Knockdown of NogoA prevents MPP+-induced neurotoxicity in PC12 cells via the mTOR/STAT3 signaling pathway

JIANBIN ZHONG[1*], XIE LI[1*], LIMEI WAN[1], ZHIBANG CHEN[1], SIMIN ZHONG[1], SONGHUA XIAO[2] and ZHENGWEN YAN[2]

[1]Department of Neurology, Zeng Cheng People's Hospital and Boji Hospital of Sun Yat-sen University; [2]Department of Neurology, Sun Yat-sen Memorial Hospital of Sun Yat-sen University, Guangzhou, Guangdong 511300, P.R. China

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Abstract. NogoA is a myelin-associated protein, which is important in the inhibition of axonal fiber growth and in regeneration following injury of the mammalian central nervous system. A previous study suggested that NogoA may be key in the process of Parkinson's disease (PD), which is the second most common chronic neurodegenerative disorder worldwide. The regulatory mechanism underlying the effect of NogoA on the process of PD remains to be fully elucidated. The present study aimed to investigate the effect and underlying mechanism of NogoA on cellular viability, apoptosis and autophagy induced by 1-methyl-4-phenylpyridinium (MPP^+) in PC12 cells, a commonly used *in vitro* PD model. PC12 cells were treated with 1 mM MPP^+ for 24 h and the cells were harvested for western blotting. The results demonstrated that the protien expression levels of NogoA were increased in the PC12 cells treated with MPP^+. Subsequently, NogoA small interfering RNA was synthesized and transfected into PC12 cells to silence the expression of NogoA. NogoA knockdown significantly reduced the MPP^+-induced decrease in cell viability and apoptosis, detected using a cell counting kit-8 and flow cytometric analysis, respectively. Interference in the expression of NogoA increased the MPP^+-induced decrease in mitochondrial membrane potential, determined quantitatively by flow cytometry using JC-1 dye, and the protein levels of Beclin-1. In addition, MPP^+ treatment activated the mammalian target of rapamycin (mTOR)/signal transducer and activator of transcription 3 (STAT3) signaling pathway. Knockdown of NogoA significantly inhibited the expression levels of mTOR and STAT3. Furthermore, overexpression of NogoA had similar neurotoxic effects on the PC12 cells as MPP^+ treatment. Treatment with rapamycin, an inhibitor of the mTOR/STAT3 signaling pathway had a similar effect to that of NogoA knockdown in the MPP^+-treated PC12 cells. Taken together, the results from the present study demonstrated that NogoA may regulate MPP^+-induced neurotoxicity in PC12 cells via the mTOR/STAT3 signaling pathway and provided an explanation regarding the regulatory mechanism of NogoA on the process of PD.

Introduction

Parkinson's disease (PD), the second most common chronic neurodegenerative disorder following Alzheimer's disease, affects individuals >60 years old, and has a prevalence of ~5,000,000 individuals worldwide (1). PD is characterized primarily by the progressive degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (2) and the appearance of cytoplasmic Lewy body inclusions in the surviving neurons (3-5). Due to a reduction in the concentration of striatal dopamine, patients diagnosed with PD eventually lead to the development of an array of motor disorders, including bradykinesia, resting tremor, rigidity and postural instability (6). Furthermore, PD progressively affects multiple neuronal systems centrally and peripherally, leading to numerous non-motor symptoms, including olfactory deficits, anxiety and affective disorders, autonomic and digestive dysfunction and, in particular, memory impairments (7,8). Several risk factors have been suggested to be associated with the etiology of the disease, including age, genetics, oxidative stress, mitochondrial dysfunction and environmental factors (9). However, increasing evidence has demonstrated that abnormal autophagy is a fundamental processes contributing to neuronal death in DA neurons, and the inhibition of autophagy through pharmacological or genetic methods may be a critical therapeutic strategy for PD.

