergic neuron death (Bartels et al., 2011; Gadad et al., 2011; Cremades et al., 2012). This process underlies PD pathogenesis (Venda et al., 2010; Kalia et al., 2013; Ryan et al., 2014). Although monoubiquitinated and polyubiquitinated α-synuclein are present in Lewy bodies, the regulation of α-synuclein ubiquitination and its role in the formation of Lewy bodies are not fully understood (Engelender, 2008; Lee et al., 2008). Recent studies show that the E3 ubiquitin ligase seven in absentia homolog 1 (SIAH-1) mediates α-synuclein monoubiquitination (Engelender, 2008; Lee et al., 2008). SIAH-1 also plays a key role in Lewy body formation and pathogenesis in PD, contributing to α-synuclein aggregation and intracellular inclusion formation. The intracellular inclusions and aggregates cannot be cleared by the ubiquitin-proteasome pathway, and must therefore rely on the autophagy degradation pathway (Engelender, 2008; Lee et al., 2008).

Despite the data linking SIAH-1 to α-synuclein aggregation and degradation, the function of SIAH-1 in the autophagy-lysosomal pathway remains unknown. Furthermore, it is not clear whether SIAH-1-mediated ubiquitination directs α-synuclein to the ubiquitin-proteasome pathway or the autophagy pathway. SIAH-1 may activate or downregulate autophagy *via* the p53 pathway, thereby promoting or inhibiting the degradation of α-synuclein. To clarify the role of SIAH-1 in α-synuclein degradation, we induced autophagy and inhibited SIAH-1 function using an anti-SIAH-1 antibody. We then examined the effects on SIAH-1 activity, p53 expression and on the ubiquitin proteasome pathway and the autophagy-lysosomal degradation pathway.

Materials and Methods

Cell culture and treatments

The rat pheochromocytoma (PC12) cell line was purchased

from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). All cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (C11995; Life Technologies, Carlsbad, CA, USA). For experiments, cells were seeded in culture flasks, or 24 or 96-well plates until 60–70% confluence. Cells were divided into six groups. In the control group, the normal growth of cells was observed. In the rapamycin (RAPA) group, cells were treated with 0.2 μg/mL RAPA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 24 hours. In the anti-SIAH-1 group, cells were treated with 4 μg/mL anti-SIAH-1 antibody (Santa Cruz Biotechnology) for 24 hours. In the 1-methyl-4-phenylpyridinium (MPP^+) group, cells were treated with 0.5 mM MPP^+ (Santa Cruz Biotechnology) for 24 hours. In the MPP^+ RAPA group, cells were treated with MPP^+ for 24 hours then RAPA for 24 hours. In the MPP^+ anti-SIAH-1 group, cells were treated with MPP^+ for 24 hours then anti-SIAH-1 antibody for 24 hours.

3-(4,5-Cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay for cell viability

Cells were cultured in 96-well plates with RPMI-1640 medium containing 10% fetal bovine serum at a density of 1×10^5/mL, in a volume of 200 μL/well. Cells in the exponential phase of growth were incubated with MPP^+, RAPA and SIAH-1 antibody for 24 hours. The culture medium was refreshed, and 20 μL MTT solution (final concentration of 0.5 mg/mL) was added to each well. Cells were incubated at 37°C for an additional 4 hours in the dark. After incubation, the medium containing MTT was removed, and 150 μL dimethyl sulfoxide was added to each well to dissolve the formazan dye crystals on a shaker for 15 minutes. The optical density was measured at 492 nm with a microplate reader (Model 680; Bio-Rad, CA, USA). Cell viability was expressed as a percentage of the value in the control group.

