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Wnt/β-catenin signaling plays an essential role in α7 nicotinic receptor-mediated neuroprotection of dopaminergic neurons in a mouse Parkinson's disease model

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder with an incidence second only to Alzheimer's disease. The main pathological feature of PD is the death of dopaminergic neurons in the substantia nigra pars compacta. Nicotinic receptor agonists are neuroprotective in several PD models and there is considerable evidence that α7 nicotinic acetylcholine receptors (α7-nAChRs) are important therapeutic targets for neurodegenerative diseases. However, the involvement of α7-nAChRs and underlying signaling mechanisms in PD pathogenesis are unclear. The objective of the present study was to explore the potential functions of α7-nAChRs in PD pathology, and to determine whether these effects are exerted via Wnt/β-catenin signaling in a mouse PD model. In the *in vivo* study, α7-nAChR knockout (α7-KO) reversed the beneficial effects of nicotine on motor deficits, dopaminergic neuron loss, astrocyte and microglia activation, and reduced striatal dopamine release induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Injury to SH-SY5Y cells by 1-methyl-4-phenylpyridinium treatment was also ameliorated by nicotine, and this effect was abolished by methyllycaconitine (MLA), a selective α7-nAChR antagonist, or by siRNA-mediated α7-nAChR knockdown. Furthermore, nicotine increased expression levels of Wnt/β-catenin signaling proteins in the PD mouse model or in the SH-SY5Y cells treated by 1-methyl-4-phenylpyridinium, and these effects were also reversed by MLA or α7-siRNA treatment *in vivo* or *in vitro*. These results suggest that endogenous α7-nAChR mechanisms play a crucial role in a mouse PD model via regulation of Wnt/β-catenin signaling.

1. Introduction

Parkinson's disease (PD) is the most common movement disorder, afflicting approximately 1% of all people over the age of 65 [1]. The motor deficits of PD result from the selective loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc) [2], but the exact pathogenic mechanisms are still unknown. L-DOPA replacement therapy is still the first-line treatment for PD, but it cannot prevent disease progression, and long-term use is associated with many serious adverse reactions. Therefore, etiological and neurobiological studies of PD are needed to identify novel targets for drug development, thereby improving patient quality of life and reducing the burden on families and the healthcare system.

Epidemiological studies show that the risk of PD is significantly lower in smokers than non-smokers [3–5]. Indeed, tobacco use is the most potent environmental factor affecting PD susceptibility [4], with smokers having a 50% lower incidence of PD [6]. A large number of studies suggest that nicotine may be the key to explaining this association, although the detailed mechanisms are not yet clear. Recent studies have shown that nicotine may protect SNpc DAergic neurons by activating nicotinic acetylcholine receptors (nAChRs), ligand-gated ion channels of variable subunit composition. The α-bungarotoxin -sensitive α7-nAChR has attracted widespread attention due possible contributions to neurodegenerative and neuropsychiatric diseases. The α7-nAChR is widely distributed

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in the human brain, with high expression in hippocampus, geniculate nuclei, thalamus, and cortex [7–9], where α7-nAChR activation is implicated in development, neuronal survival, synaptic plasticity, and regulation of neurotransmitter release. Further, α7-nAChRs are implicated in anxiety, learning, memory, and movement, with consequent implications for neurological and neuropsychiatric diseases such as schizophrenia [10], Alzheimer's disease [11], PD [8,12,13], and traumatic brain injury [14].

The Wingless-type MMTV integration site (Wnt) proteins are important mediators of cell-to-cell communication and intracellular signaling associated with CNS development [15]. Recently, several Wnt ligands were shown to regulate the trafficking of α7-nAChRs to the plasma membrane in mature hippocampal neurons in culture [16]. Moreover, Wnt signaling through the Wnt-β-catenin pathway (Wnt1 or Wnt3a) is crucial for several aspects of midbrain DAergic neuron development. Wnt1 may serve a broader array of functions, such as patterning in the midbrain, differentiation of SNpc progenitors, and DAergic neuron survival [17]. However, the involvement of the Wnt-β-catenin pathway in the neuroprotective actions of α7-nAChR remains obscure.

In the present study, we examined possible α7-nAChR-mediated neuroprotection of DAergic neurons via the Wnt-β/catenin pathway in the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated α7-nAChR knockout (α7-KO) mice and 1-methyl-4-phenylpyridinium ion (MPP^+)-treated SH-SY5Y cells, common *in vivo* and *in vitro* models of PD pathogenesis. Our study provides direct evidence that Wnt/β-catenin signaling is a critical effector of α7-nAChR-induced protection of DAergic neurons.

2. Materials and methods

2.1. Reagents and antibodies

MPTP, MPP+, Nicotine, methyllycaconitine (MLA), streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human neuroblastoma SH-SY5Y cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), Opti-MEM, non-essential amino acids (NEAA) and penicillin were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Sciencell (Carlsbad, CA, USA). Protease inhibitor cocktail was purchased from Life Technologies Corp. (Carlsbad, CA, USA). α7-nAChR antibody was purchased from Abcam (Cambridge, MA, USA). Wnt1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). pSer9-GSK3β antibody and β-catenin antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Dopamine transporter (DAT) antibody and β-actin antibody were purchased from Millipore (Millipore, MA, USA). All other drugs were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals

Alpha7-nAChR knockout mice (male, 10–12 weeks old, weigh, 24–28 g, C57BL/6J background) were purchased from the Jackson Laboratory (B6.129S7-charna7tm1bay, number 003232; Bar Harbor, ME, USA). Mice were maintained in the Animal Resource Centre of the Faculty of Medicine, Nanjing Medical University. The mice were fed standard rodent chow and water *ad libitum*. Room temperature was maintained at 24 ± 2 °C and 50−60% humidity under a 12 h:12 h light:dark cycle. The animals were acclimated to the experimental environment for 3 days before experiments. The absences of α7-nAChR mRNA and protein in KO mice were confirmed by RT-PCR and western blot analysis, respectively. All experiments were approved by the Animal Care and Use Committee of Nanjing Medical University.

2.3. Grouping and treatment

Wild-type (WT) C57BL/6J mice and α7-KO mice were randomly divided into treatment groups as described below. The WT and α7-KO MPTP treatment groups received four intraperitoneal (i.p.) injections of 20 mg/kg MPTP-HCl in sterile saline (0.9% vol. NaCl) at 2-h intervals on the same day, while matched control mice received equal-volume i.p. saline. The MPTP plus nicotine (MPTP + Nic) groups received 1.0 mg/kg nicotine per day (i.p. in sterile saline) for 21 consecutive days prior to MPTP as well as 1.0 mg/kg [*i.p.*] 1 h before each of the four MPTP injections. In the MPTP + Nic group, administration of nicotine (*i.p.*, 1.0 mg/kg, once daily) was continued for 7 days after the MPTP injections.

