



Activation of $\alpha 7$ -nAChRs Promotes the Clearance of α -Synuclein and Protects Against Apoptotic Cell Death Induced by Exogenous α -Synuclein Fibrils

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Misfolding and abnormal aggregation of α -synuclein (α Syn) have been shown to increase the risk of developing Parkinson's disease (PD). Finding some way to reduce the aggregation of α Syn is particularly important for the treatment of PD. The main route in prion-like α Syn spreading is the cholinergic innervated vagus nervous system and central cholinergic neurons. Since the degenerative changes and death of cholinergic neurons also run through the pathological process of PD, we hypothesize an involvement of the cholinergic system in α Syn aggregation. The $\alpha 7$ nicotinic acetylcholine receptors ($\alpha 7$ -nAChRs) are one of the most abundant nAChRs in the mammalian brain. Using nicotine and a selective $\alpha 7$ -nAChRs agonist PNU-282987, we found a protective effect of $\alpha 7$ -nAChRs on the cell damage induced by α Syn-PFF (preformed fibrils) through inhibiting apoptotic cell death. We further discovered an additive effect of $\alpha 7$ -nAChRs on the clearance of α Syn in normal and α Syn stably transduced SH-SY5Y cells. Moreover, using $\alpha 7$ -nAChRs knockout mice, we noticed that $\alpha 7$ -nAChRs deficiency increased the deposition of α Syn and aggravated the loss of dopaminergic neurons in a chronic MPTP mouse model of PD. Our findings for the first time indicated that $\alpha 7$ -nAChRs activation exhibited a neuroprotective effect on α Syn pathology and aggregation by promoting the clearance of α Syn.

Keywords: α -synuclein, apoptosis, Parkinson's disease, PNU-282987, nicotinic acetylcholine receptor

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's Disease (Nussbaum and Ellis, 2003). This disease is more common in the elderly, and the prevalence increases with age. As previously reported (Raza et al., 2019), about 1–3% of the population over the age of 65, and its number is going to rise from 8.7 to 9.3 million by 2030. The typical clinical manifestations of PD are motor symptoms (resting tremor, slow movement, rigidity, difficulty walking, and gait) and non-motor symptoms (autonomic disorders, smell, vision, sleep, cognition, and affective disorders) (Meissner et al., 2011; Rascol et al., 2011; Schapira and Jenner, 2011).

The pathological characteristics of PD are the progressive loss of dopamine neurons in the substantia nigra and the appearance of Lewy bodies (LBs) and Lewy neurites (LNs) in neurons (Dickson, 2018). LBs and LNs are intraneuronal inclusions composed of α -synuclein (α Syn) aggregates. The presence of α Syn in cytoplasmic inclusions represents aberrant cytologic localization since α Syn is a natively unfolded protein enriched in presynaptic terminals and plays a role in synaptic vesicle release (Burré et al., 2010). Numerous studies have shown that α Syn misfolding and abnormal aggregation can cause severe damage to neurons and other cells, leading to mitochondrial damage (Devi et al., 2008; Luth et al., 2014), endoplasmic reticulum stress (Colla et al., 2012), synaptic dysfunction (Huang et al., 2019), autophagy, and lysosomal dysfunction (Nguyen et al., 2019). While misfolding and abnormal aggregation of α Syn is the pathological mechanisms of PD, finding some way to reduce the aggregation of α Syn is particularly important for the treatment of PD.

Many studies recently indicate that α Syn is transported in the long-distance and bi-directional gut-brain axis (Kim et al., 2019). This main transport route is the cholinergic innervated vagus nervous system and central cholinergic neurons (Travagli et al., 2020). In the distal gut, α Syn is selectively expressed in most of the intestinal cholinergic axons. Moreover, in addition to the progressive death of dopaminergic neurons, the degenerative changes and death of cholinergic neurons also run through the pathological process of PD (Perez-Lloret et al., 2016). These findings suggest an involvement of the cholinergic system in α Syn aggregation and transmission.

In the central nervous systems, cholinergic neurons and nicotinic acetylcholine receptors (nAChRs) participate in diverse functions. A large number of epidemiological studies showed a lower incidence of PD in tobacco smokers (Hernán et al., 2002; Thacker et al., 2007; Ascherio and Schwarzschild, 2016). Nicotine, a key ingredient in tobacco products, has neuroprotective effects of dopaminergic neuronal loss in PD models via acting on nAChRs (Quik et al., 2014; Perez, 2015). Among these nAChRs, α 7-nAChRs are one of the most abundant nAChRs (Quik et al., 2015). Our previous studies found that α 7-nAChRs activation has a protective effect on cell damage caused by 1-methyl-4-phenylpyridinium (MPP⁺) (Xu et al., 2019). However, the potential roles of α 7-nAChRs in the cellular toxicity of α Syn aggregates have remained unknown.

In the present study, we prepared α Syn-PFF (preformed fibrils) formed from purified recombinant human wild-type α Syn and constructed SH-SY5Y cells overexpressing human wild-type α Syn. Since SH-SY5Y cells express endogenous α 7-nAChRs subtypes (Peng et al., 1997), we used a non-selective nAChR agonist nicotine and a selective α 7-nAChR agonist PNU-282987 to test whether their addition to SH-SY5Y cells challenged with α Syn-PFF could afford neuroprotection. We found that α 7-nAChRs activation protected against apoptosis induced by α Syn-PFF and reduced the levels of α Syn in normal SH-SY5Y cells and stably transduced cells. Furthermore, using α 7-nAChRs knockout (KO) mice, we discovered that α 7-nAChRs deficiency increased the deposition of α Syn and aggravated the loss of dopaminergic neurons in a chronic regimen

of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and probenecid (MPTP/p). Our findings for the first time indicate that α 7-nAChRs activation can reduce α -synucleinopathies by promoting the clearance of α Syn.

MATERIALS AND METHODS

Reagents

The ToxinEraser™ Endotoxin Removal Kit (#L00338) and ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (#L00350C) were purchased from GenScript (Piscataway, NJ, USA). MPTP, MPP⁺, nicotine, PNU-282987, methyllycaconitine citrate (MLA), puromycin, probenecid, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), JC-1, and TH (Tyrosine hydroxylase) antibody (#T1299) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The cOmplete™ EDTA-free protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail were purchased from Roche Applied Science (Indianapolis, IN, USA). LDH detection kit was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Hoechst 33342 was purchased from AAT Bioquest (Sunnyvale, CA, USA). Annexin V-FITC apoptosis detection kit was purchased from Vazyme (Nanjing, China). Nissl staining kit was purchased from Keygen Biotech (Nanjing, China). Antibodies of Bcl-2 (#3498), Bax (#2772), and cleaved caspase-3 (#14220) were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). α Syn antibody (#610787) was purchased from BD Biosciences (San Jose, CA, USA). Monoclonal anti phosphorylated α Syn antibody (pSyn#64; 015-25191) was purchased from Wako Pure Chemical Industries (Osaka, Japan). β -actin antibody (YFMA0052) was purchased from Yifeixue Biotech (Nanjing, China). 3,3'-diaminobenzidine (DAB) kit was purchased from Beyotime (Shanghai, China).