Western blot analysis

Western blot analysis was performed as previously described by our group (Cai et al., 2009). Cells were lysed and sonicated in lysis buffer. After electrophoresis, samples were transferred onto a polyvinylidene difluoride membrane (Millipore, Temecula, CA, USA), and then immunoblotted with the following antibodies: goat polyclonal anti-SIAH-1 (1:100; sc-5505; Santa Cruz Biotechnology), rabbit polyclonal anti-α-synuclein (1:1,000; 2642; Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal anti-light chain 3 (LC3) (1:1,000; ab62721; Abcam, Cambridge, MA, USA), rabbit polyclonal anti-E1 (1:1,000; 4891S; Cell Signaling Technology), rabbit polyclonal anti-P53 (1:1,000; 2642; Cell Signaling Technology), and mouse monoclonal β-actin (1:1,000; A3854; Sigma-Aldrich, St Louis, MO, USA). Subsequently, the following horseradish peroxidase-conjugated secondary antibodies were added: polyclonal goat anti-rabbit secondary antibody for LC3, α-synuclein and E1; polyclonal donkey anti-goat secondary antibody for SIAH-1; and polyclonal goat anti-mouse secondary antibody for β-actin (1:1,000; Beyotime Biotechnology, Jiangsu, China). Images were captured using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA), and band intensities were calculated by densitometric analysis using Image J software (NIH, Bethesda, MD, USA).

Semi-quantitative analysis of mRNA by reverse transcription (RT)-PCR

Total RNA was extracted from PC12 cells using TRIzol (Life Technologies). First-strand cDNA was synthesized using PrimeScript RT Enzyme Mix I (RR037A; Takara, Otsu, Japan). Primer pairs for the amplification of cDNA for LC3, α-synuclein, SIAH-1 and β-actin were designed (**Table 1**). cDNA amplification was performed using DyNAmo SYBR green qPCR kits (Finnzymes Oy, Espoo, Finland). Amplification was performed using an iCycler iQ Multicolor Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The expression levels of LC3, SIAH-1 and α-synuclein were normalized to that of β-actin.

Immunofluorescence microscopy

PC12 cells were seeded onto non-coated 12-mm coverslips and treated with MPP^+ (0.5 mM, 24-hour exposure) and goat polyclonal SIAH-1 antibody (4 μg/mL, 24 hours), and then fixed in ice-cold 4% paraformaldehyde for 15 minutes. The cells were incubated with rabbit polyclonal anti-LC3 antibody (1:250), goat polyclonal anti-SIAH-1 antibody (1:500) or rabbit polyclonal anti-α-synuclein antibody (1:250) for 1.5 hours at 37°C. Cells were then treated with polyclonal mouse anti-rabbit secondary antibody for LC3 and α-synuclein or polyclonal mouse anti-goat secondary antibody for SIAH-1 (1:1,000; Beyotime Biotechnology) for 1 hour at 37°C. Thereafter, cells were stained with 4′,6-diamidino-2-phenylindole (0.3 μg/mL) for 15 minutes, and examined with a laser scanning confocal microscope (TCS SP2 CLSM; Leica, Wetzlar, Germany). Images were collected and processed using the imaging software provided with the Leica TCS system.

Statistical analysis

All experiments were performed in triplicate, and the results are presented as the mean ± SD. Statistical analysis was performed using SPSS 12.0 software (SPSS, Chicago, IL, USA). Multiple group comparisons were performed using one-way analysis of variance and Fisher's least significant difference test (equal variances assumed). A value of $P < 0.05$ was considered statistically significant.

Results

SIAH inhibition increased cell viability

We assessed PC12 cell viability after a 24-hour treatment with RAPA (used to induce autophagy) and SIAH-1 antibody. We found no significant effect on viability under normal growth conditions ($P > 0.05$). As expected, MPP^+-treated cells showed reduced viability. RAPA and SIAH-1 antibody each significantly increased viability in cells treated with MPP^+ ($P = 0.0008$ and 0.0014, respectively). These results indicate that RAPA and SIAH-1 antibody improve viability