NogoA is a myelin-associated protein, which is important in inhibiting axonal fiber growth and in the regeneration that occurs following injury to the mammalian central nervous

Correspondence to: Dr Limei Wan, Department of Neurology, Zeng Cheng People's Hospital and Boji Hospital of Sun Yat-sen University, 1 Guangming East Road, Zengcheng, Guangzhou, Guangdong 511300, P.R. China
E-mail: wanlimei2015@yeah.net

*Contributed equally

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system (10). It is expressed at high levels by oligodendrocytes and subpopulations of neurons during early neuronal development, and is downregulated during adulthood in the majority of regions, with the exclusion of the hippocampus (11-13). Previous genetic studies have reported that NogoA is involved in schizophrenia (14,15) and synaptic plasticity (16,17), and is a negative regulator of central nervous system angiogenesis (18). However, NogoA knockout or deficiency mice leads to the exhibition of a variety of behavioral alterations, including decreased anxiety, behavioral inflexibility and impairments in short-term memory (19,20). These symptoms are similar to those in patients with PD, and these results suggest that NogoA is key in the process of PD. However, the physiological role of neuronal NogoA in patients with PD remains to be elucidated. NogoA has an A-amino-acid-residue extracellular domain and an axon growth inhibiting domain, which is important in the inhibition of neurite outgrowth in the central nervous system through binding to the NogoA receptor (NgR) expressed on the neuron (21-23).

To further investigate the function of NogoA for PD, PC12 cells were treated with 1-methyl-4-phenylpyridinium (MPP^+) as a model of PD *in vitro*, and NogoA was found to be upregulated following MPP^+ treatment. To further investigate the effect and regulatory mechanisms of NogoA, the PC12 cells were exposed to MPP^+ following pretreatment with NogoA small interfering (si)RNA or negative control siRNA. The results demonstrated that NogoA knockdown reduced MPP^+-induced neurotoxicity and inhibited the expression levels of mTOR and STAT3 in the PC12 cells. Furthermore, NogoA overexpression had similar effects on the PC12 cells as MPP^+ treatment. Treatment with rapamycin, an inhibitor of the mTOR/STAT3 signaling pathway, had similar effects in the MPP^+ treated PC12 cells as those observed following NogoA siRNA transfection. The results suggested that NogoA may regulate MPP^+-induced neurotoxicity in PC12 cells via the mTOR/STAT3 signaling pathway. Our results further demonstrated that NogoA may be a potential target protein for PD treatment. In addition, our results provided a novel explanation regarding the mechanism of NogoA on the regulation of PD.

Materials and methods

Cell culture and PD model. PC12 rat pheochromocytoma cell line was obtained from American Type Culture Collection (Manassas, VA, USA). The PC12 cells were cultured in a type I collagen-coated culture flask (BD Biosciences, Franklin Lakes, NJ, USA) in RPMI-1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 10% heat-inactivated horse serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were maintained at 37˚C in a humidified incubator supplied with 5% CO_2. To establish the PD model, 1 mM MPP^+ (Sigma-Aldrich, St. Louis, MO, USA) was added to the medium for 24 h.

siRNA and NogoA overexpression. The siRNA sequence used for NogoA was 5'-GAGGCAGAUUAUGUUACA ATT-3' (Ribobio, Guangzhou, China). The full-length of NogoA was cloned and inserted into the plasmid expression vector, pcDNA3.1 (Promega, Madison, WI, USA). The transfection of siRNAs and plasmids was performed using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Treatment groups. The PC12 cells were divided into four groups, as follows: i) negative control (NC) group, which was pretreated with 200 nmol/l NC siRNA for 24 h prior to exposure to 1 mM MPP^+ for 24 h; ii) siRNA group, which was pretreated with 200 nmol/l NogoA siRNA for 24 h prior to exposure to 1 mM MPP^+ for 24 h; iii) overexpression group, which was transfected with NogoA overexpression vector for 48 h; and iv) rapamycin group, which was pretreated with mTOR inhibitor, rapamycin (25 mmol/l; Sigma-Aldrich) for 24 h prior to exposure to 1 mM MPP^+ for 24 h. The PC12 cells were seeded at $1x10^3$/well in a 96-well plate for the cell proliferation assay, $4x10^5$/well in a 6-well plate for flow cytometric analysis and western blotting. Then cells were treated according to the experimental design. Cells were harvested for each assay.