2.4. Behavioral tests

Motor deficits in mice after MPTP treatment were assessed with the pole test as described [18]. Briefly, a straight wood pole with a diameter of 10 mm and a height of 500 mm topped by a cork ball 25-mm in diameter was used to assess climbing ability. A mouse was placed on the cork ball with head facing upward. Three times were recorded: the time from beginning of movement to the time when the mouse's head was fully downward, the time when the mouse had climbed halfway down the pole, and the time from halfway down to the bottom of the pole. Motor performance was scored as follows: 3 for times of less than 3 s, 2 if less than 6 s, and 1 if longer than 6 s. Pole climbing ability was tested 1, 3, and 7 days after MPTP administration.

2.5. Immunohistochemistry

After perfusion via the left ventricle, brain tissues were quickly removed and fixed overnight in 4% paraformaldehyde. The tissue samples were then dehydrated by submersion in 20% sucrose for 3 days and 30% sucrose for another 3 days. After sucrose-gradient dehydration, 30-μm thick frozen sections were prepared according to the mouse brain map and stored in glycerol: phosphate buffered saline (PBS) (1:1 v/v) solution at −20 °C. Sections were incubated with primary antibody against tyrosine hydroxylase (TH) (1:3000, Sigma), glial fibrillary acidic protein (GFAP) (1:800; Millipore Corp., Billerica, MA, USA), or Mac-1 (polyclonal, 1:100, CD11b, AbD; Serotec, Oxford, UK) at 4 °C overnight, followed by 1 h incubation with corresponding horseradish peroxidase-conjugated secondary antibodies at room temperature. Then, 3,3′-diaminobenzidine tetrahydrochloride was used as a color substrate. The total numbers of TH-immunoreactive (-IR) neurons, GFAP-IR astrocytes, and Mac-1-IR microglia in the SNpc were determined stereologically using the optical fractionator method [12,19,20]. Briefly, TH-IR neurons, GFAP-IR astrocytes, and Mac-1-IR microglia were counted in the SNpc of every fourth section (30 μm) throughout the entire extent of the SNpc. Each midbrain section was viewed at low power (×10 objective), and the SNpc was outlined in accordance with the established anatomical landmark. Then, at a random starting point, the number of TH positive neurons, GFAP positive astrocytes and Mac-1-IR microglia was counted at high power (×100, oil immersion). To avoid double counting of cells with unusual shapes, each type of cell (TH-IR neurons, GFAP-IR astrocytes and Mac-1-IR microglia) was counted only when its nucleus was optimally visualized, which occurred in only one focal plane. After all the appropriate cells were counted, the total numbers of TH-IR neurons, GFAP-IR astrocytes, and Mac-1-IR microglia in the SNpc were calculated using the formula described by West [21]. Sampling grid dimensions were 120 × 120 × 5 mm (x, y, and z axes, respectively).

2.6. Measurement of dopamine (DA) levels in the striatum

DA in dialyzed samples was measured using high performance liquid chromatography with a LC-20A pump (Shimadzu, Kyoto, Japan), and a RF-20A fluorescence detector (Shimadzu, Kyoto, Japan). Chromatographic separation of dopamine was performed using a Thermo Hypersil GOLD C18 column (4.6 mm × 150, i.d., 5 μm; Thermo Fisher Scientific Inc., MA, USA). Mobile phase A phase consisted of 30 mM citric acid, 40 mM Sodium acetate anhydrous, 0.2 mM EDTA-2Na, and 0.4 mM sodium octane sulfonate (pH = 3.9). and phase B of methanol. Phase ratio was A: B = 86:14, and flow rate was 1.0 mL/min. Dopamine concentration was calculated based on the peak area standard curve using a Lab Solutions LC-solution Version 1.2 Workstation (Shimadzu, Kyoto, Japan).

2.7. Cell culture and treatment

Nicotine, MPP^+, and MLA were dissolved in Hank's buffered saline at neutral pH (7.0). Cells were cultured in DMEM containing 10% fetal bovine serum, 1% non-essential amino acids, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were grown in sterile plastic culture flasks at 1×10^5/cm^2. Before experiments, cells were harvested and plated in six-well or 96-well plates, and cultured in DMEM containing 10% fetal bovine serum for 24 h. The medium was replaced with serum-free medium for drug treatments. In MPP^++Nicotine group cultures, nicotine was added 1 h before MPP^+. In MLA-treated groups, MLA was added 30 min before nicotine. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 24 h.

2.8. MTT assay

Cell viability was measured by the MTT assay. Briefly, the medium was removed from cultures treated as described and replaced with 200 μL of 0.5 mg/mL MTT dissolved in PBS. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 4 h. Subsequently, 200 μL DMSO (Aladdin Industrial Corporation, Shanghai, China) was added and cultures incubated at room temperature for 15 min. The absorbance of each well was measured at 450 nm.

2.9. siRNA transfection

Human α7-nAChR siRNA (sense: 5′-GUCUUGGACAGAUCA CUAUTT-3′; antisense: 5′-AUAGUGAUCUGUCCAAGACTT-3′) and control siRNA (sense: 5′-UUCUCCGAACGUGUCACGUdTdT-3′; antisense: 5′-ACGUGACACGUUCGGAGAAdTdT-3′) were purchase from Shanghai GenePharma (Shanghai, China). One day prior to transfection, SH-SY5Y cells were treated with 0.25% pancreatin, and inoculated into six-well plates (1×10^6 cells/well). Cells were cultured to 60–80% confluence, washed with serum-free DMEM three times and then 1.5 mL Opti-MEM (Gibco, Cat. No. 31985) serum-free medium was added to each well. Cells were transfected with α7-nAChR siRNA or control siRNA in six-well plates (1 μg each) using Lipofectamine 2000 (Invitrogen, Cat. # 11668) according to the manufacturer's instructions. After a 6 h transfection, the supernatant was removed and replaced with complete medium and culture was continued for a further 24 h.

2.10. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Alpha7-nAChR mRNA levels were determined using qRT-PCR. Total RNA (10 μg) was extracted from SH-SY5Y cells. The primers for α7-nAChR (HQP054678) were obtained from GeneCopoeia, Inc. (Rockville MD, USA). Glyceraldehyde-3- phosphate dehydrogenase (GAPDH) mRNA (TaqMan, GAPDH control reagents; Applied Biosystems, Foster City, CA, USA) was used for sample standardization. qRT-PCR was performed using the SYBR-Green PCR Core reagents kit (Applied Biosystems, Foster City, CA, USA).

2.11. Immunocytochemistry

Cells were washed in PBS and fixed with 4% paraformaldehyde for 10 min, treated with 1% bovine serum albumin in PBS for 1 h, and then incubated for 12 h at 4 °C with anti-α7-nAChR. Following incubation with primary antibodies, cells were washed and incubated for 1 h at room temperature with fluorophore-conjugated secondary antibodies. Stained cells were photographed using a fluorescence microscope (Nikon Optical TE2000-S, Tokyo, Japan).