Purification of Recombinant Human Wild-Type α Syn and Preparation of α Syn PFF

Recombinant human wild-type α Syn was prepared and purified as previously described (Hua et al., 2017). The expression of monomeric α Syn-His was confirmed by Western analysis (**Supplementary Figure 1A**). According to the manufacturer's instruction, bacterial endotoxins were removed using the ToxinEraser™ Endotoxin Removal Kit. The endotoxin level was determined using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit. Endotoxin-free α Syn contains less than 0.12 EU/ml of endotoxin. The concentration of monomeric α Syn was prepared above to 5 mg/ml with sterile phosphate buffer saline (PBS) and shook at 37°C, 1,000 rpm for seven days. The liquid containing the α Syn monomers appeared turbid after fibril formation (**Supplementary Figure 1B**). Sonicated α Syn-PFF was prepared via sonicating six times before each experiment (total 10 s, 1 s on, 1 s off). Thioflavin T (ThT) assay (**Supplementary Figure 1C**), SDS-PAGE (**Supplementary Figure 1D**), and electron microscope (**Supplementary Figure 1E**) were used to identify α Syn morphologies before subsequent experiments.

The majority of sonicated α Syn-PFF was less than 50 nm (Supplementary Figure 1F). The average length of α Syn-PFF was 41.96 ± 0.80 nm.

Cell Culture and Treatment

Human SH-SY5Y neuroblastoma cells were cultured as previously described (Hua et al., 2017). The medium was replaced with a serum-free medium containing PNU-282987 or nicotine. After one hour, $1 \mu\text{M}$ α Syn-PFF or $500 \mu\text{M}$ MPP⁺ was added into the medium for 24 h. The selective α 7-nAChRs antagonist MLA was added one hour before the addition of PNU-282987 or nicotine.

MTT Assay and Lactate Dehydrogenase (LDH) Assays

The MTT assay and LDH-based cytotoxicity assays were used to measure cell death and survival, as previously described (Hua et al., 2017).

Hoechst 33342 Staining

After washing with PBS three times and fixed in 4% paraformaldehyde for 30 min, SH-SY5Y cells were stained with Hoechst 33342 ($10 \mu\text{g}/\text{mL}$, 20 min). The cells with highly-condensed and brightly-stained nuclei under a fluorescence microscope (Zeiss AX10; Carl Zeiss Microscopy GmbH, Jena, Germany) were regarded as apoptotic cells. The apoptotic index was defined as the ratio of the apoptotic cell number to the total cell number.

Flow Cytometry

According to the manufacturer's instructions, the apoptosis of SH-SY5Y cells was detected by Annexin V-FITC Apoptosis Detection kits. The SH-SY5Y cells treated with different drugs were trypsinized and washed three times with PBS. After centrifugation, the cells were resuspended in $500 \mu\text{l}$ binding buffer with $5 \mu\text{l}$ Annexin V-FITC plus $5 \mu\text{l}$ Propidium Iodide (PI). After mixed gently and reacted for 15 min in the dark at room temperature, samples were analyzed in a flow cytometer (Guava EasyCyte; Millipore, Billerica, MA, USA).

JC-1 Staining

The dissipation of mitochondrial electrochemical potential gradient is an early apoptosis event (Tuszkorn et al., 2019). The digested cells were washed three times with PBS, stained with JC-1 dye solution at 37°C for 30 min, and finally detected the fluorescence intensity of the cells with a Guava EasyCyte flow cytometer to analyze the mitochondrial membrane potential.

Western Blot Analysis

In general, the cells were lysed with RIPA lysis buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1% Nonidet P-40, 1mM sodium orthovanadate) containing protease inhibitors and phosphatase inhibitors, and the proteins were extracted from the cells.

For detergent-solubility fractionation of phosphorylated α Syn (pS129- α Syn), the cells first were lysed with 1% Triton X-100 (TX-100) extraction buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1% Triton X-100) containing protease inhibitors and phosphatase inhibitors, sonicated, and centrifuged at $100,000 \text{g}$

for 30 min. The supernatant was collected as the Triton X-100 - soluble fraction. Then, the pellet was dissolved in 2% SDS RIPA buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1% Nonidet P-40, 1mM sodium orthovanadate, and 1% SDS). The supernatant was collected as the SDS soluble fraction.

The proteins were denatured in SDS sample buffer, separated by 10% SDS-PAGE, and transferred to PVDF membranes. Blots were blocked with 5% skimmed milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h and then washed three times with TBST buffer for 10 min each. The blots were then incubated with the following primary antibodies: Bcl-2 (1:1000), Bax (1:1000), cleaved caspase-3 (1:1000), α Syn (1:500), pS129- α Syn (1:200), or β -actin (1:10000) overnight at 4°C . The membranes were washed four times with TBST for 10 min each time, then incubated the secondary antibody, and washed four times with TBST. Finally, the membrane was observed and analyzed with Tanon 5200 (Tanon Science and Technology Co. Ltd, Shanghai, China).

Construction and Culture of α Syn Stable Transgenic Cell Line

To generate a stable transgenic cell line expressing the human wild-type α Syn, SH-SY5Y cells were transfected with lentivectors pLenti-EF1a-EGFP-F2A-Puro-CMV-SNCA-Myc-His or pLenti-EF1a-EGFP-F2A-Puro-CMV-MCS according to the procedure recommended by the manufacturer (Obio Technology, Shanghai, China). The stable transfectants were selected in $2 \mu\text{g}/\text{ml}$ puromycin and further maintained in $1 \mu\text{g}/\text{ml}$ puromycin added to SH-SY5Y growth media.

Animals and Treatment

Alpha7-nAChRs KO mice (male, 10-12 weeks old, weighing 24-28 g, C57BL/6J background) were purchased from the Jackson Laboratory (B6.129S7-charna7tm1bay, number 003232; Bar Harbor, ME, USA). Wild-type (WT) C57BL/6J mice (male, 10-12 weeks old, weighing 24-28 g) were purchased from the Animal Core Facility of Nanjing Medical University (Nanjing, Jiangsu, China). The mice were fed standard rodent food and water ad libitum. The room temperature was maintained at $24 \pm 2^\circ\text{C}$, and the daily light and dark time were 12 h each. All animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (IACUC).

For the chronic MPTP regimen, KO and WT mice received ten doses of MPTP ($20 \text{mg}/\text{kg}$ in saline, *s.c.*) with probenecid ($250 \text{mg}/\text{kg}$ *i.p.*, twice a week for 5 weeks) as previously described (Sun et al., 2016). The mice in the control group were treated with normal saline and the same dose of probenecid. The mice were sacrificed 3.5 days after the first injection and 7 days after the last injection (42 days after the first injection).

Nissl Staining and Immunohistochemistry

After mice were perfused with 4% paraformaldehyde, brains were dissected out, maintained in 4% paraformaldehyde overnight, and then cryopreserved in 30% sucrose in PBS and stored at -70°C until used. Twenty-micrometer coronal sections were cut and stained with Nissl according to the instructions. The sections were incubated with anti-TH antibody (1:4000) or anti- α Syn antibody (1:200) overnight at 4°C , and then incubated

with HRP conjugated secondary antibodies (1:10000, Beyotime, Shanghai, China) at room temperature for 1 h. Finally, the brain slices were stained in a solution of DAB according to the manufacturer's instructions.