Table 1 Primer pairs for the amplification of cDNA transcripts

Gene	Sequence (5′–3′)	Product size (bp)
LC3		
Upstream	GAG TGG AAG ATG TCC GGC TC	200
Downstream	CCA GGA GGA AGA AGG CTT GG	
α-synuclein		
Upstream	CCT CAG CCC AGA GCC TTT C	256
Downstream	CCT CTG CCA CAC CCT GCT T	
SIAH-1		
Upstream	CTG TCG CCC CAA ACT TAC AT	232
Downstream	CAA GGA GCC TTG CCA CTT AC	
E1		
Upstream	CCC TAC ATG ACC AAG GCA CT	187
Downstream	CCC ACT CGA AGC TGT TCT TC	
β-Actin		
Upstream	TCA GGT CAT CAC TAT CGG CAA T	432
Downstream	AAA GAA AGG GTG TAA AAC GCA	

LC3: Light chain 3; SIAH-1: E3 ubiquitin ligase seven in absentia homolog 1.

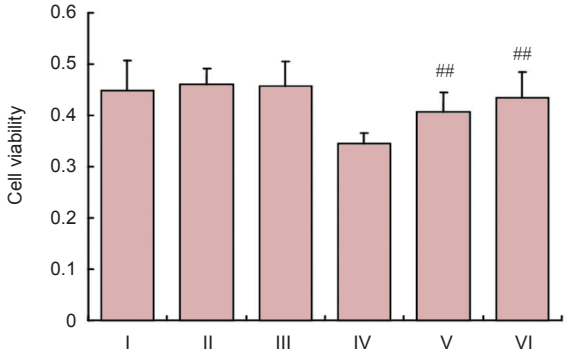

Figure 1 MTT assay for cell viability after treatment with MPP$^+$, RAPA and SIAH-1 antibody.

RAPA and SIAH-1 antibody treatment had no significant effect on cell viability under normal growth conditions ($P > 0.05$). In the MPP$^+$ group, RAPA and SIAH-1 antibody significantly increased cell viability (##$P < 0.01$, *vs.* MPP$^+$ group; mean ± SD, $n \geq 6$, one-way analysis of variance followed by Fisher's least significant difference *post hoc* test). I: Control group; II: RAPA group; III: anti-SIAH-1 group; IV: MPP$^+$ group; V: MPP$^+$ RAPA group; VI: MPP$^+$ anti-SIAH-1 group. MTT: 3-(4,5-Cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MPP$^+$: 1-methyl-4-phenylpyridinium; RAPA: rapamycin; SIAH-1: E3 ubiquitin ligase seven in absentia homolog 1.