2.12. Western blot analysis

One week after MPTP administration, brain tissues were rapidly removed on ice, added 1:10 (v/v) to buffer containing protease inhibitor cocktail (Life Technologies Corp., Carlsbad, CA, USA), sonicated, and then incubated on ice for 40 min. Cell lysates were centrifuged at 12000*g* for 15 min and the supernatants retained as whole-cell protein samples. A 1 μL sample of lysate was used for measuring the total protein concentration. Samples were mixed with 5 × loading buffer and denatured in boiling water for 5 min before storage at −20 °C for future use.

According to the BCA quantification results, 20 μg protein was loaded on each lane of 10% polyacrylamide gels and separated by SDS-PAGE (80 V). Separated proteins were electrotransferred to polyvinylidene fluoride membranes (300 mA constant current for 90 min). Blotted membranes were incubated in TBST (pH 7.4, 10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) with 5% non-fat milk powder for 1 h and then in 5% BSA–TBST containing one of the following primary antibodies overnight at 4 °C: anti-DAT (1:1000), anti-α7-nAChR (1:200), anti-Wnt1 (1:200), anti-pSer9-GSK3β (1:1000), anti-β-catenin (1:1000), or anti-β-actin (1:1000). Immunolabeled membranes were washed three times in TBST and incubated with secondary antibody for 1 h, followed by three washes with TBST. Finally, immunoblots were scanned by a gel imaging system (GelMax Imager; Ultra-Violet Products Ltd., Upland, CA, USA). Band density was measured using GelPro Analyzer software (Media Cybernetics, Inc., Bethesda, MD, USA).

2.13. Statistical analyses

All values are presented as mean ± standard error of the mean. Treatment group means were compared by one-way or two-way analysis of variance. When ANOVA showed significant differences among groups, pair-wise comparisons were conducted using the Newman-Keuls *post hoc* test. For all analyses, $P < 0.05$ was considered statistically significant. All statistical analyses were performed using IBM SPSS software (Version 21.0).

3. Results

3.1. α7-nAChR knockout abolished nicotine-mediated improvement of motor dysfunction in the MPTP mouse model

Nicotine treatment protects against the neurological and behavioral deficits induced by MPTP in mice. To investigate the contribution of α7-nAChRs to nicotine-mediated suppression of motor deficits, pole climbing performance was compared between MPTP-treated WT and α7-KO mice following vehicle or nicotine pretreatment (MPTP and MPTP + Nic groups). Untreated control

WT and α7-KO mice displayed no motor deficits as measured by pole descent times. However, a significant prolongation of the total time for climbing down the pole was observed in both WT and α7-KO mice 3 h and 24 h after MPTP treatment. Nicotine pretreatment ameliorated this motor deficit in MPTP-treated WT mice. In contrast, the prolonged motor deficits observed in MPTP-treated α7-KO mice were not ameliorated by nicotine pretreatment ($^{※※}P < 0.01$ on days 1 and 3, $^{※}P < 0.05$ on day 7 compared to the MTPT + Nic WT group) (Fig. 1).

3.2. α7-nAChR knockout eliminated nicotine-mediated protection of dopaminergic neurons in the SNpc of the MPTP mouse model

Consistent with motor function tests, nicotine protected DAergic neurons against MPTP toxicity in WT mice but not in α7-KO mice. Numbers of DAergic neurons in SNpc were determined in all treatment groups by TH staining. Results revealed no difference in SNpc TH-IR cell number between untreated WT and α7-KO mice, while MPTP administration decreased TH-IR cells by 73% and 74%, respectively, in SNpc of WT and α7-KO mice ($^{**}P < 0.01$, Fig. 2). Nicotine pretreatment significantly increased SNpc TH-IR cell number in MPTP-lesioned WT mice (MPTP + Nic group) compared to MPTP WT mice ($^{\#\#}P < 0.01$, Fig. 2), while cell number was significantly lower in MPTP + Nic α7-KO mice compared to MPTP + Nic WT mice ($^{※※}P < 0.01$, $^{**}P < 0.01$, Fig. 2). Thus, nicotine does not protect against DAergic neuron loss induced by MPTP in the absence of α7-nAChRs.

3.3. α7-nAChR knockout abolished the inhibitory effect of nicotine on MPTP-induced astrocytes and microglia activation in the SNpc

Since neuro-inflammation is crucial for PD pathogenesis, we next evaluated the effects of nicotine on astrocyte and microglia activation in MPTP-lesioned WT and α7-KO mice as determined by GFAP and MAC-1 staining, respectively. As shown in Fig. 3, only faintly immunoreactive GFAP-IR astrocytes and MAC-1-IR microglia were observed in the SNpc of untreated WT and α7-KO mice. MPTP treatment led to 2.99-fold and 2.77-fold elevations of GFAP-IR astrocytes and MAC-1-IR microglia, respectively, in the SNpc of WT mice ($^{**}P < 0.01$ compared to untreated WT mice), and to 3.14-fold and 2.40-fold elevations, respectively, in α7-KO mice ($^{**}P < 0.01$ compared to untreated α7-KO mice). Pretreatment with nicotine (1.0 mg/kg) inhibited MPTP-induced astrocyte and microglia activation in WT mice ($^{\#\#}P < 0.01$ compared to MPTP WT mice), while there were still significant differences in SNpc GFAP-IR astrocyte and MAC-1-IR microglia numbers between the MPTP + Nic WT mice and MPTP + Nic α7-KO mice ($^{※※}P < 0.01$). These results indicate that α7-nAChRs are necessary for suppression of MPTP-induced astrocyte and microglia activation by nicotine.

3.4. α7-nAChR knockout abolished the improvement effect of nicotine on MPTP-induced suppression of dopamine release in the striatum

To examine if α7-nAChRs are necessary for preservation of DAergic neuron function under MPTP treatment, basal DA release in striatum was measured by High performance liquid chromatography. As shown in Fig. 4A, striatal DA was significantly lower in MPTP-lesioned WT and α7-KO mice compared to corresponding untreated controls. Further, nicotine increased striatal DA in MPTP-treated WT but not in MPTP-treated α7-KO mice.

We next examined if α7-nAChRs are required for the nicotine-mediated maintenance of striatal DAT expression, which enables re-uptake of DA from the synaptic cleft into the pre-synaptic neuron. Western blotting demonstrated reduced DAT expression in both WT and α7-KO mice after MPTP administration ($^{**}P < 0.01$ compared to corresponding untreated groups, Fig. 4B), and nicotine reversed this effect only in WT mice ($^{\#\#}P < 0.01$ vs. MPTP WT mice, Fig. 4B), while DAT expression in MPTP + Nic α7-KO mice remained suppressed compared to the MPTP + Nic WT group ($^{※※}P < 0.01$, Fig. 4B). Together, these results demonstrate that α7-nAChRs contribute to nicotine-mediated elevation of dopamine release and DAT expression in the striatum of MPTP PD model mice.