Cell proliferation assay. Cell proliferation was monitored using a Cell Counting Kit-8 (CCK8; Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's protocol. The PC12 cells were seeded at $1x10^3$ per well in a 96-well plate and treated according to the experimental design. CCK8 reagent (10 μl) was added to each well and the plate was incubated for 4 h at 37˚C. The absorbance was measured at a wavelength of 490 nm using a Vmax Kinetic ELISA microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Each sample was assayed in triplicate.

Cell apoptosis assay. The PC12 cells were harvested and washed twice with phosphate-buffered saline (PBS; Hyclone, Logan, UT, USA). According to the manufacturer's protocol, $5x10^5$ cells were collected and 500 μl binding buffer from a KGA107 kit (Nanjing KeyGen Biotech Co., Ltd, Nanjing, China) was added to suspend the cells. Annexin V-fluorescein isothiocyanate (5 μl) was added, followed by 5 μl of propidium iodide. Following incubation for 10 min at room temperature in the dark, the cells were analyzed immediately using a BD FACSCalibur™ (BD Accuri C6; BD Biosciences).

Assessment of changes in mitochondrial membrane potential ($\Delta\Psi m$). Changes in the $\Delta\Psi$m were determined quantitatively using flow cytometry, using Molecular Probes ™ JC-1 dye (200 nmol/l; Invitrogen; Thermo Fisher Scientfic, Inc.) according to the manufacturer's protocol. PC12 cells ($5x10^4$) were seeded in 6-well plates. Following treatment, the cells were collected, washed twice with PBS, and resuspended in 500 ml JC-1 working solution for 15-20 min. Cells were centrifuged at 1,000 rpm for 5 min at room temperature and the supernatant was removed. The cells were resuspended in 500 μl 1X incubation buffer and changes in fluorescence were detected by flow cytometry (Ex / Em =488 nm/530 nm. The JC-1 dye has an excitation of 480 nm and an emission of 580/535 nm) and forms aggregates, which result in a red emission in normal polarized mitochondria, whereas it forms monomers emitting green fluorescence on the depolarized mitochondrial membrane. The $\Delta\Psi$m depolarization is an early

characteristic of apoptosis (24). The percentages of cells with green fluorescence (JC-1 monomers), which indicate depolarized cells, were measured.

Western blot analysis. The PC12 cells ($2x10^6$) were collected and washed twice with ice-cold PBS. The cell pellets were suspended in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) for 40 min on ice and the lysates were centrifuged at 12,000 x g at 4˚C for 15 min. Total protein concentration was determined using a BCA Protein Assay kit (Beyotime Institute of Biotechnology). Equal quantities (30 μg) of protein in the supernatant were resolved by 10% SDS polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Pall Life Sciences, Port Washington, NY, USA). The membranes were blocked for 1.5 h at 37˚C with 5% non-fat milk, prior to incubation with rabbit anti-rat NogoA polyclonal antibody (1:1,000, cat. no. ab62024), rabbit anti-rat NgR polyclonal antibody (1:1,000, cat. no. ab189792), mouse anti-rat mTOR monoclonal antibody (1:200, cat. no. ab87540), mouse anti-rat STAT3 monoclonal antibody (1:5,000), or rabbit anti-rat polyclonal Beclin 1 antibody (1:100, cat. no. ab55878; all primary antibodies purchased from Abcam, Cambridge, MA, USA). Following washing with Tris-buffered saline with 0.5% Tween-20 (TBST), the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:20,000, cat. no. BA1055) or goat anti-mouse IgG (1:25,000, cat. no. BA1050; all secondary antibody purchased from BosterBio, Wuhan, China) at 37˚C for 40 min. Following further washing with TBST, the membrane was assayed using enhanced chemiluminescence (EMD Millipore, Billerica, MA, USA) and recorded on X-ray films. The protein bands were quantified by densitometry using QuantityOne software (Bio-Rad, Hercules, CA, USA), and the values were expressed relative to GAPDH.