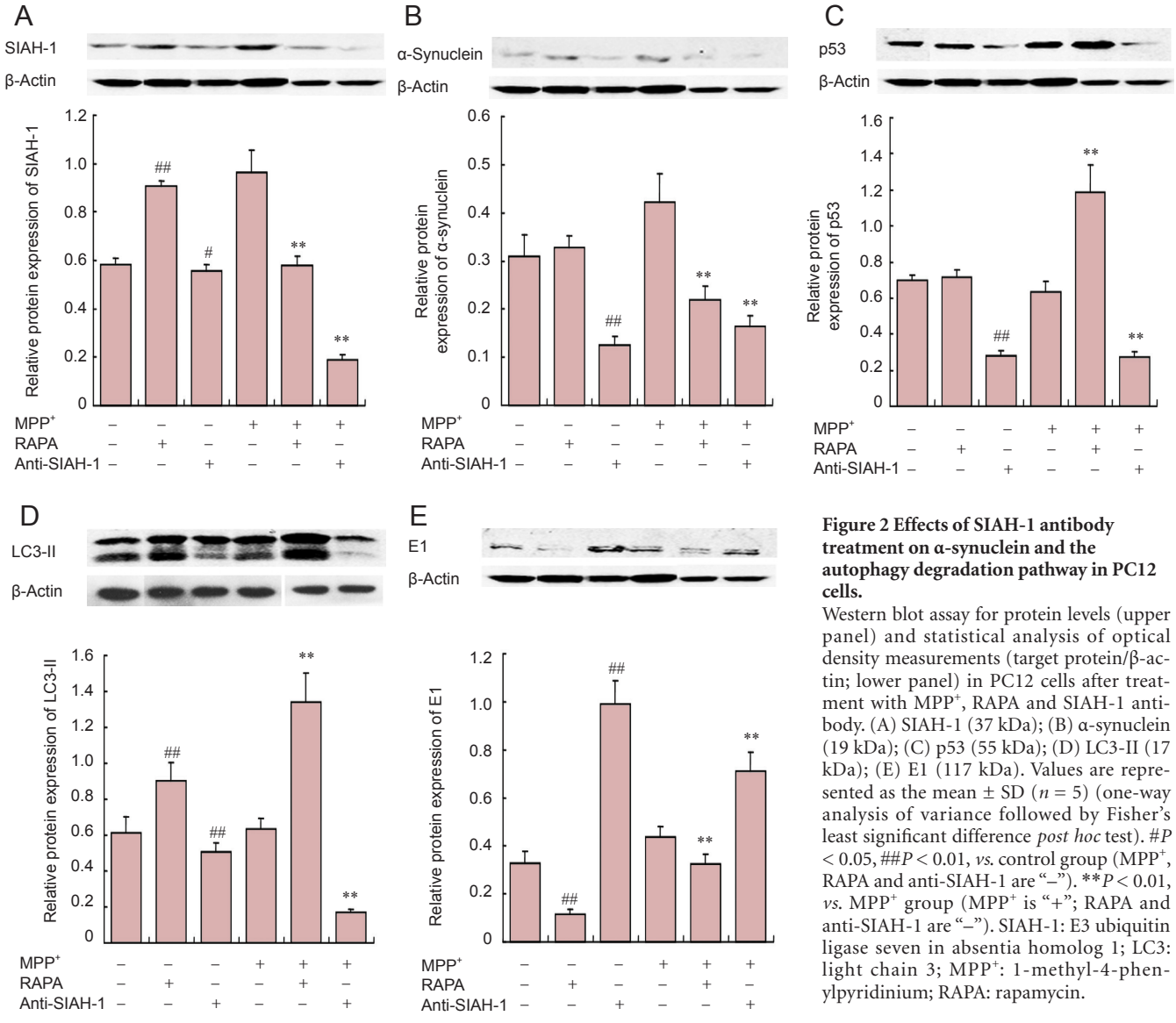

Figure 2 Effects of SIAH-1 antibody treatment on α-synuclein and the autophagy degradation pathway in PC12 cells.

Western blot assay for protein levels (upper panel) and statistical analysis of optical density measurements (target protein/β-actin; lower panel) in PC12 cells after treatment with MPP$^+$, RAPA and SIAH-1 antibody. (A) SIAH-1 (37 kDa); (B) α-synuclein (19 kDa); (C) p53 (55 kDa); (D) LC3-II (17 kDa); (E) E1 (117 kDa). Values are represented as the mean ± SD ($n = 5$) (one-way analysis of variance followed by Fisher's least significant difference *post hoc* test). #$P < 0.05$, ##$P < 0.01$, *vs.* control group (MPP$^+$, RAPA and anti-SIAH-1 are "–"). **$P < 0.01$, *vs.* MPP$^+$ group (MPP$^+$ is "+"; RAPA and anti-SIAH-1 are "–"). SIAH-1: E3 ubiquitin ligase seven in absentia homolog 1; LC3: light chain 3; MPP$^+$: 1-methyl-4-phenylpyridinium; RAPA: rapamycin.

in MPP^+-stressed cells (**Figure 1**).