3.5. α7-nAChR knockout eliminated nicotine-mediated activation of Wnt/β-catenin signaling in the SNpc of the MPTP mouse model

It has been reported that signaling through the Wnt/β-catenin pathway (Wnt1 or Wnt3a) is crucial for midbrain DAergic neuron development, survival, and MPTP-induced PD pathology [15]. Midbrain SNpc tissues from MPTP-lesioned WT mice showed lower expression levels of Wnt1 (51%), p-Ser9-GSK-3β (52%, which is the inactive form), and β-catenin (41%) than untreated WT mice, all of which were restored by nicotine (1.0 mg/kg) treatment. Similarly, MPTP-lesion the α7-KO mice exhibited roughly equivalent reductions in Wnt1 (50%), p-Ser9-GSK-3β (47%), and β-catenin (45%); however, nicotine did not restore expression levels of Wnt/β-catenin signaling as there were no significant differences between MPTP α7-KO mice and MPTP + Nic α7-KO mice ($^{※※}P > 0.05$, Fig. 5).

3.6. The α7-nAChR-specific antagonist blocked nicotine-mediated protection of SH-SY5Y cells against MPP^+

In principle, the neuroprotective effects of α7-nAChR signaling on MPTP-treated PD model mice could be mediated by direct effects on neurons or by indirect effects, such as suppression of neuroinflammation. To assess direct effects, we examined nicotine-mediated protection against MPP^+ toxicity in cultured DAergic neurons. To study the contribution of α7-nAChRs, receptors were blocked pharmacologically using the α7-nAChR-specific antagonist MLA. SH-SY5Y cell pretreated with nicotine (1, 10 or 100 μM) 24 h before MPP^+ (500 μM) stimulation exhibited dose-dependent increases in cell viability compared to cells treated with MPP^+ alone as measured by MTT assay ($^{**}P < 0.01$, Fig. 6A). In contrast, nicotine-mediated protection against MPP^+ was blocked by MLA (100 nM) ($^{※※}P < 0.01$ vs. MPP^+ alone, Fig. 6B). Then, we evaluated potential contributions of the Wnt1/β-catenin signaling pathway to nicotine/α7-nAChR-mediated protection by measuring Wnt1, p-Ser9-GSK-3β, and β-catenin expression levels in MPP^+, Nic + MPP^+, and Nic + MPP^++MLA treatment groups of SH-SY5Y cells. As shown in Fig. 6C, MPP^+ treatment reduced the expression levels of Wnt1 (50%), p-Ser9-GSK-3β (57%), and β-catenin (50%), while expression levels of all three proteins were rescued by nicotine (30 min pretreatment of 10 μM nicotine) to 94%, 95%, and 94% of control, respectively. Furthermore, the α7-nAChR antagonist MLA (100 nM) blocked this upregulation of Wnt1, p-Ser9-GSK-3β, and β-catenin in SH-SY5Y cells.

3.7. α7-nAChR knockdown blocked nicotine-mediated protection of SH-SY5Y cells against MPP^+

Furthermore, α7-nAChR receptors were knocked down by a targeted siRNA. First, RT-PCR, western blot, and immunofluorescence assays were used to demonstrate the knockdown efficiency and specificity of siRNA α7-nAChR silencing. As shown in Fig. 7A, α7-nAChR-targeted siRNA (α7-siRNA) silencing significantly reduced α7-nAChR mRNA levels in SH-SY5Y cells to 53% of control cells treated with neg-siRNA. Moreover, α7-nAChR protein expression in SH-SY5Y cells was also significantly inhibited after α7-siRNA silencing as revealed by western blot and immunofluorescence

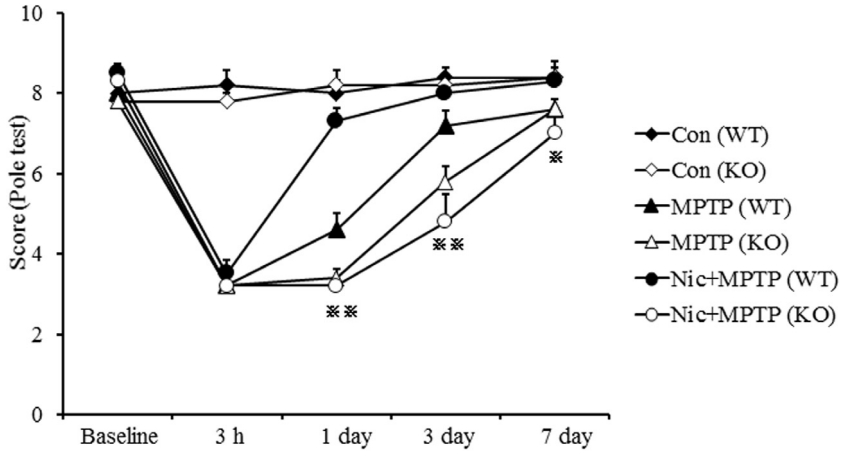

Fig. 1. Rescue of MPTP-induced motor dysfunction by nicotine depends on α7-nAChR expression. Pole test scores for each animal were calculated 3 h, 1 day, 3 days, and 7 days following MPTP treatment. Data are presented as mean ± SEM of 10 individual experiments. $^{※}P < 0.05$ and $^{※※}P < 0.01$ indicate significant differences between wild-type (WT) mice and α7-nAChR knockout (α7-KO) mice treated with MPTP alone or MPTP plus nicotine (1 mg/kg).

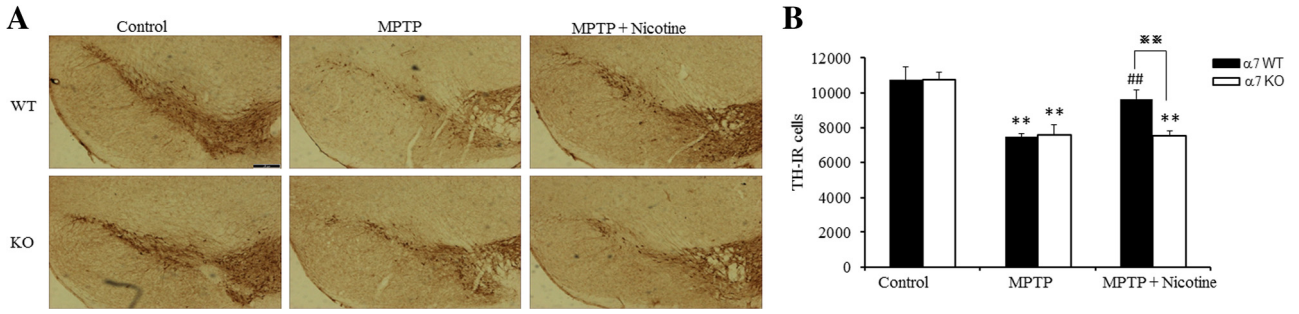

Fig. 2. Nicotine-mediated protection of mouse substantia nigra pars compacta (SNpc) dopaminergic neurons against MPTP toxicity depends on α7-nAChR expression. (A) Tyrosine hydroxylase (TH)-immunoreactive (IR) neurons in the mouse SNpc. Scale bar: 200 μm. (B) Stereological cell counts of TH-IR neurons in the mouse SNpc. Data are presented as mean ± SEM of six individual experiments. $^{**}P < 0.01$ vs. the WT control group; $^{\#\#}P < 0.01$ vs. the WT MPTP treatment group. $^{※※}P < 0.01$ indicates significant differences between WT mice and α7-KO mice treated with nicotine (1 mg/kg) and MPTP.