Statistical analysis. Statistical analyses were performed using the SPSS 19.0 software package (IBM SPSS, Armonk, NY, USA). All numerical data were analyzed using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

MPP^+ treatment increases the expression of NogoA and activates the mTOR/STAT3 signaling pathway. To investigate the role of NogoA in the MPP^+-induced PD model, PC12 cells were treated with 1 mM MPP^+ for 24 h. The protein expression levels of NogoA were detected and, as shown in Fig. 1, the protein expression level of NogoA was markedly upregulated following MPP^+ treatment. The expression levels of NgR were also detected, and western blotting revealed that the protein expression level of NgR was also increased following MPP^+ treatment. These results indicated that NogoA may be involved in MPP^+-induced neurotoxicity in PC12 cells. To further investigate the possible regulatory mechanism of NogoA in MPP^+-induced PD model, the expression levels of mTOR and STAT3, which are key proteins in of the mTOR/STAT3 signaling pathway, were also detected. As shown in Fig. 1, the protein expression levels of mTOR and STAT3 were markedly upregulated following MPP^+ treatment. This result indicated that the mTOR/STAT3 signaling pathway may be involved in MPP^+-induced neurotoxicity in PC12 cells.

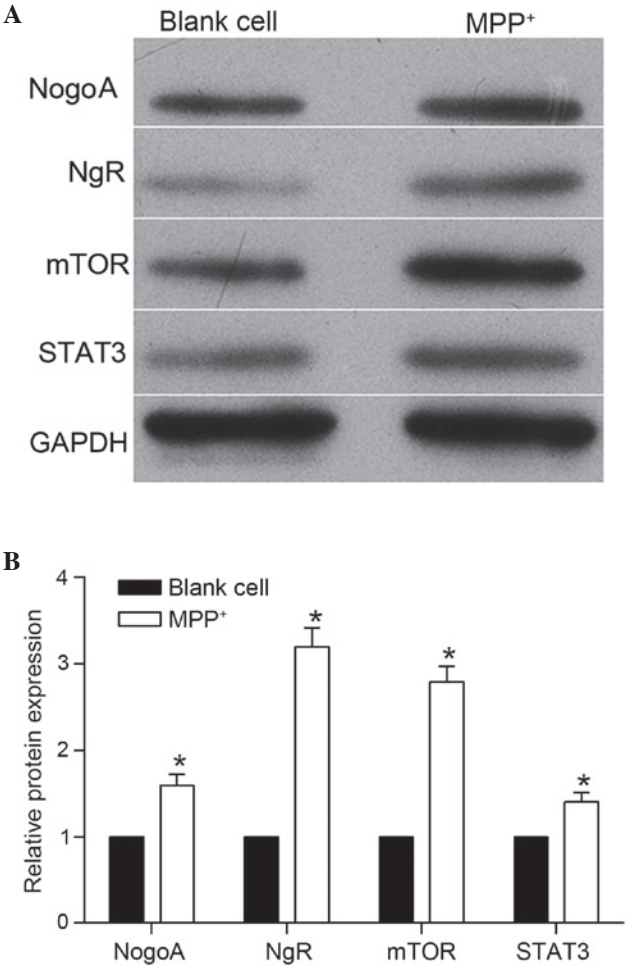

Figure 1. Expression levels of NogoA, NgR, mTOR and STAT3 following MPP^+ treatment in PC12 cells were determined by western blotting. (A) Representative western blotting showing the protein expression levels. (B) Quantification of the expression levels, expressed as the fold increase ($^*P<0.05$, compared with the blank cell). MPP^+, 1-methyl-4-phenylpyridinium; NgR, NogoA receptor; mTOR, mechanistic target of rapamycin; STAT3, signal transducer and activator of transcription 3.

Knockdown of NogoA inhibits the decrease in cell viability induced by MPP^+ in PC12 cells. To evaluate the role of NogoA, NogoA knockdown and overexpression models were established via transfection with NogoA siRNA or a NogoA overexpression vectors. As shown in Fig. 2A and B, the expression of NogoA was successfully decreased and overexpressed in the knockdown and overexpression models, respectively. A CCK8 assay was performed to evaluate the effects of NogoA against the MPP^+-induced significant decrease in cell viability (Fig. 2C). The results indicated that 1 mM MPP^+ significantly decreased the viability of the PC12 cells in the NC group. However, the viabilities of the PC12 cells pretreated with NogoA siRNA for 24 h were markedly increased, compared with the NC group. The cell viability in the NogoA overexpression group was similar to that in the NC group, whereas the cell viability in the rapamycin treated group was markedly increased, compared with the NC group. Therefore,