MPP^+ increased α-synuclein expression and SIAH-1 levels

MPP^+ treatment not only increased the expression of α-synuclein ($P < 0.01$) and E1 ($P < 0.01$), but also that of SIAH-1 ($P < 0.01$). We found a nonsignificant increase in LC3-II expression ($P = 0.17$) (**Figure 2**). In addition, we observed significant α-synuclein aggregation and localization in the cytoplasm after MPP^+ treatment. SIAH-1 immunofluorescence staining was significantly increased after 24 hours of MPP^+ treatment (**Figure 3**).

Effects of autophagy induction on SIAH levels and α-synuclein degradation

Autophagy was induced by rapamycin treatment, and SIAH-1 levels and α-synuclein degradation were measured. LC3-II levels were significantly increased ($P < 0.01$), while E1 expression was decreased ($P < 0.01$), and SIAH-1 levels were increased ($P < 0.01$), with no significant change in P53 or α-synuclein expression ($P = 0.536$). Additionally, LC3-II and E1 expression levels were increased in the MPP^+ group ($P < 0.01$), while E1 expression was reduced significantly ($P < 0.01$). RAPA treatment significantly diminished SIAH-1 and α-synuclein levels ($P < 0.01$; **Figure 2**).

Decreasing SIAH activity reduced α-synuclein levels and inhibits the autophagy degradation pathway

SIAH activity was inhibited by treating cells with SIAH-1 antibody. LC3-II expression was decreased ($P = 0.004$), and E1 expression was increased significantly ($P < 0.01$). Both p53 and α-synuclein levels decreased significantly ($P < 0.01$). A consistent trend was found in the MPP^+ group, where SIAH-1 levels significantly decreased after treatment with SIAH-1 antibody ($P < 0.01$). Furthermore, LC3-II expression decreased ($P < 0.01$) and E1 expression increased ($P < 0.01$). Finally, p53 and α-synuclein levels decreased significantly ($P < 0.01$; **Figure 2**).

Inhibition of SIAH-1 altered SIAH-1, E1, LC3-II and α-synuclein mRNA levels

To confirm western blot assay results, we measured mRNA levels of SIAH-1, E1, LC3-II and α-synuclein after SIAH-1 antibody treatment. Consistent with western blot results, we observed decreased mRNA expression levels of SIAH-1, LC3-II and α-synuclein ($P < 0.05$), and increased E1 mRNA levels ($P = 0.0086$; data not shown).

MPP^+ treatment elevated E1 mRNA levels ($P = 0.0302$). However, α-synuclein levels increased ($P = 0.0030$). In addition, MPP^+ treatment increased LC3-II and SIAH-1 mRNA levels ($P < 0.01$). SIAH-1 antibody treatment reversed these trends and significantly reduced SIAH-1 transcript levels ($P < 0.0001$). In contrast, E1 mRNA expression increased ($P = 0.0170$; data not shown).

α-Synuclein, SIAH-1 and LC3 colocalized in PC12 cells

We explored the relationship between SIAH-1, α-synuclein and LC3 in the cells after SIAH-1 antibody treatment by examining their localization using immunofluorescence. Immunoreactivities of α-synuclein, SIAH-1 and LC3 decreased after SIAH-1 antibody treatment. In addition, colocalization of α-synuclein and LC3 was lost. However, α-synuclein and SIAH-1 retained their colocalization. Furthermore, α-synuclein aggregates were significantly reduced (**Figure 4**).