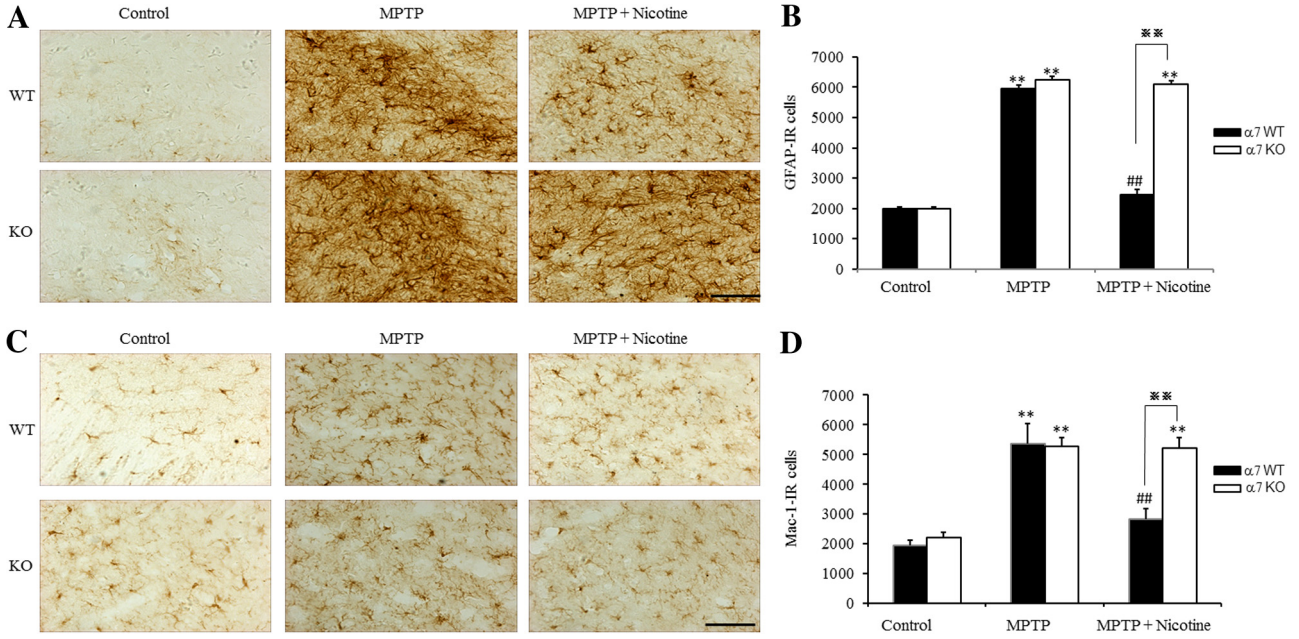

Fig. 3. Nicotine-mediated suppression of MPTP-induced astrocyte and microglia activation in the mouse SNpc is dependent on α7-nAChR expression. (A) Astrocytes in mouse SNpc. Scale bar: 40 μm. (C) Microglia in mouse SNpc. Scale bar: 40 μm. (B) Stereological cell counts of astrocytes in the mouse SNpc. (D) Stereological cell counts of microglia in the mouse SNpc. The data are presented as mean ± SEM of six individual experiments. $^{**}P < 0.01$ vs. the WT control group; $^{\#\#}P < 0.01$ vs. the WT MPTP treatment group. $^{※※}P < 0.01$ indicates significant differences between WT mice and α7-KO mice treated with nicotine (1 mg/kg) and MPTP.

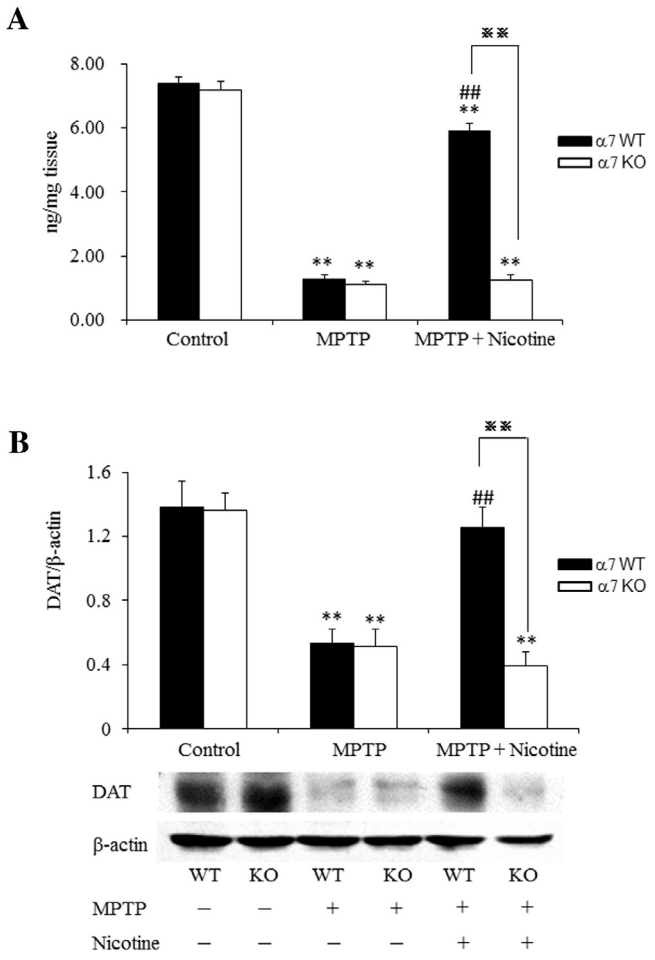

Fig. 4. Preservation of striatal basal dopamine release by nicotine following MPTP treatment is dependent on α7-nAChRs. (A) High performance liquid chromatography analysis of DA release in the mouse striatum. (B) Western blot analysis of DA transporter (DAT) expression in the striatum of the acute MPTP mouse model. The data are presented as mean ± SEM of 10 individual experiments. $^{**}P < 0.01$ vs. the WT control group; $^{\#\#}P < 0.01$ vs. the WT MPTP treatment group. $^{※※}P < 0.01$ indicates significant differences between WT mice and α7-KO mice treated with nicotine (1 mg/kg) and MPTP.

(Fig. 7B, C). Our data have also shown that α7-nAChR knockout mice do not express α7-nAChR protein (Fig. 7B).