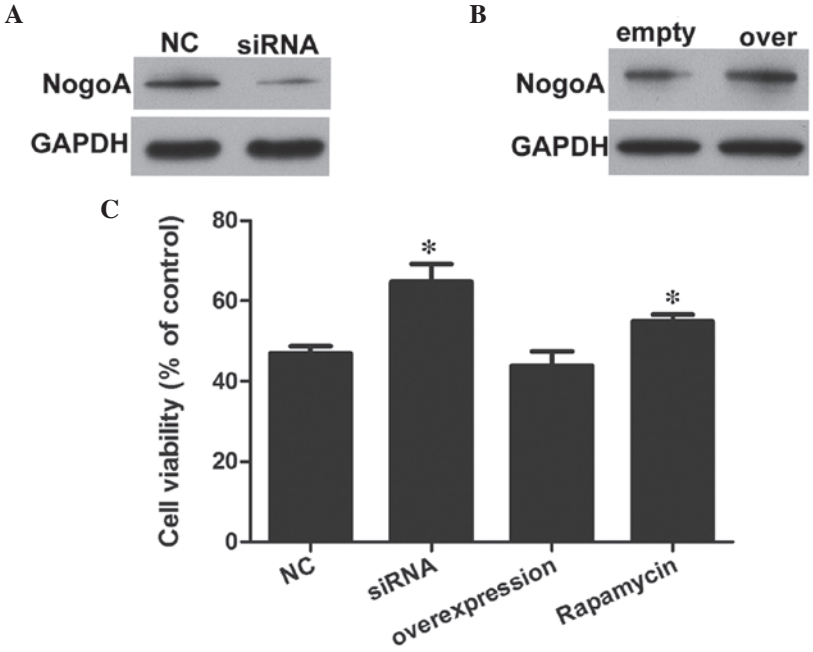

Figure 2. Effect of NogoA knockdown on the viability of PC12 cells induced by MPP^+. (A) Protein expression of NogoA following transfection with NC and NogoA siRNA. (B) Protein expression of NogoA following transfection with pcDNA3.1 NogoA overexpression vector or pcDNA3.1 empty vector. (C) Viability was determined using a Cell Counting Kit-8 assay. The NC group was pretreated with 200 nmol/l NC siRNA for 24 h, prior to exposure to 1 mM MPP^+ for 24 h; the siRNA group was pretreated with 200 nmol/l NogoA siRNA for 24 h, prior to exposure to 1 mM MPP^+ for 24 h; the overexpression group was transfected with NogoA overexpression vector for 48 h; the rapamycin group was pretreated with rapamycin for 24 h, prior to exposure to 1 mM MPP^+ for 24 h. Samples were examined in triplicate and data are presented as the mean ± standard deviation. $^*P<0.05$, vs. NC group. NC, negative control; siRNA, small interfering RNA; MPP^+, 1-methyl-4-phenylpyridinium; empty, empty vector; over, overexpression vector.

