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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).