We found that α7-nAChR knockdown blocked nicotine-mediated protection of SH-SY5Y cells against MPP^+ (MPTP + Nic α7-KO mice group compared to MPTP + Nic WT mice group, $^{※※}P < 0.01$, Fig. 8A). Thus, α7-nAChR activation by nicotine appears to directly protect DAergic neurons against MPP^+ toxicity. As shown in Fig. 8B, MPP^+ treatment reduced the expression levels of Wnt1, p-Ser9-GSK-3β, and β-catenin in SH-SY5Y cells transfected with neg-siRNA or α7-siRNA. Nicotine restored expression levels of Wnt1, p-Ser9-GSK-3β, and β-catenin to 98%, 84%, and 100%, respectively, in neg-siRNA-transfected SH-SY5Y cells, but had no effect on α7-siRNA-transfected SH-SY5Y cells. These results strongly suggest that Wnt/β-catenin signaling may be involved in nicotine-mediated neuroprotection mechanism through α7-nAChR activation.

4. Discussion

In this study, α7-nAChR knockout abolished nicotine-mediated protection of SNpc DAergic neurons against MPTP toxicity in mice, a widely studied model of PD pathogenesis. Further, α7-KO blocked nicotine-mediated maintenance of DAergic function (striatal DA release) and striatal DA uptake, as well as nicotine-mediated protection against MPP^+ toxicity in cultured DAergic SH-SY5Y neurons. Knockout of α7-nAChRs also blocked the improvement in motor coordination by nicotine treatment in PD model mice, which may reflect maintained reductions in DA and DAT levels in striatum. α7-nAChR deficiency reduced activation of the Wnt/β-catenin signaling pathway by nicotine as evidenced by reversal of nicotine-induced Wnt, pSer9-GSK3β, and β-catenin upregulation in the mouse SNpc and SH-SY5Y cell line. These findings strongly suggest that α7-nAChRs may mediate the protective effects of nicotine and thus may be involved in the pathogenesis of PD via regulation of the Wnt/β-catenin signaling pathway.

Both ABT-107, a specific α7-nAChR agonist, and nicotine increased DA release and DAT expression in the ipsilateral striatum of 6-OHDA model rats (Bordia et al., 2015). However, ABT-107 may act on other nicotinic receptors. Therefore, nicotinic receptor knockout mice may be more appropriate for examining the relationship between specific nicotinic receptor subtypes and DAergic neuron function under both physiological and pathological

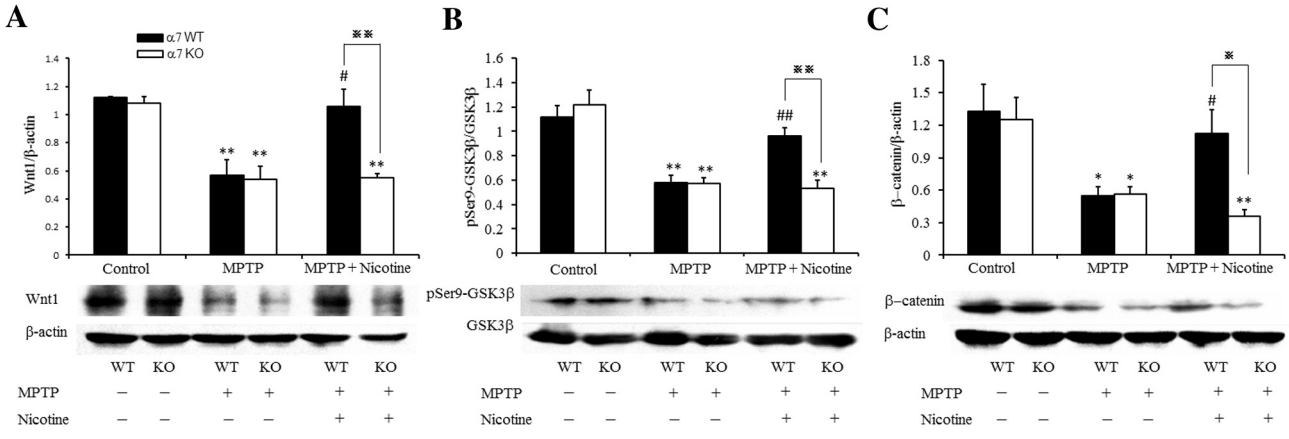

Fig. 5. Effect of nicotine on MPTP-induced inhibition of the Wnt/β-catenin signaling pathway in the mouse SNpc. (A–C) Western blot analysis of Wnt1 (A), p-Ser9-GSK3β (B), and β-catenin (C) expression levels in mouse SNpc. The data are presented as mean ± SEM of five independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the WT control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. the WT MPTP treatment group. $^{※}P < 0.05$ and $^{※※}P < 0.01$ indicate significant differences between WT mice and α7-KO mice treated with nicotine (1 mg/kg) and MPTP.