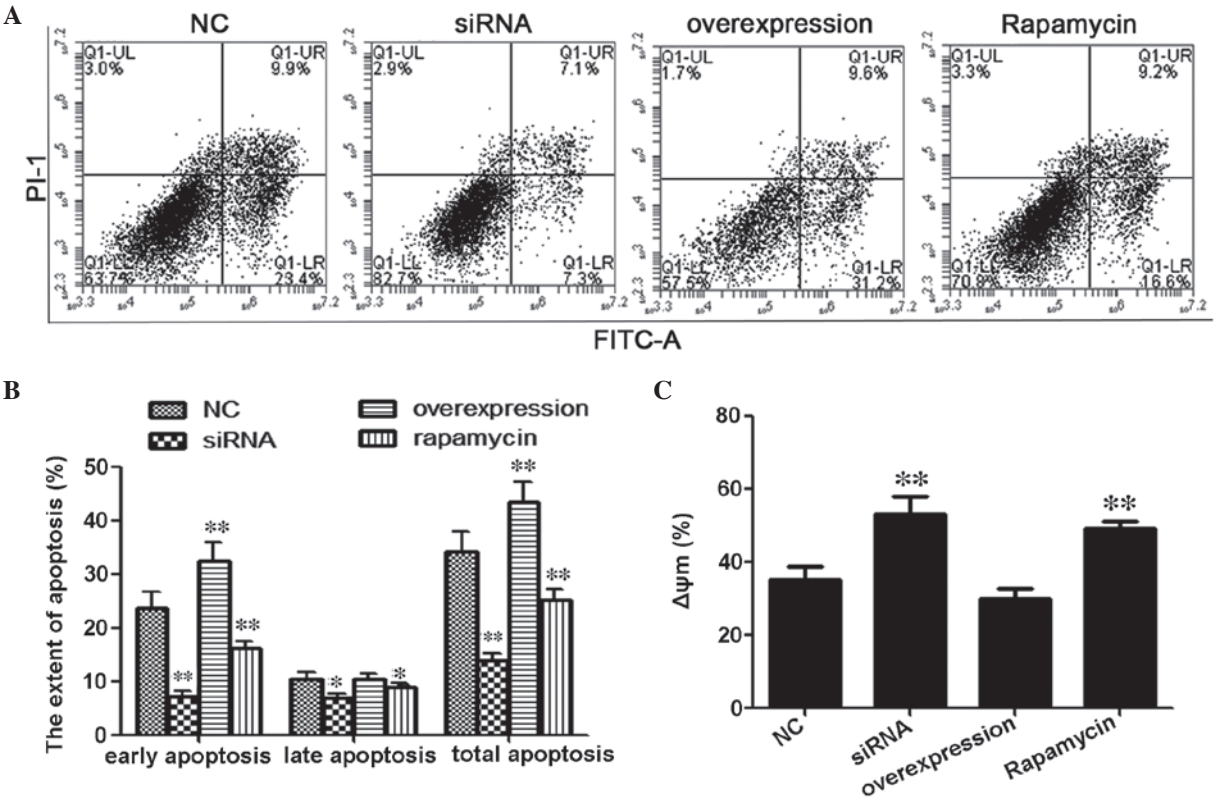

Figure 3. Effect of NogoA silencing on the apoptosis of PC12 cells induced by MPP^+. The cells were examined using flow cytometry with Annexin FITC/PI staining. (A) Representative flow cytometric analysis graphs for each group. (B) Extent of apoptosis among the experimental groups. The apoptotic ratio combined the early and late apoptosis percentage. (C) Cell ΔΨm was determined using a JC-1 assay. The NC group was pretreated with 200 nmol/l NC siRNA for 24 h, prior to exposure to 1 mM MPP^+ for 24 h; the siRNA group was pretreated with 200 nmol/l NogoA siRNA for 24 h, prior to exposure to 1 mM MPP^+ for 24 h; the overexpression group was transfected with NogoA overexpression vector for 48 h; the rapamycin group was pretreated with rapamycin for 24 h, prior to exposure to 1 mM MPP^+ for 24 h. Data are presented as the mean ± standard deviation. $^{**}P<0.01$, vs. the NC group. NC, negative control; siRNA, small interfering RNA; MPP^+, 1-methyl-4-phenylpyridinium; ΔΨm, mitochondrial membrane potential; FITC, fluorescein isothiocyanate; PI, propidium iodide.