Discussion

The ubiquitin-proteasome and autophagy-lysosomal pathways are two main pathways for clearing proteins and organelles in eukaryotic cells (Jimenez-Sanchez et al., 2012; Kuang et al., 2013; Nixon, 2013). The proteasome is a barrel-shaped multi-protein complex that degrades short-lived nuclear and cytosolic proteins (Jimenez-Sanchez et al., 2012; Sadanandom et al., 2012; Gu and Enenkel, 2014). Proteasome substrates are forced to linearize and travel through the narrow cylindrical pore of the proteasome, preventing the clearance of oligomers and aggregates (Verhoef et al., 2002; Mizushima and Komatsu, 2011). The E3 ubiquitin ligase SIAH-1 monoubiquitinates α-synuclein and promotes aggregation and toxicity in cells (Rott et al., 2008, 2011; Engelender, 2012). The α-synuclein monoubiquitination leads to α-synuclein aggregation and the formation of intracellular inclusions in dopaminergic neurons (Szargel et al., 2009; Alvarez-Castelao and Castano, 2011). Therefore, we hypothesized that the inhibition of SIAH-1 would suppress α-synuclein monoubiquitination and aggregation, leading to increased levels of monomers. The monomers would then likely be selectively degraded by the ubiquitin-proteasome pathway, but not by the autophagy pathway.

Several *in vitro* studies (Kalivendi et al., 2004; Su et al., 2011; Park et al., 2014), including our previous study (Cai et al., 2009), have shown that MPP^+ induces α-synuclein expression and aggregation. This results in symptoms similar to those in Parkinson's disease in experimental animals and humans (Sherer et al., 2002; Yang et al., 2009), and appear to be induced by, among others, increased levels of oxidative stress and apoptosis (Fiskum et al., 2003; Bernstein and O'Malley, 2013; Schildknecht et al., 2013). However, it is unclear how oxidative stress alters SIAH activity or its function in α-synuclein degradation.

In this study, SIAH-1 protein and mRNA expression levels were significantly increased in MPP^+-treated cells. Hara et al. (2006) showed that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treatment produces GAPDH/SIAH protein complexes in the corpus striatum, triggering the NO/GAPDH/SIAH cell death cascade. Furthermore, suppressing SIAH-1 markedly reduces cell death (Ortiz-Ortiz et al., 2010). These findings are consistent with our result that SIAH-1 antibody treatment increases cell viability in MPP^+-treated cells. This suggests that the SIAH-1 antibody alleviates cellular stress. However, it remains unclear how the SIAH-1 antibody prevents the MPP^+-induced reduction in cell viability.

Recent studies show that SIAH-1-mediated α-synuclein monoubiquitination leads to the formation of α-synuclein aggregates and inclusions (Rott et al., 2008; Szargel et al., 2009). SIAH-1 mediates the ubiquitination of α-synuclein