Stereology

As previously reported (Sun et al., 2016), the numbers of Nissl, TH, and α Syn positive cells in the substantia nigra pars compacta (SNpc) were assessed using an unbiased stereological procedure with an optical fractionator (MicroBrightField Inc., Williston, VT). The regions of SNpc in the sections were outlined at low magnification (40 \times). The counting frame size was 40 \times 40 μ m, and the sampling grid size was 80 \times 80 μ m. The sampling scheme was designed to have a coefficient of error (CE) less than 10 % to get reliable results. All stereological analyses were carried out under the 200 \times objective of an Olympus BX52 microscope (Olympus America Inc., Melville, NY).

Statistical Analysis

All data were expressed as mean \pm S.E.M. Statistical analysis was performed by two-way or one-way analysis of variance (ANOVA) followed by Tukey *post-hoc* test using GraphPad Prism software (San Diego, CA, USA). *Post-hoc* power calculations were performed G*Power 3.1.9.7 software with $\alpha = 0.05$. $P < 0.05$ was considered to be statistically significant.

RESULTS

Activation of $\alpha 7$ -nAChRs Alleviated α Syn-PFF-Induced SH-SY5Y Cells Damage

To determine the involvement of $\alpha 7$ -nAChRs in α Syn pathology, we investigated the effects of PNU-282987 and nicotine on α Syn-PFF-induced cell injury. In SH-SY5Y cells, 1 μ M α Syn-PFF produced a reduction (79.8 \pm 0.8 %, $p < 0.001$) in cell proliferation by MPP⁺ used a positive control (76.1 \pm 1.8 %, $p < 0.001$), while 1 μ M PNU-282987 or 1 μ M nicotine alone

did not affect the cell proliferation (Figure 1A). Pretreatment with PNU-282987 (0.01 μ M: 80.2 \pm 1.4 %, $p > 0.999$; 0.1 μ M: 86.5 \pm 1.3 %, $p = 0.0526$; 1 μ M: 93.6 \pm 0.6 %, $p < 0.0001$) or nicotine (0.01 μ M: 86.4 \pm 0.8 %, $p = 0.0573$; 0.1 μ M: 89.8 \pm 1.7 %, $p = 0.0010$; 1 μ M: 95.5 \pm 1.2 %, $p < 0.0001$) significantly enhanced cell viability in a dose-related manner compared to the α Syn-PFF group (Figure 1A). *Post-hoc* analysis indicated that this experiment had a power of 90.6 % to detect a significant difference among the groups.

The increased leakage of LDH from cells into the medium indicates cell death. In SH-SY5Y cells, α Syn-PFF (134.3 \pm 10.2%, $p = 0.0398$) and MPP⁺ (138.6 \pm 5.5%, $p = 0.0145$) increased LDH leakage compared to the control cells, while pretreatment with PNU-282987 (0.01 μ M: 108.4 \pm 6.3 %, $p = 0.2267$; 0.1 μ M: 98.0 \pm 3.2 %, $p = 0.0251$; 1 μ M: 83.7 \pm 10.4 %, $p = 0.0008$) or nicotine (0.01 μ M: 107.2 \pm 2.8 %, $p = 0.1816$; 0.1 μ M: 101.2 \pm 2.1%, $p = 0.0522$; 1 μ M: 88.4 \pm 10.38 %, $p = 0.0022$) attenuated this LDH leakage in a dose-related manner compared to the α Syn-PFF group (Figure 1B). There was no significant difference in the leakage of LDH with 1 μ M PNU-282987 or 1 μ M nicotine alone. *Post-hoc* analysis indicated that this experiment had a power of 81.1 % to detect a significant difference among the groups.

These results showed that $\alpha 7$ -nAChRs activation could alleviate the α Syn-PFF-induced damage of SH-SY5Y cells.

Activation of $\alpha 7$ -nAChRs Reversed α Syn-PFF-Induced Apoptosis in SH-SY5Y Cells

Since apoptosis plays a vital role in the neurotoxicity of α Syn, we calculated the α Syn-PFF-induced apoptotic changes with PNU-282987 and nicotine. A flow cytometric analysis for apoptosis indicated that 1 μ M α Syn-PFF caused significant apoptosis