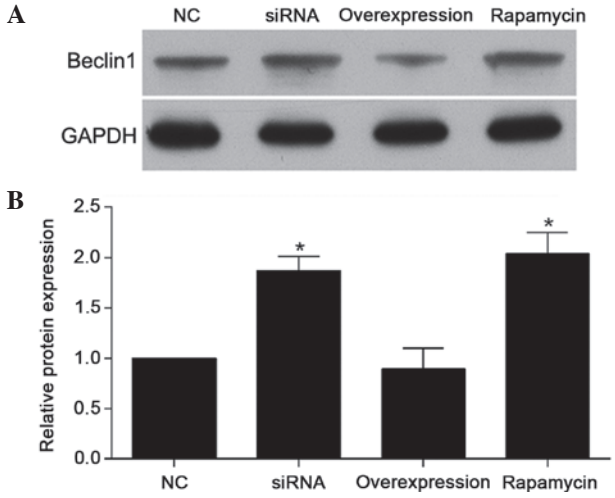

Figure 4. Effect of NogoA knockdown on the expression of Beclin-1. (A) Representative western blotting of Beclin-1. (B) Quantification of the expression levels of Beclin-1 in each group, presented as fold-increase. The NC group was pretreated with 200 nmol/l NC siRNA for 24 h, prior to exposure to 1 mM MPP^+ for 24 h. The siRNA group was pretreated with 200 nmol/l NogoA siRNA for 24 h, prior to exposure to 1 mM MPP^+ for 24 h. The overexpression group was transfected with NogoA overexpression vector for 48 h and the rapamycin group was pretreated with rapamycin for 24 h, prior to exposure to 1 mM MPP^+ for 24 h. $^*P<0.05$, vs. NC group. NC, negative control; siRNA, small interfering RNA; MPP^+, 1-methyl-4-phenylpyridinium.

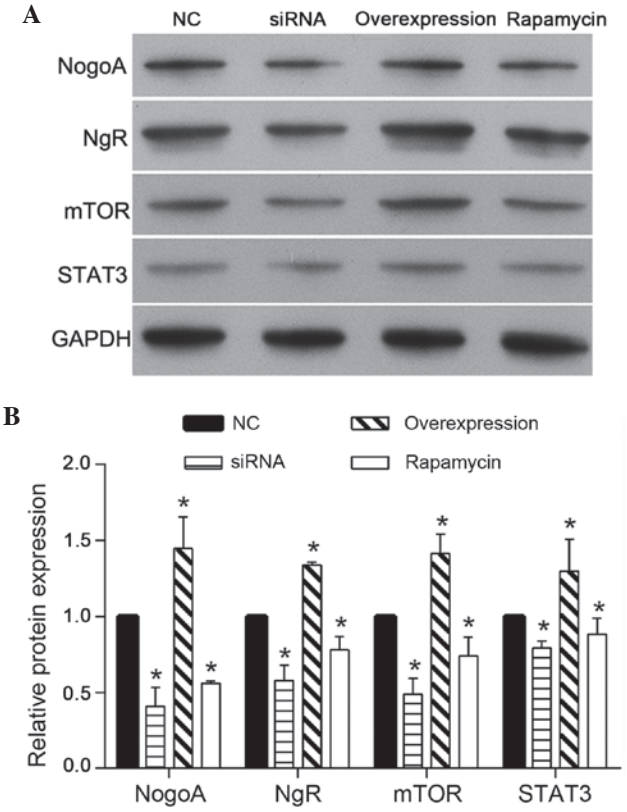

Figure 5. Protein expression levels of NogoA, NgR, mTOR and STAT3 in each group of PC12 cells. (A) Representative western blotting of NogoA, NgR, mTOR and STAT3 protein expression. (B) Quantification of the expression levels of NogoA, NgR, mTOR and STAT3 in each group, presented as the fold-increase. The NC group was pretreated with 200 nmol/l NC siRNA for 24 h, prior to exposure to 1 mM MPP^+ for 24 h. The siRNA group was pretreated with 200 nmol/l NogoA siRNA for 24 h, prior to exposure to 1 mM MPP^+ for 24 h. The overexpression group was transfected with the NogoA overexpression vector for 48 h and the rapamycin group was pretreated with rapamycin for 24 h, prior to exposure to 1 mM MPP^+ for 24 h. $^*P<0.05$, vs. NC group. NC, negative control; siRNA, small interfering RNA; MPP^+, 1-methyl-4-phenylpyridinium.

pretreatment with either the NogoA siRNA or mTOR/STAT3 signaling pathway inhibitor had neuroprotective effects against MPP^+-induced cytotoxicity.

Knockdown of NogoA inhibits the decrease in cell autophagy induced by MPP^+ in PC12 cells. To examine the effect of NogoA and rapamycin treatment on MPP^+-induced autophagy, the expression level of Beclin-1 was also detected (Fig. 4). The results demonstrated that the expression levels of Beclin-1 were increased in the NogoA siRNA group and rapamycin group, compared with the NC group. In addition, the effect of NogoA overexpression on the expression of Beclin-1 was similar to the effect observed in the NC group, which was pretreated with 200 nmol/l NC siRNA for 24 h prior to exposure to 1 mM MPP^+ for 24 h.