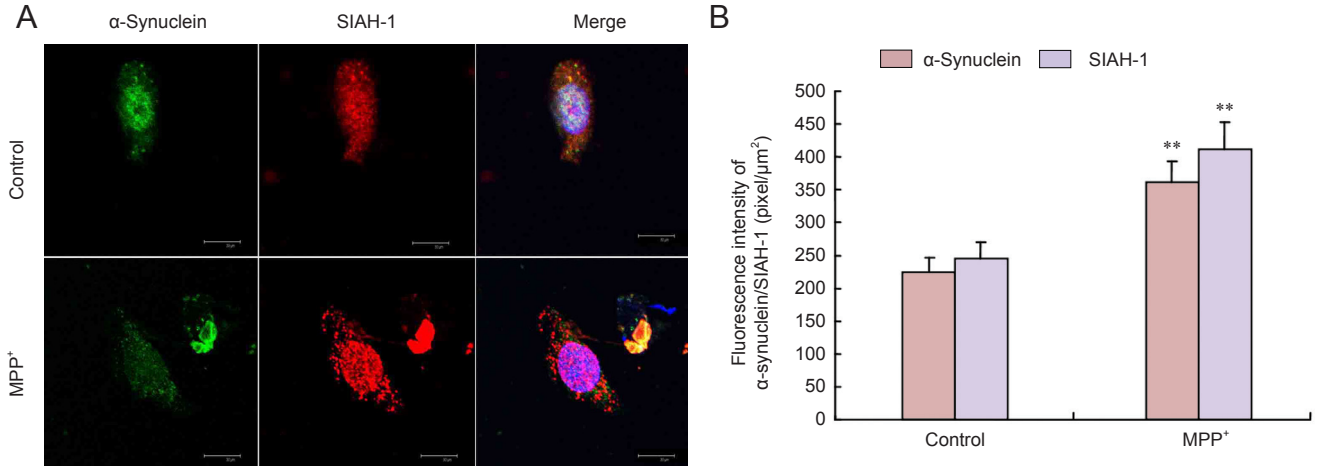

Figure 3 Change in α-synuclein and SIAH-1 in PC12 cells in response to MPP$^+$.
(A) Immunolabeling for α-synuclein (green) and SIAH-1 (red) or DAPI staining (blue) in PC12 cells. Image overlays are shown in the third column. MPP$^+$ treatment caused an increase in SIAH-1 and α-synuclein fluorescence intensities (laser scanning confocal microscope; scale bars: 30 μm). (B) Quantification of fluorescence intensities of α-synuclein and SIAH-1. At least 30 cells were included for analysis from five images per group. Values are presented as the mean ± SD (n = 5; one-way analysis of variance followed by Fisher's least significant difference *post hoc* test). **P < 0.01, *vs.* control group (normal growth of cells). SIAH-1: E3 ubiquitin ligase seven in absentia homolog 1; MPP$^+$: 1-methyl-4-phenylpyridinium; DAPI: 4′,6-diamidino-2-phenylindole.