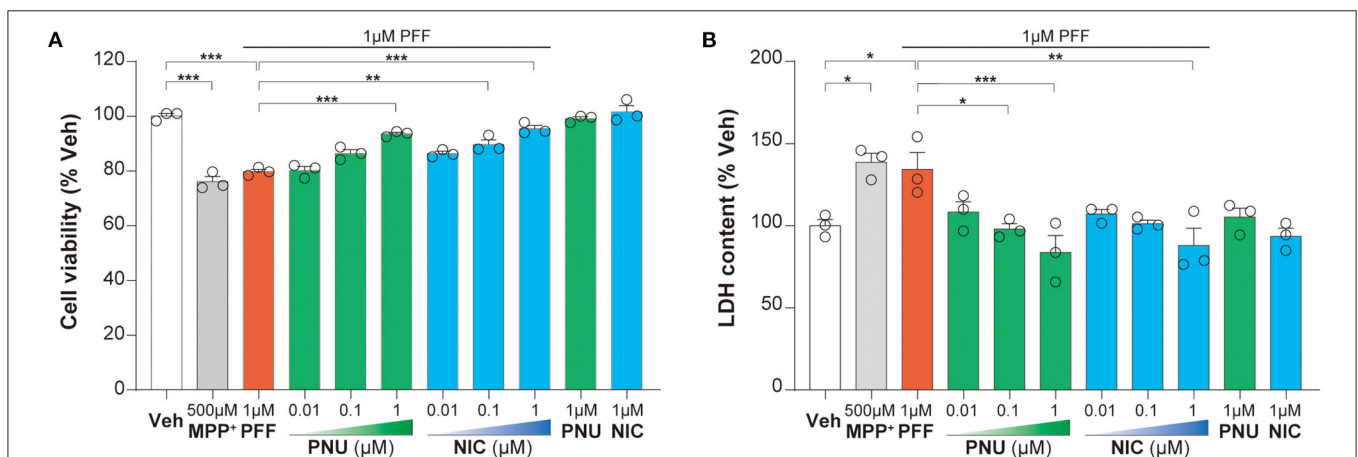
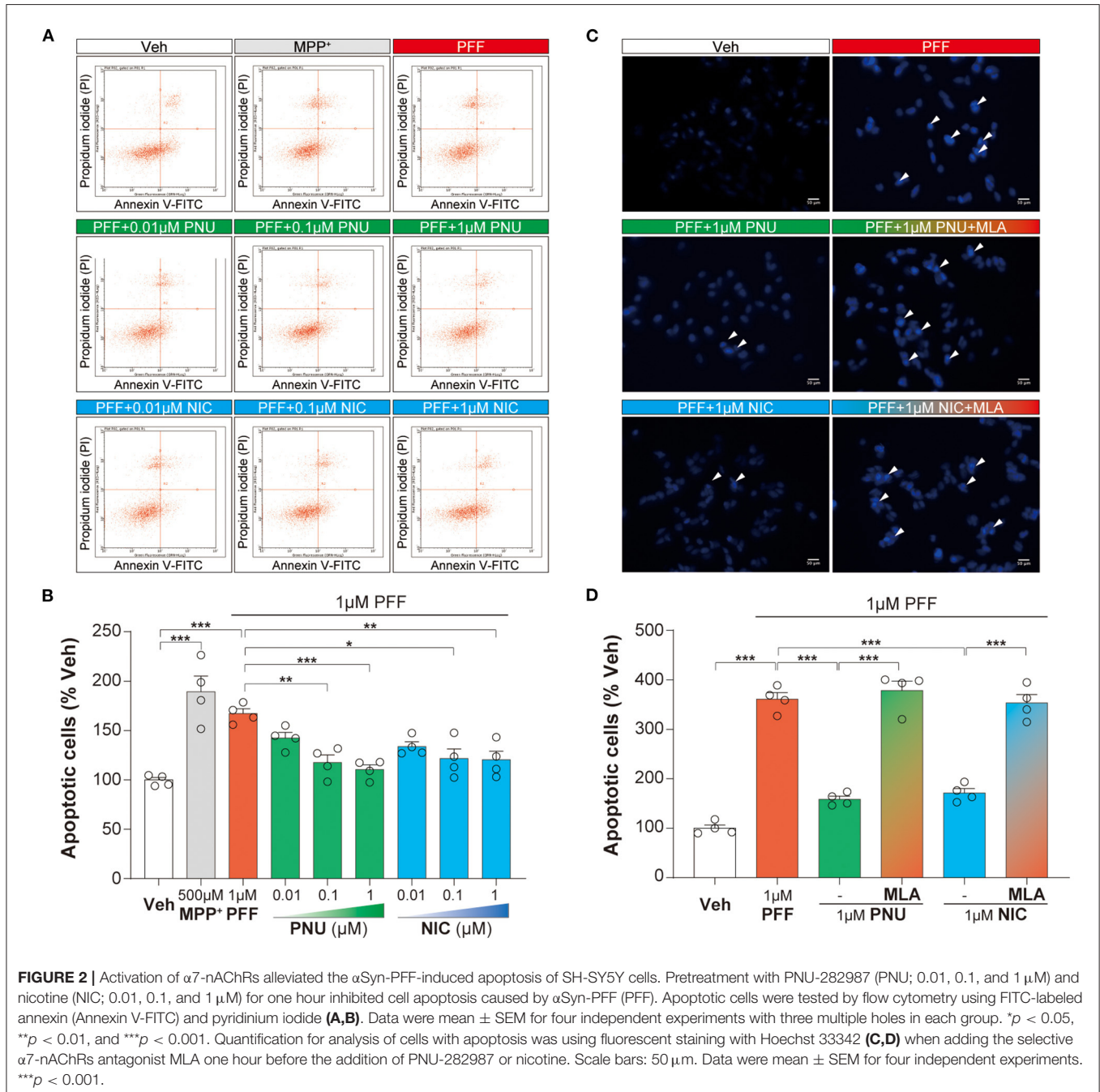


FIGURE 1 | Activation of $\alpha 7$ -nAChRs alleviated the α Syn-PFF-induced damage of SH-SY5Y cells. Pretreatment with PNU-282987 (PNU; 0.01, 0.1, and 1 μ M) and nicotine (NIC; 0.01, 0.1, and 1 μ M) for one hour protected against cell damage caused by α Syn-PFF (PFF). Cell viability was measured using the MTT assay (A), whereas the level of cell death was determined by LDH assay (B). Data were mean \pm SEM for three independent experiments with three multiple holes in each group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

($167.1 \pm 4.8 \%$, $p < 0.0001$), which was reversed in a dose-related manner by pretreated with PNU-282987 (0.01 μM : $142.4 \pm 5.6 \%$, $p = 0.4403$; 0.1 μM : $117.7 \pm 7.5 \%$, $p = 0.0044$; 1 μM : $110.5 \pm 4.8 \%$, $p = 0.0009$) or nicotine (0.01 μM : $133.8 \pm 4.7 \%$, $p = 0.1207$; 0.1 μM : $121.6 \pm 9.7 \%$, $p = 0.0105$; 1 μM : $120.3 \pm 8.8 \%$, $p = 0.0078$, **Figures 2A,B**). *Post-hoc* analysis indicated that this experiment had a power of 91.9 % to detect a significant difference among the groups. Then, 1 μM PNU-282987 and 1 μM nicotine were chosen for the following experiment.

Similarly, Hoechst 33342 staining assay revealed that 1 μM $\alpha\text{Syn-PFF}$ increased the rate of apoptotic cells ($361.1 \pm 12.9 \%$, $p < 0.0001$) compared to the control group. When pretreated with 1 μM PNU-282987 ($158.6 \pm 6.3 \%$, $p < 0.0001$) and 1 μM nicotine ($171.4 \pm 8.9 \%$, $p < 0.0001$), the rate of apoptotic cells was significantly reduced (**Figures 2C,D**). These anti-apoptotic effects of $\alpha 7$ -nAChRs activation were expectedly reversed by pretreatment with 100 nM MLA (PNU-282987 + MLA: $378.1 \pm 19.3 \%$, $p < 0.0001$; nicotine + MLA: $353.2 \pm 17.1 \%$, $p < 0.0001$; **Figures 2C,D**). *Post-hoc* analysis indicated that