NogoA silencing and rapamycin treatment decrease the expression levels of NgR, mTOR and STAT3. To investigate the regulatory mechanism of NogoA on PC12 cells treated with MPP^+, the protein expression levels of NgR, mTOR and STAT3 were detected using western blotting. As shown in Fig. 5, the expression levels of NgR, mTOR and STAT3 were decreased in the NogoA siRNA and rapamycin groups, compared with the NC group. In addition, the effect of NogoA overexpression was similar to the effect observed in the NC group, which was pretreated with 200 nmol/l NC siRNA for 24 h prior to exposure to 1 mM MPP^+ for 24 h.

Discussion

In the present study, NogoA was found to be upregulated by MPP^+ treatment, which suggested that NogoA may be involved in MPP^+-induced neurotoxicity in PC12 cells. The present study subsequently investigated the effect of NogoA knockdown on MPP^+-induced cell proliferation, autophagy and apoptosis in the PC12 cells. The results demonstrated that NogoA knockdown inhibited MPP^+-induced apoptosis, and decreases cell viability and autophagy. These results suggested that a reduction in NogoA levels may prevent MPP^+-induced neurotoxicity in PC12 cells.

NogoA, particularly neuronal NogoA, regulates the modulation of neuronal and synaptic plasticity, and adult neurogenesis in the adult brain (25-27). A previous study suggested that the Nogo signaling pathway is important in neuropsychiatric diseases of neurodevelopmental origin, notably schizophrenia and bipolar disorder (28). Based on these results, NogoA appears to be an important regulator in the nervous system. PC12 cells treated with MPP^+ have been frequently used as a PD model to investigate the molecular mechanisms of PD *in vitro* (29,30). The results of the present study indicated that the knockdown of NogoA prevented MPP^+-induced neurotoxicity in the PC12 cells, and it was hypothesized that NogoA may be involved in regulating the development of PD and may assist in further elucidating the role of NogoA in regulating PD.

mTOR is a serine-threonine protein kinase, which regulates multiple intracellular processes in response to extracellular

signals, nutrient availability, the energy status of the cell and stress (31). In the nervous system, mTOR regulates the differentiation, survival and development of neurons (31). The mTOR-dependent signaling pathway is regulated by various types of protein in the neuronal cell membrane, however, the regulatory protein in patients with PD remains to be elucidated. In the present study, MPP^+ treatment was found to activate the mTOR/STAT3 signaling pathway, and that this activation was inhibited following a decrease in the expression of NogoA. Furthermore, the effect of NogoA siRNA on the mTOR/STAT3 signaling pathway was similar to that of rapamycin. Therefore, it was hypothesized that NogoA is a regulatory protein of the mTOR/STAT3 signaling pathway on the cell membrane in PC12 cells treated by MPP^+.

The mTOR signaling pathway also regulates the cellular processes of autophagy and apoptosis (32,33). Autophagy and apoptosis are two important cellular processes with complex and associated protein networks (34). In the PC12 cells, the level of autophagy, indicated by the protein expression of Beclin-1, increased following NogoA siRNA or rapamycin treatment, whereas the rate of apoptosis decreased. These results indicated that NogoA hass dual functions in regulating autophagy and apoptosis via the mTOR/STAT3 signaling pathway.

In conclusion, the present study demonstrated that NogoA was upregulated by MPP^+ treatment, and that NogoA knockdown inhibited the MPP^+-induced apoptosis, and decrease in cell viability and autophagy. In addition, the mTOR/STAT3 signaling pathway was involved in the regulation of NogoA on MPP^+-induced PC12 cells. Therefore, NogoA may regulate MPP^+-induced neurotoxicity in PC12 cells via the mTOR/STAT3 signaling pathway. The present results further demonstrated that NogoA may be a potential target protein for PD treatment. In addition, these results provided a novel mechanism regarding NogoA on regulating the process of PD.

Acknowledgements

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