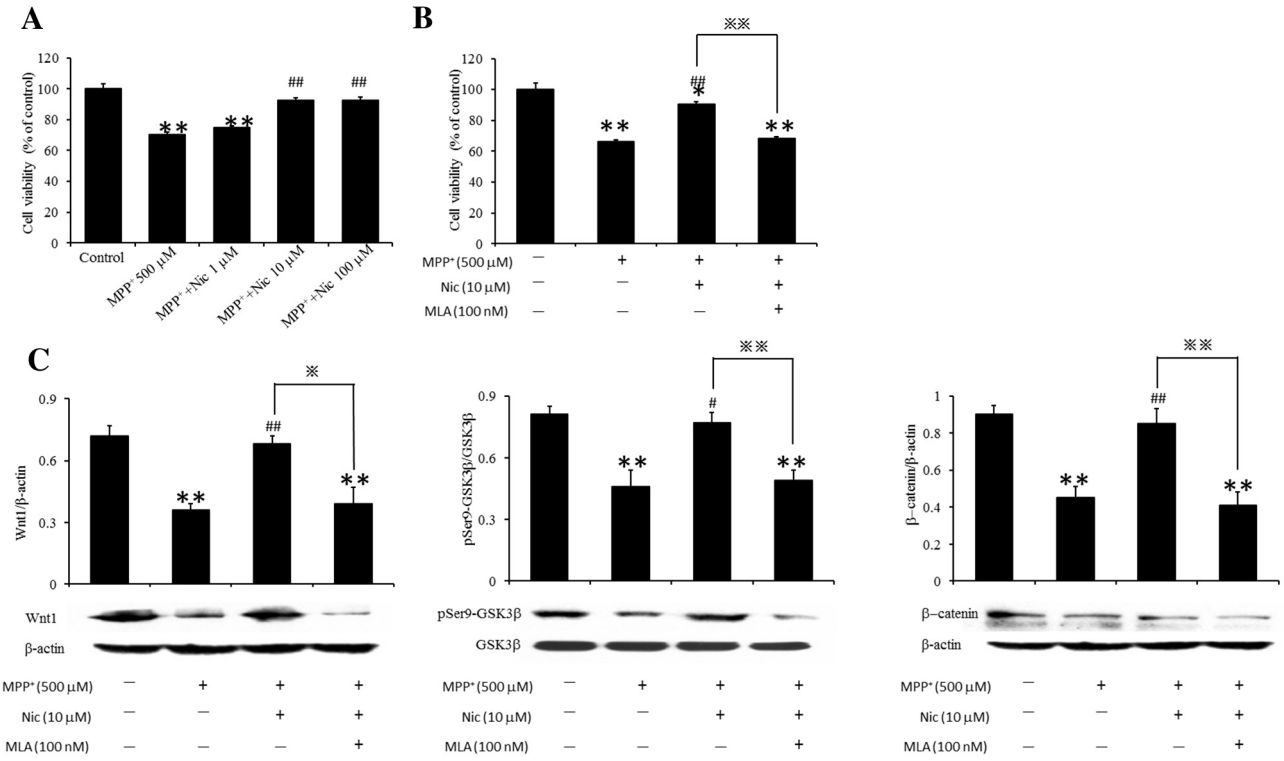

Fig. 6. The α7-nAChR-specific antagonist blocked nicotine-mediated protection of SH-SY5Y cells against MPP^+. (A) Nicotine dose-dependently suppressed the decrease in SH-SY5Y cell viability induced by MPP^+ (500 μM). (B) The α7-nAChR-selective antagonist methyllycaconitine (MLA) abolished the protective effects of nicotine against MPP^+ (500 μM) toxicity. (C) SH-SY5Y cells were pretreated with 10 μM nicotine in the absence or presence of MLA (100 nM) for 30 min and then challenged with MPP^+ for 24 h. After treatment, cells were harvested, and levels of Wnt1, p-Ser9-GSK3, and β-catenin analyzed. Upper panels show densitometric analysis, while the lower panels show representative blots. The data are presented as mean ± SEM of five independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. the MPP^+ treatment alone group, $^{※}P < 0.05$ and $^{※※}P < 0.01$ indicate significant differences between the MPP^+ treatment alone group and nicotine (10 μM) + MPTP treatment group.

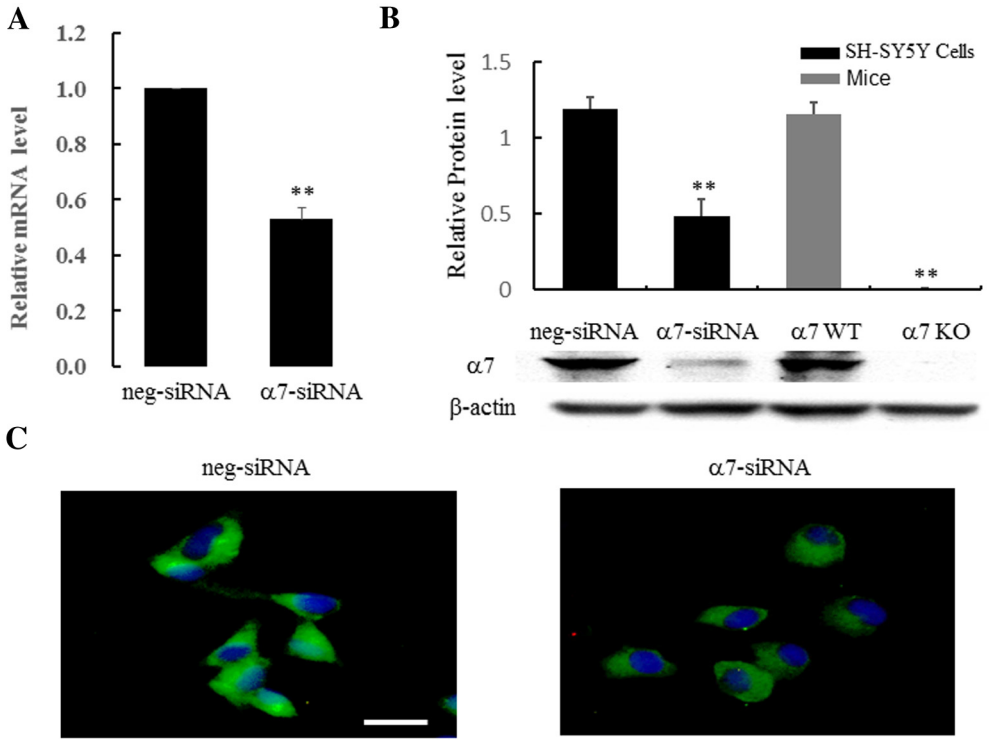

Fig. 7. Modulation of α7-nAChR expression in SH-SY5Y cell cultures by siRNA transfection. (A) Transfection efficiency of siRNA in SH-SY5Y cells was assayed by quantitative real-time PCR. Expression levels of α7-nAChR mRNA were compared between cultures transfected with targeted siRNA or the negative siRNA. (B) Downregulation of α7-nAChR protein expression as assessed by Western blot (compared to the negative siRNA and the WT control mouse). (C) Expression of the α7-nAChR in SH-SY5Y cell cultures as assessed by anti-α7-nAChR fluorescence immunostaining under confocal microscopy. Scale bar: 50 μm $^{**}P < 0.01$ vs. the control group.