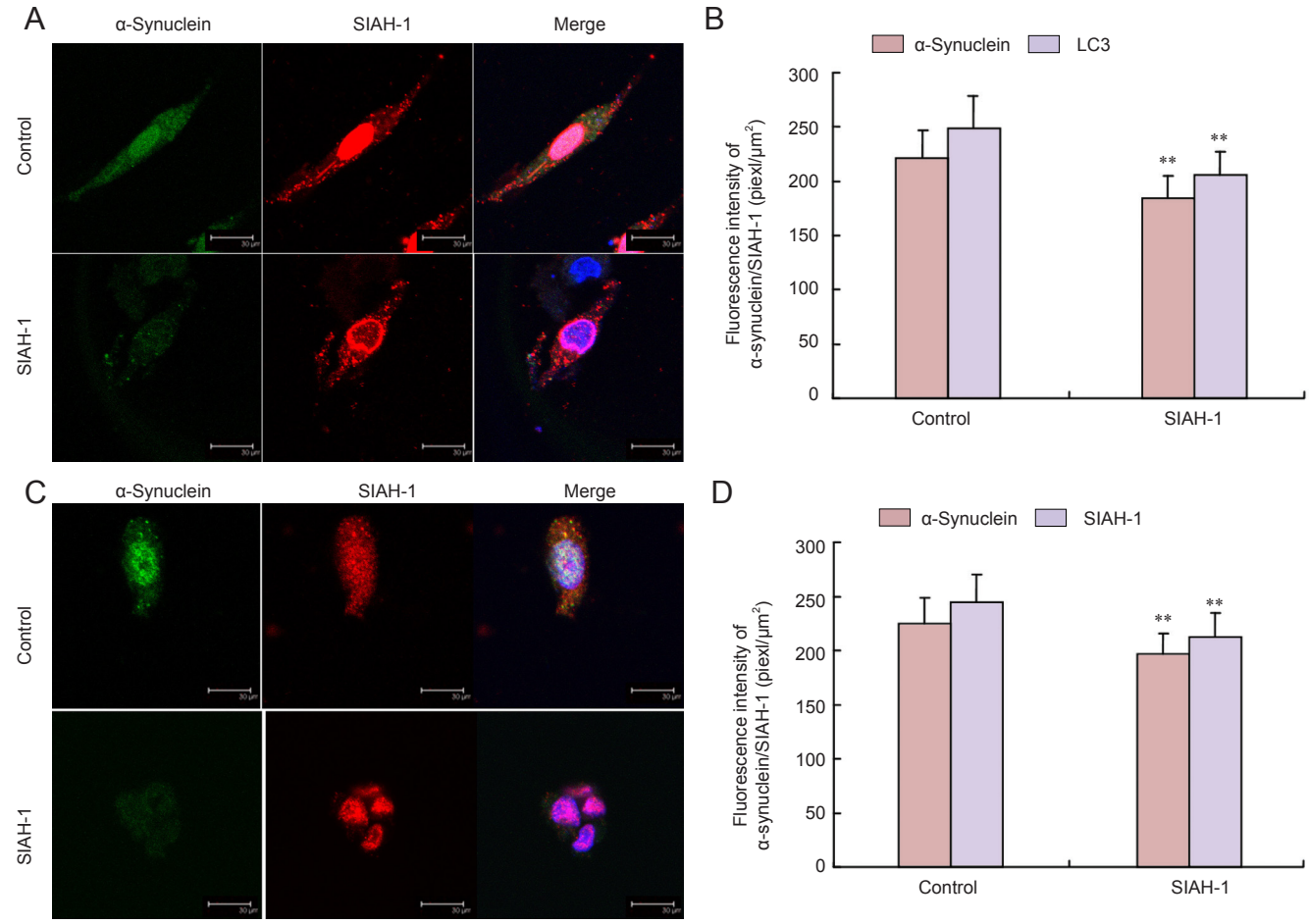

Figure 4 Colocalization of α-synuclein, SIAH-1 and microtubule-associated protein LC3 in PC12 cells after SIAH-1 antibody treatment.
(A, C) Laser scanning confocal microscope; scale bars: 30 μm. Immunoreactivity for α-synuclein, SIAH-1 and LC3 decreased after treatment with SIAH-1 antibody, and colocalization of α-synuclein and LC3 was lost, but α-synuclein and SIAH-1 retained their colocalization. (A) Immunostaining for α-synuclein (green), microtubule-associated protein LC3 (red) and DAPI (blue). (B) Quantification of immunostaining results in A. (C) Immunostaining for α-synuclein (green), SIAH-1 (red) and DAPI (blue). (D) Quantification of immunostaining results in C. Values are presented as the mean ± SD (n = 5; one-way analysis of variance followed by Fisher's least significant difference *post hoc* test). **P < 0.01, *vs.* control group (normal growth of cells). SIAH-1: E3 ubiquitin ligase seven in absentia homolog 1; LC3: light chain 3; DAPI: 4′,6-diamidino-2-phenylindole.

at a single lysine, promoting aggregation and cytotoxicity (Engelender, 2008; Rott et al., 2008). Our results show that treatment with SIAH-1 antibody reduces α-synuclein levels in normal and MPP^+-treated cells. In addition, our immunofluorescence results revealed that α-synuclein aggregates were significantly reduced as well by the antibody. However, the mechanisms by which SIAH-1 antibody reduces α-synuclein levels and plays a protective role in cells remain to be elucidated.

In summary, in this study, we found that MPP^+ increases SIAH activity and impairs the clearance of α-synuclein by inducing the upregulation and aggregation of α-synuclein. Co-treatment with SIAH-1 antibody clearly had neuroprotective effects. Our findings suggest that SIAH-1 plays a key role in the pathogenesis of Parkinson's disease. Furthermore, SIAH-1 may be a potential new therapeutic target for neurodegenerative diseases characterized by α-synuclein aggregates.

Acknowledgments: *We are very grateful to the staff from Cyrus Tang Hematology Center and the Institute of Neuroscience at Soochow University in China for their helps. We also thank Dr. Austin Cape for careful reading and insightful suggestions.*

Author contributions: *ZLC, FPW and XML designed this study. JX, SRX and YYL performed experiments. YJZ, XZZ and XW analyzed the data. ZLC and JX wrote the paper. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

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Copyedited by Patel B, Hindle A, Wang J, Qiu Y, Li CH, Song LP, Zhao M