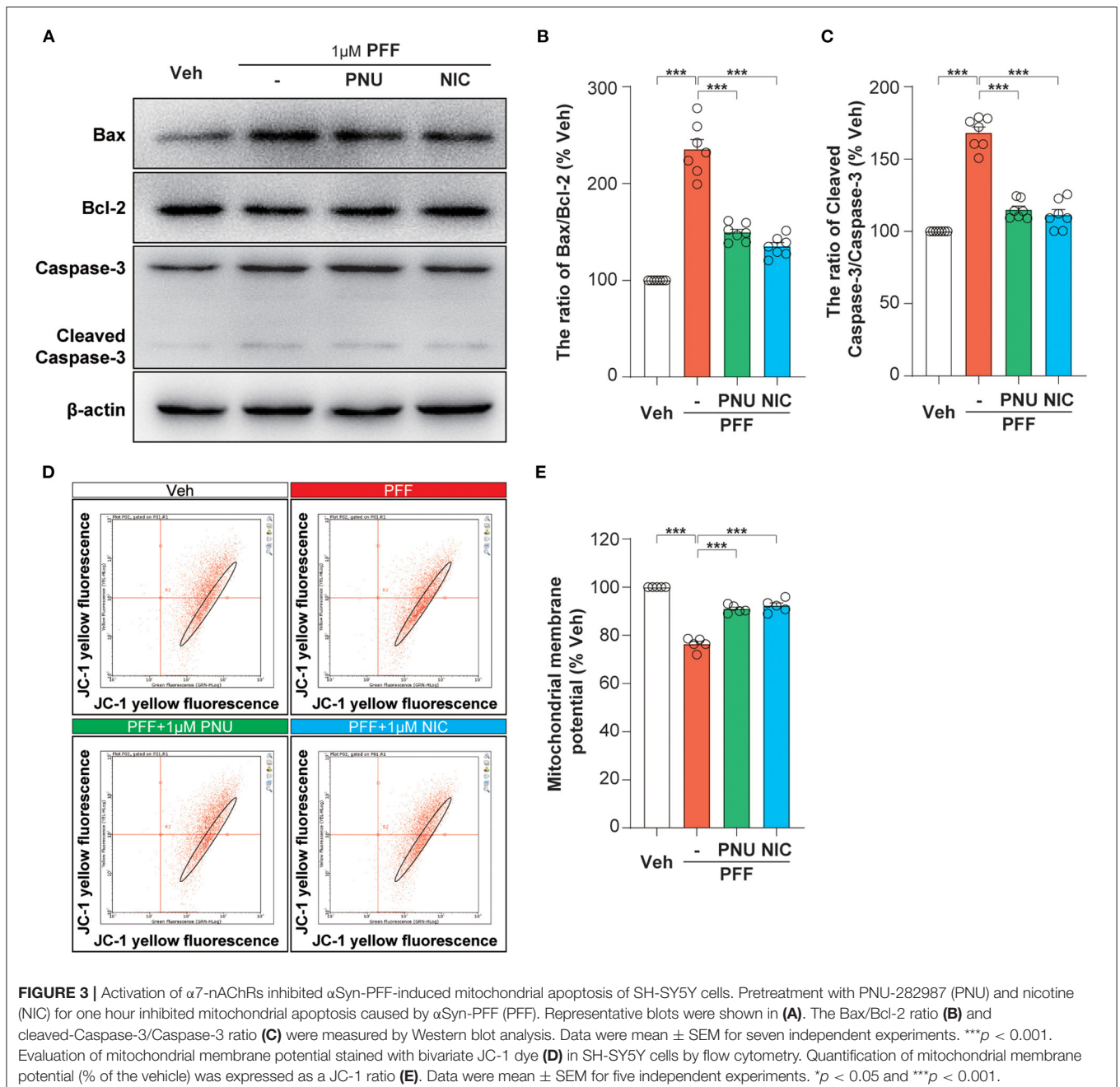


this experiment had a power of 83.1 % to detect a significant difference among the groups. These findings confirmed that $\alpha 7$ -nAChRs activation produced anti-apoptotic effects on α Syn-PFF-induced neurotoxicity.

Activation of $\alpha 7$ -nAChRs Inhibited α Syn-PFF-Induced Mitochondrial Apoptosis in SH-SY5Y Cells

To examine the expression levels of apoptosis-associated proteins exposed to α Syn-PFF, we observed the levels of Bax, Bcl-2, and cleaved-caspase-3 analyzed by Western blot assay (Figure 3A). The treatment of α Syn-PFF stimulated the ratio

of Bax/Bcl-2 (235.3 ± 10.2 %, $p < 0.0001$, Figure 3B) and the expression of cleaved-caspase-3 (168.1 ± 4.1 %, $p < 0.0001$, Figure 3C), compared to the control group. Pretreatment with PNU-282987 or nicotine inhibited the α Syn-PFF-induced alterations of Bax/Bcl-2 ratio (PNU-282987: 149.4 ± 3.4 %, $p < 0.0001$; nicotine: 135.0 ± 3.9 %, $p < 0.0001$; Figure 3B) and cleaved-caspase-3 (PNU-282987: 114.9 ± 2.5 %, $p < 0.0001$; nicotine: 111.4 ± 3.7 %, $p < 0.0001$; Figure 3C). *Post-hoc* analysis indicated that these experiments had a power of 86.2 % for Bax/Bcl-2 ratio and a power of 73.0 % for cleaved-caspase-3 to detect a significant difference among the groups.



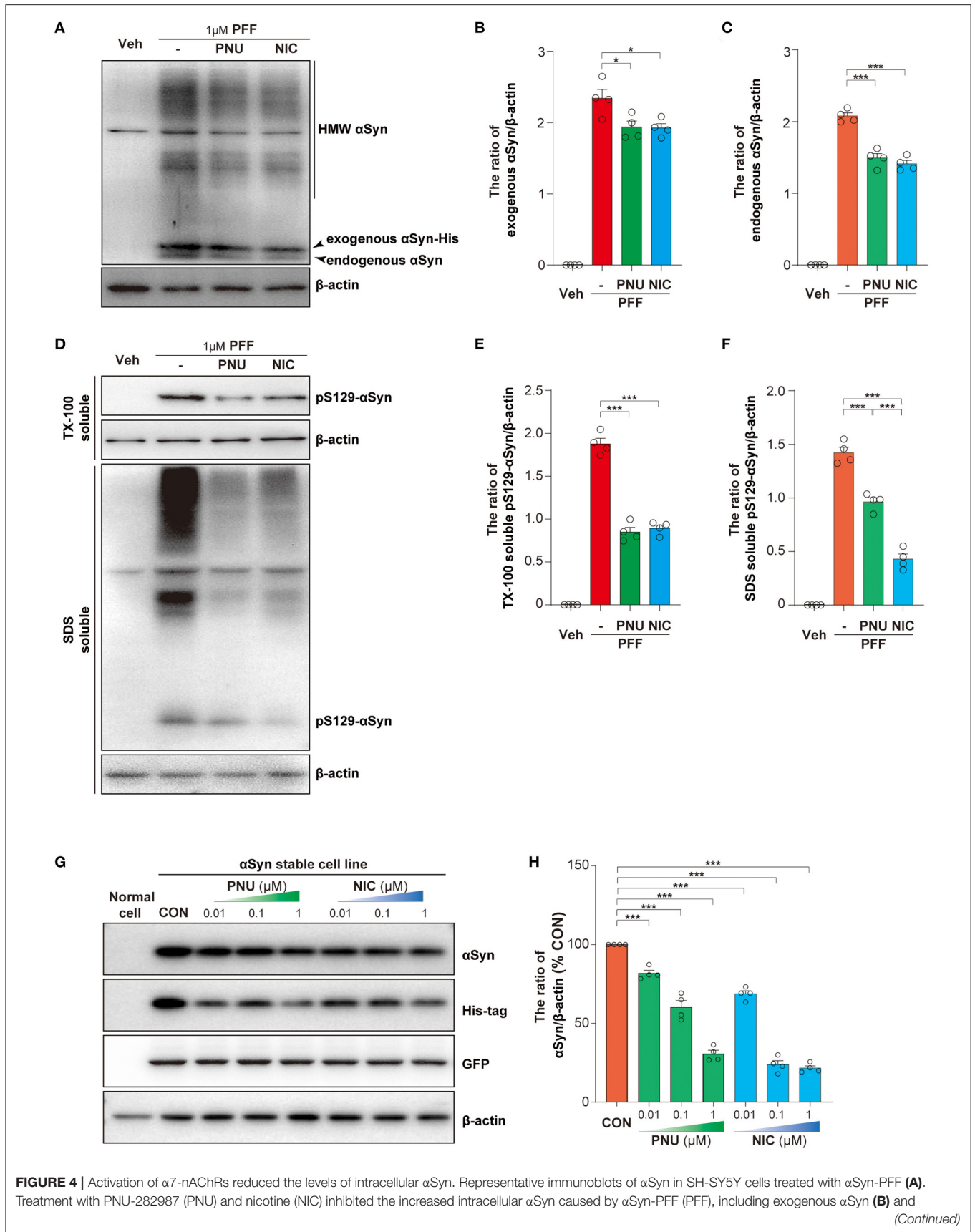


FIGURE 4 | endogenous α Syn (C). Representative immunoblots of phosphorylated α Syn (pS129- α Syn) in SH-SY5Y cells treated with α Syn-PFF (D). Treatment with PNU-282987 (PNU) and nicotine (NIC) inhibited the phosphorylation of α Syn caused by α Syn-PFF (PFF), including in the Triton X-100 (TX-100)-soluble fraction (E) and the SDS-soluble fraction (F). Representative immunoblots of α Syn, His-tag, and GFP after the treatment of PNU or NIC (G). Quantification of α Syn (% of the non-treated stable cell line) was expressed as an α Syn/ β -actin ratio (H). Data were mean \pm SEM for four independent experiments. * $p < 0.05$ and *** $p < 0.001$.