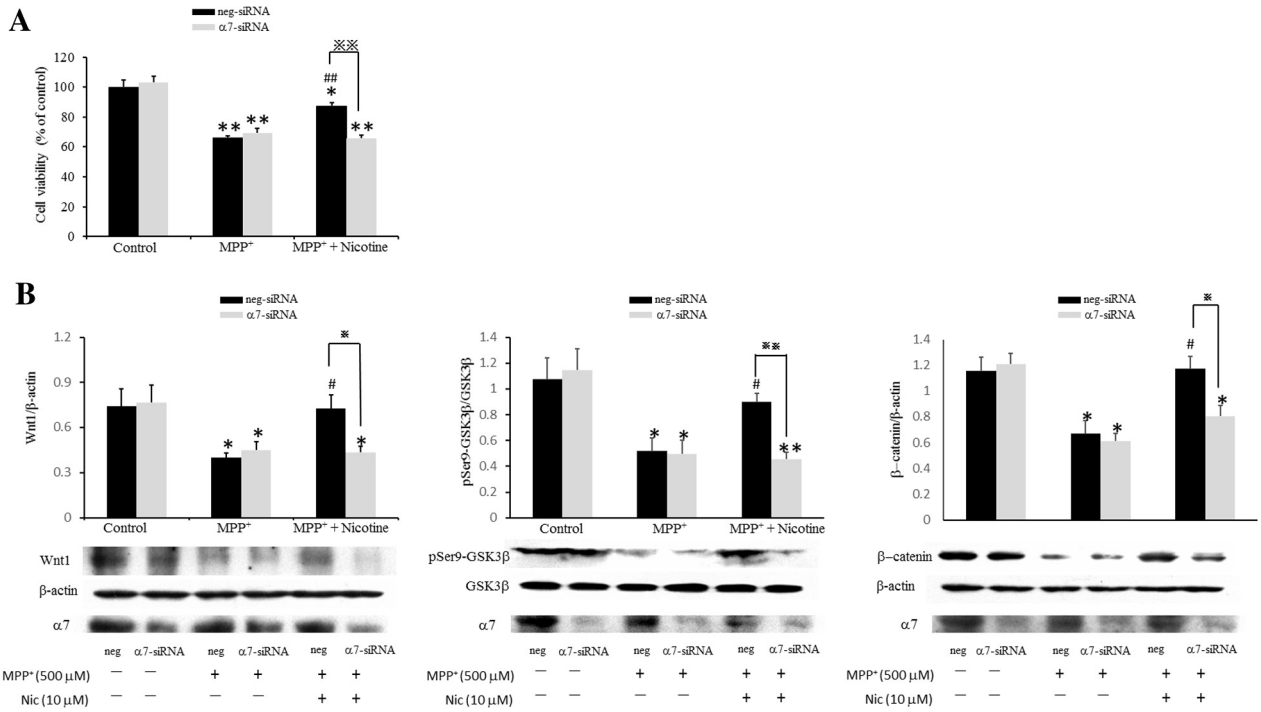

Fig. 8. α7-nAChRs knockdown blocked nicotine-mediated protection of SH-SY5Y cells against MPP^+. (A) Knockdown of α7-nAChR suppressed the protective effects of nicotine against 500 μM MPP^+. (B) SH-SY5Y cells were transfected with negative-siRNA or α7-nAChR siRNA, treated with 500 μM MPP^+ with or without nicotine pretreatment, and then harvested for analysis of Wnt1, p-Ser9-GSK3 and β-catenin expression. Upper panels show densitometric analysis, while the lower panels show representative blots. The data are presented as mean ± SEM of five independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. the MPP^+ treatment alone group, $^{※}P < 0.05$ and $^{※※}P < 0.01$ indicate significant differences between negative siRNA-transfected cells and α7-nAChR siRNA-transfected cells treated with 10 μM nicotine and 500 μM MPP^+.

conditions. Hence, we compared nicotine-mediated protection between WT and α7-KO mice and found that the absence of α7-nAChRs largely abolished the protective effects of nicotine on striatal DA in the MPTP model, which may in turn have caused DAT levels to remain underexpressed.

Over the past 50 years, many epidemiological studies involving a variety of different populations and experimental designs have suggested a significant negative association between smoking and PD incidence, and a growing body of evidence suggests a vital role for nAChRs in this relationship. There are multiple nAChR isoforms in the brain, and several have been implicated in these beneficial effects of nicotine. Our previous study revealed that stimulation of α7-nAChRs significantly inhibited astrocyte activation and tumor necrosis factor-α release induced by lipopolysaccharide, which was associated with Erk1/2 and p38 MAPK signaling pathways (Liu et al., 2012). Indeed, α7-nAChRs function through the "cholinergic anti-inflammatory pathway" (de Jonge and Ulloa, 2007), although the underlying mechanisms remain unclear. Here we demonstrate that suppression of neuroinflammation by nicotine is dependent on the presence of α7-nAChRs, although the contributions of other receptor subtypes cannot be excluded.

Sensory input induced by infection or injury is transmitted to the brainstem via the vagus nerve and output signals are sent to spleen and other effector organs by efferent nerves to trigger the inflammatory reflex. The neurotransmitters produced by peripheral autonomic nerves further promote the release of acetylcholine from CD4+ T cells to complete chemical transmission of nervous signals to immune cells, and α7-nAChRs play an important role in modulation of this pathway (Olofsson et al., 2012). Further, α7-nAChRs are associated with a variety of inflammatory diseases, such as atherosclerosis, diabetes, neuropathy, osteoarthritis, sepsis, chronic obstructive pulmonary disease, and inflammatory bowel disease, and so are promising therapeutic targets (Filippini et al., 2012). Accumulating evidence points to dysregulation of Wnt signaling in PD pathogenesis [22], and Wnts are known as important regulators of inflammation [23–25]. In the brain, both astrocytes and microglia express Wnt receptors and respond to Wnt with either pro- or anti-inflammatory activity [26,27]. However, crosstalk between the nicotinic anti-inflammatory pathway and Wnt/β-catenin pathway as it relates to nigrostriatal DAergic neuron injury/repair is still unclear. So we provide evidence that α7-nAChR/Wnt/β-catenin signaling suppresses neuro-inflammation, which may contribute to protection of SNpc DAergic neurons in PD.

We found that nicotine plays an important role in MPTP-induced inhibition of the canonical Wnt/β-catenin signaling pathway in the mouse SNpc via α7-nAChR. We then used the selective α7-nAChR antagonist MLA and siRNA-mediated α7-nAChR knockdown to inhibit α7-nAChR activation in MPP^+-treated SH-SY5Y cells, and the results showed that either pharmacological blockade or downregulation of α7-nAChR expression could abolish the nicotine-induced increases in cell viability during MPP^+ exposure. Similarly, the effects of nicotine on the Wnt/β-catenin pathway were also reversed by α7-nAChR inhibition in SH-SY5Y cells. These findings indicate that α7-nAChRs are required for nicotine-mediated protection of DAergic neuron and that Wnt/β-catenin signaling may be a critical downstream effector of this process.

While these results strongly suggest that nicotine-mediated suppression of PD symptoms in a mouse model depends on α7-nAChR stimulation and Wnt/β-catenin signaling, many questions remain answered. It is unknown whether the Wnt/β-catenin pathway is essential for any or all nicotine/α7-nAChR-mediated responses or activation of downstream cytoprotective pathways (e.g., anti-apoptotic signals). Recently, it was reported that α7-nAChRs play a detrimental role in host defense against CNS inflammation caused by microbial pathogens (e.g., meningitic

pathogens) and non-microbial factors (e.g., methamphetamine) via the NF-κB signaling pathway [28], which may be involved in regulation of the molecular marker S100B during various CNS disorders [29]. However, neuro-inflammation may be more pathogenic in PD than during infection. The present study showed that α7-nAChRs are critical for inhibition of astrocyte and microglial activation by nicotine in response to MPTP treatment. These findings suggest that α7-nAChR activation is required for suppression of neuro-inflammatory responses through the Wnt/β-catenin pathway, thereby identifying several novel therapeutic targets for future investigations of PD pathobiology.

The present study revealed that nicotine-induced improvements in the MPTP model of PD are dependent on α7-nAChRs and likely also on Wnt/β-catenin signaling. Further studies are needed to clarify the detailed molecular mechanisms before the α7-nAChR can be exploited as an effective therapeutic target for PD. Nevertheless, our findings highlight the important roles of α7-nAChRs and Wnt/β-catenin in an animal model of PD and suggest the potential utility of α7-nAChR agonists as novel treatments.

Author contributions

1. Study conception and design: YL, JH 2. Acquisition, analysis and/or interpretation of data: YL, SH, BY, YF, XQ, YC, JH 3. Drafting/revision of the work for intellectual content and context: YL, YC, JH 4. Final approval and overall responsibility for the published work: YC, JH.

Conflicts of interest

The authors state that no conflicts of interest exist.

Disclaimer

The authors, their immediate families, and any research foundations with which they are affiliated have not received any financial payments or other benefits from any commercial entity related to the subject of this article.

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