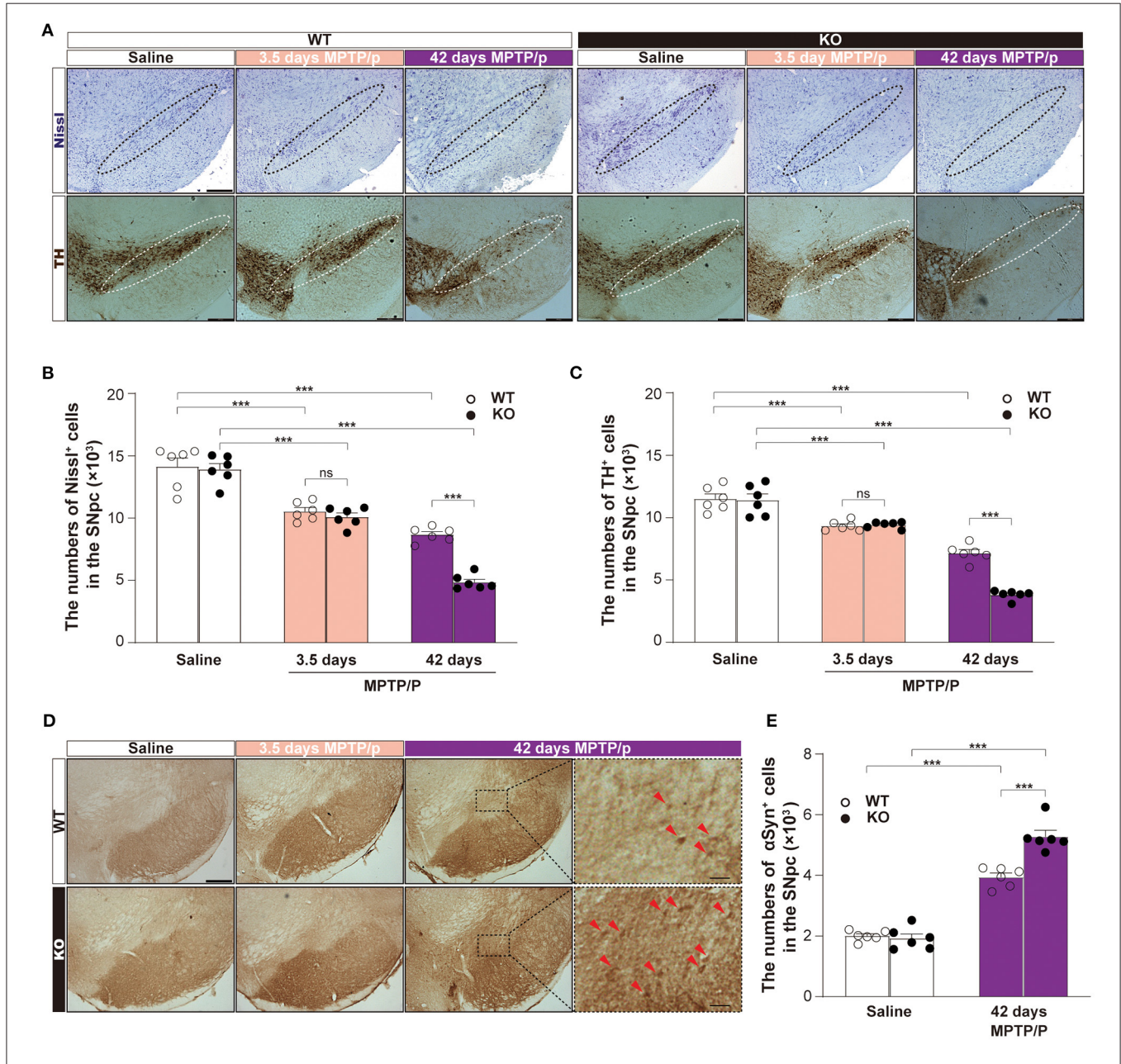


FIGURE 5 | $\alpha 7$ -nAChRs deficiency aggravated the loss of dopamine neurons and increased the deposition of α Syn in a chronic MPTP mouse model of PD. Representative pictures for Nissl-positive staining and TH-positive staining (A) were shown. Scale bar, 250 μ m. Nonbiased stereological analysis of Nissl-positive staining cells (B) and TH-positive staining (C) in the SNpc from saline- and MPTP/p-treated $\alpha 7$ -nAChRs knockout (KO) and wild-type (WT) mice. Data were mean \pm SEM obtained with six mice of each group. *** $p < 0.001$. Representative pictures for α Syn-positive staining cells in the midbrain (D). Scale bar, 250 μ m. Forty-two days MPTP/p enlarged show the enlargement of α Syn-positive staining cells (red arrow) in SNpc. Scale bar, 50 μ m. Nonbiased stereological analysis (E) of α Syn-positive staining cells in the SNpc from saline- and MPTP/p-treated $\alpha 7$ -nAChRs KO and WT mice. Data were mean \pm SEM obtained with six mice of each group. *** $p < 0.001$. MPTP/p: 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine/probenecid; SNpc, substantia nigra pars compacta.

Bcl-2 and Bax are potent regulators of apoptosis via regulating the permeability of the mitochondrial membrane. The α Syn-PFF-induced alteration of the mitochondrial membrane potential was further determined by the molecular probe JC-1 (Figure 3D). Bivariate analysis of mitochondrial membrane potential showed that a loss of the mitochondrial membrane potential ($76.3 \pm 1.2\%$, $p < 0.0001$) in α Syn-PFF-treated cells was alleviated by the pretreatment with PNU-282987 ($90.9 \pm 0.7\%$, $p < 0.0001$) or nicotine ($92.3 \pm 1.2\%$, $p < 0.0001$; Figure 3E). *Post-hoc* analysis indicated that these experiments had a power of 90.3% to detect a significant difference among the groups. We conclude that $\alpha 7$ -nAChR stimulation contributes to the limitation of the mitochondrial pathway of apoptosis induced by α Syn-PFF.

Activation of $\alpha 7$ -nAChRs Suppressed the Accumulation of α Syn in SH-SY5Y Cells and Stable Transgenic Cell Line

To further explore the protective mechanism of $\alpha 7$ -nAChRs on cell apoptosis, we first calculated the levels of intracellular α Syn. As previously reported (Luk et al., 2009; Apetri et al., 2016), α Syn-PFF exogenously added was taken up by SH-SY5Y cells and then induced the increase of endogenous α Syn (Figure 4A). Interestingly, compared with α Syn-PFF alone (exogenous: 2.34 ± 0.12 ; endogenous: 2.08 ± 0.04), the pretreatment of PNU-282987 or nicotine led to fewer amounts of HMW exogenous α Syn with a concomitant reduction of both monomeric exogenous α Syn (PNU-282987: 1.94 ± 0.08 , $p = 0.0185$; nicotine: 1.93 ± 0.06 , $p = 0.0149$; Figure 4B) and endogenous α Syn (PNU-282987: 1.49 ± 0.06 , $p < 0.0001$; nicotine: 1.41 ± 0.05 , $p < 0.0001$; Figure 4C). *Post-hoc* analysis indicated that these experiments had a power of 78.8% for exogenous α Syn and 78.6% for endogenous α Syn to detect a significant difference among the groups.

Phosphorylation of α Syn at serine-129 is thought to be a marker of α Syn pathology. As showed in Figure 4D, α Syn-PFF exogenously induced the increase of pS129- α Syn in the TX-100-soluble fraction (1.88 ± 0.06) and the SDS-soluble fraction (1.42 ± 0.05). The pretreatment of PNU-282987 or nicotine inhibited the phosphorylation of α Syn in the TX-100-soluble fraction (PNU-282987: 0.85 ± 0.05 , $p < 0.0001$; nicotine: 0.89 ± 0.04 , $p < 0.0001$; Figure 4E) and the SDS-soluble fraction (PNU-282987: 0.96 ± 0.04 , $p < 0.0001$; nicotine: 0.43 ± 0.05 , $p < 0.0001$; Figure 4F). *Post-hoc* analysis indicated that these experiments had a power of 78.9% for α Syn in the TX-100-soluble fraction and 78.8% for α Syn in the SDS-soluble fraction to detect a significant difference among the groups.

We next established an α Syn stable transgenic cell line and examined whether $\alpha 7$ -nAChRs activation promoted the clearance of α Syn species. The stable transgenic cell line containing a non-fusion GFP and a C-terminal Myc-His-tag was confirmed by Western blotting analysis (Supplementary Figure 2A) and immunofluorescence (Supplementary Figure 2B). We confirmed that $1 \mu\text{M}$ PNU-282987 (Supplementary Figure 2C) or $1 \mu\text{M}$ nicotine (Supplementary Figure 2D) alone did not affect cell proliferation for 24 h or 48 h of treatment. Both PNU-282987 and nicotine significantly reduced α Syn and His-tag expressions,

not GFP expression (Figure 4G). Compared with non-treated stable transgenic cell line, PNU-282987 ($0.01 \mu\text{M}$: $81.8 \pm 1.9\%$, $p = 0.002$; $0.1 \mu\text{M}$: $60.5 \pm 4.0\%$, $p < 0.0001$; $1 \mu\text{M}$: $30.6 \pm 2.3\%$, $p < 0.0001$) and nicotine ($0.01 \mu\text{M}$: $68.8 \pm 2.0\%$, $p < 0.0001$; $0.1 \mu\text{M}$: $23.8 \pm 2.5\%$, $p < 0.0001$; $1 \mu\text{M}$: $21.6 \pm 1.5\%$, $p < 0.0001$) reduced α Syn expressions in a dose-dependent manner (Figure 4H). *Post-hoc* analysis indicated that these experiments had a power of 93.8% for α Syn expressions to detect a significant difference among the groups. These results indicate that $\alpha 7$ -nAChRs activation may directly accelerate the clearance of α Syn.

$\alpha 7$ -nAChRs Deficiency Aggravated the Loss of Dopamine Neurons and Increased the Deposition of α Syn in a Chronic MPTP Mouse Model of PD

To confirm the potential role of $\alpha 7$ -nAChRs in α Syn pathology and clearance *in vivo*, we established the chronic MPTP mouse models of PD using $\alpha 7$ -nAChRs KO mice and WT mice. According to the immunohistochemical staining of the midbrain (Figure 5A) and stereological counting, there was no difference in the numbers of Nissl-positive neurons (KO: 13905 ± 463 , WT: 14132 ± 684 , $p = 0.9717$; Figure 5B) and TH-positive neurons (KO: 11399 ± 501 , WT: 11495 ± 402 , $p = 0.5927$; Figure 5C) in the SNpc between KO and WT mice. However, MPTP treatment markedly decreased the numbers of Nissl-positive neurons [Two-way ANOVA, genotype: $F_{(1,30)} = 20.33$, $p < 0.0001$; Treatment: $F_{(2,30)} = 159.8$, $p < 0.0001$; interaction: $F_{(2,30)} = 12.22$, $p = 0.0001$] and TH-positive neurons [Two-way ANOVA, genotype: $F_{(1,30)} = 20.45$, $p < 0.0001$; Treatment: $F_{(2,30)} = 199.4$, $p < 0.0001$; interaction: $F_{(2,30)} = 20.14$, $p < 0.0001$]. Interestingly, the reduced numbers of Nissl-positive neurons (10542 ± 303 , $p = 0.8255$) and TH-positive neurons (9419 ± 104 , $p = 0.9971$) in the SNpc of KO mice showed no difference than that of WT mice (Nissl: 10093 ± 313 , TH: 9342 ± 159) 3.5 days after the first injection. Then, $\alpha 7$ -nAChRs deficiency aggravated the loss of Nissl-positive neurons (KO: 4857 ± 243 , WT: 8665 ± 240 , $p < 0.0001$) and TH-positive neurons (KO: 3817 ± 150 , WT: 7156 ± 283 , $p < 0.0001$) 42 days after the first injection. Moreover, the chronic MPTP/p treatment increased the deposition of α Syn in the midbrain 42 days after the first injection (Figure 5D). Unlike 3.5 days after the first injection, the mice in 42 days developed α Syn-positive staining in the SNpc. Compared with matched WT mice (3944 ± 132), KO mice showed more α Syn-positive staining (5279 ± 206 , $p < 0.001$) in the SNpc [Two-way ANOVA, genotype: $F_{(1,20)} = 18.15$, $p = 0.0001$; Treatment: $F_{(1,20)} = 325.2$, $p < 0.0001$; interaction: $F_{(1,20)} = 23.34$, $p = 0.0001$; Figure 5E]. These results indicate that $\alpha 7$ -nAChRs play an important role in the process of MPTP-induced midbrain neuron loss via regulating the clearance of α Syn *in vivo*.

DISCUSSION

Numerous studies have shown the neuroprotective effects of nicotine in the PD model through $\alpha 7$ -nAChRs (Kawamata and

Shimohama, 2011; Nicholatos et al., 2018). In particular, *in vitro* and *in vivo* studies have shown that nicotine can strongly prevent neurotoxicity induced by selective toxic agents such as rotenone, 6-OHDA, and MPTP by activating α 7-nAChRs expressed on DA neurons (Kawamata and Shimohama, 2011). Previous studies in our laboratory have also shown that the use of nicotine and PNU-282987 can inhibit MPP⁺-induced apoptosis of SH-SY5Y cells through the ERK/p53 signaling pathway (Xu et al., 2019). Here, using the toxic α Syn-PFF to model SH-SY5Y cells, we at first revealed that α 7-nAChRs activation could significantly reduce the cytotoxicity of α Syn oligomeric species by inhibiting the mitochondrial apoptosis pathway. Then, we found an additive effect of α 7-nAChRs on the clearance of α Syn *in vitro* and *in vivo*. Thus, we demonstrated for the first time that α 7-nAChRs exhibited a neuroprotective effect on α Syn pathology and aggregation via promoting α Syn clearance.

Pathological α Syn is reported to trigger apoptosis in various cell types, especially mitochondria-dependent apoptosis (Yasuda and Mochizuki, 2010; Yasuda et al., 2013). Recent studies suggest that α Syn oligomers can localize to mitochondria, interact with ATP synthase, and open the mitochondria permeability transition pore (mPTP) (Ludtmann et al., 2018). The mPTP opening results in the depolarization of mitochondrial membrane potential and the activation of caspase-3, leading to apoptotic death. In agreement with these previous reports, our findings demonstrated increased cellular toxicity in SH-SY5Y cells upon treatment with extracellular α Syn-PFF. We previously documented an anti-apoptotic role of α 7-nAChRs via regulating the ERK/p53 signaling pathway in MPP⁺-induced cell death models (Xu et al., 2019). Similarly, the α 7-nAChRs agonist PNU-282987 and nicotine reduced apoptotic cell death induced by α Syn-PFF, indicating the α 7-nAChRs-mediated neuroprotection both in MPP⁺- and α Syn-induced PD models. Activation of α 7-nAChRs can inhibit the increased Bax/Bcl-2 ratio and cleaved-caspase-3, maintain mitochondrial membrane potential, and prevent α Syn-PFF-induced apoptosis. Thus, our findings confirm that α 7-nAChRs are effective targets for protection against cell damage in the PD model. However, the protective mechanisms of α 7-nAChRs on α Syn-PFF-induced apoptosis have not been elucidated.

Numerous studies found that the extracellular α Syn could internalize to the cells and enhance the intracellular formation of α Syn (Luk et al., 2009; Nonaka et al., 2010; Danzer et al., 2012). Pathogenic α Syn containing a mitochondrial targeting sequence is preferentially translocated and binds to mitochondria, inducing mitochondrial dysfunction and apoptosis (Wang et al., 2019). Our study revealed that exogenous α Syn-PFF could not only be taken up and accumulate in SH-SY5Y cells but induced the increase of endogenous α Syn and the phosphorylation of α Syn, which are consistent with findings from previous studies. Interestingly, we found that α 7-nAChRs activation showed the reduction of both exogenous and endogenous α Syn, which was confirmed in α Syn stable transgenic cell line and a mouse model of PD using the chronic MPTP/P treatment. In addition to regulating dopaminergic

neuronal death and apoptosis via MAPK signaling (Xu et al., 2019) or Wnt/ β -catenin signaling pathways (Liu et al., 2017), our results indicate that α 7-nAChRs play an important role in α -synucleinopathy through enhancing the clearance of α Syn.

Since smoking can reduce the incidence of PD, it is proposed whether nicotine affects the oligomerization and aggregation of α Syn. Some researchers found that nicotine *in vitro* could directly inhibit α Syn fibrillation, destabilize preformed α Syn fibrils, and stabilize soluble oligomeric α Syn (Ono et al., 2007; Hong et al., 2009). Using *in vitro* and a yeast model of PD, Kardani et al. (2017) also discovered that nicotine caused α Syn oligomerization inhibition. Recently, Federica Bono et al. (2019) showed that nicotine activated the dopamine D3R-nAChR complex and prevented α Syn accumulation in primary cultures of mouse dopaminergic neurons and human iPSC-derived dopaminergic neurons. However, chronic nicotine administration showed no effect on the levels of α Syn in the SNpc and striatum of Thy1- α Syn mice (Subramaniam et al., 2018). Besides, nicotine has intracellular machinery that governs protein folding, transport and turnover via regulating endoplasmic reticulum and mitochondrial health (Srinivasan et al., 2014). Thus, it is still controversial whether nicotine can modify the production, clearance, or aggregation of α Syn. In the present study, both PNU-282987 and nicotine dose-dependently attenuated the accumulation of α Syn after exposure to exogenous α Syn or overexpression of α Syn. Moreover, α 7-nAChRs deficiency increased the deposition of α Syn in the SNpc and aggravated dopaminergic neurons' loss after the chronic MPTP/p treatment. Previous evidence for the impairment of cholinergic systems in PD patients and animal models, together with the current effect of α 7-nAChRs agonists on α Syn accumulation, supports the hypothesis that α 7-nAChRs is involved in regulating α Syn aggregation. Future studies in the PFF-induced mice model should determine whether chronic treatment of α 7-nAChRs agonists can improve α Syn aggregation via direct activating this receptor.

There are many mechanisms for clearing neurotoxic proteins in the brain, such as the autophagy-lysosomal pathway (Wong and Cuervo, 2010), the chaperone-mediated autophagy (Djajadikerta et al., 2020), and the ubiquitin-proteasome system (Limanaqi et al., 2020). Among these, the ubiquitin-proteasome system is the main degradation pathway of α Syn under normal conditions, while the autophagy-lysosomal pathway is recruited with the increase of α Syn (Ebrahimi-Fakhari et al., 2011). It is interesting to note that, in a recent study using a mice model of inflammatory bowel disease, α 7-nAChRs deficient mice showed an impairment of the autophagic function through reduction of AMPK-mTOR-p70S6K signaling pathway (Shao et al., 2019). Since we observed increased accumulation of α Syn in the SNpc of α 7-nAChRs KO mice, activation of α 7-nAChRs may promote α Syn clearance and degradation by enhancing the ubiquitin-proteasome system or the autophagy-lysosomal pathway. Additionally, we did not rule out the possibility that α 7-nAChRs agonist directly decreased extracellular α Syn-PFF

or blocks α Syn uptake in the present study. Thus, the exact mechanisms of α 7-nAChRs on α Syn clearance are not very clear. They need more investigations, including the effects of α 7-nAChRs on the ubiquitin-proteasome system and the autophagy-lysosomal pathway in the PFF-induced mice model.

CONCLUSION

Our results found that α 7-nAChRs activation protected against apoptosis induced by α Syn-PFF, partially via inhibiting α Syn accumulation and phosphorylation. Although the exact mechanisms of α 7-nAChRs on α Syn clearance are not very clear and need more investigations, our study provides ideas for finding new targets for PD from the perspective of α Syn clearance.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

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AUTHOR CONTRIBUTIONS

YF, JZhang, and YH conceived and planned the experiments. JZhao, SX, and TT carried out cellular experiments. YuL and YaL designed and performed the animal experiment. JZhao and TT performed biochemical analysis. YH and JZhang contributed to the interpretation of the results. JZhang and YF wrote the manuscript. All authors reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2021.637319/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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