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Research Article

LncRNA NR_030777 Alleviates Paraquat-Induced Neurotoxicity by Regulating Zfp326 and Cpne5

Hongyu Yang,[*,†,2] Qingxia Lin,[*,e] Nengzhou Chen,[*,e] Zhousong Luo,[*] Chunyan Zheng,[*] Jing Li,[*] Fuli Zheng,[*,‡] Zhenkun Guo,[‡,§] Ping Cai,[‡,§,¶] Siying Wu,[§,‖,1] Yuan-Liang Wang,[*,‡,1] and Huangyuan Li[*,‡,§,1]

[*]Department of Preventive Medicine, School of Public Health, Fujian Medical University, Fuzhou 350122, China; [†]Department of Labor Hygiene and Environmental Hygiene, School of Public Health, Guangdong Pharmaceutical University, Guangzhou 510006, China; and [‡]The Key Laboratory of Environment and Health [§]Fujian Provincial Key Laboratory of Environment Factors and Cancer [¶]Department of Health Inspection and Quarantine; and [‖]Department of Epidemiology and Health Statistics, School of Public Health, Fujian Medical University, Fuzhou 350122, China

[1]To whom correspondence should be addressed at School of Public Health, Fujian Medical University, No. 1 Xuefu North Road, University Town, Fuzhou 350122, China. E-mail: sywu@fjmu.edu.cn, E-mail: wangyl@fjmu.edu.cn, and E-mail: lhy@fjmu.edu.cn.

[2]Hongyu Yang, Qingxia Lin, and Nengzhou Chen contributed equally to this study.

ABSTRACT

Paraquat (PQ) is herbicide widely used in agricultural production. It is identified as an environmental toxicant that could lead to neurodegeneration damage. Parkinson's disease (PD) is a central nervous system degenerative disease that occurs in the elderly. Main risk factors for PD include genetic and environmental variables, but its specific mechanism is still not well understood. Emerging evidence suggests that long noncoding RNAs (lncRNAs) play an important role in PD. LncRNA NR_030777 has a full length of 2208 bp and is highly conserved among species. RNA profiling showed a significant alteration in lncRNA NR_030777 expression upon PQ-induced neurotoxicity. However, little is known on the functional relevance of lncRNA NR_030777 in the development of PQ. In this study, we discovered a vital protective role of lncRNA NR_030777 in PQ-induced neurotoxicity. The expression of NR_030777 correlates with elevated level of reactive oxygen species induced by PQ. In addition, activated expression of NR_030777 alleviates neurotoxicity by regulating the expression of Zfp326 and Copine 5. We report that lncRNA NR_030777 has a vital protective role in neurotoxicity induced by environmental toxicants such as PQ. This study could serve as an exemplary case for lncRNAs to be considered as a potential target for the prevention and treatment of PQ-induced neurodegenerative disorders such as PD.

Key words: LncRNA; neurotoxicity; toxicoepigenetics; paraquat; Parkinson's disease.

Parkinson's disease's (PD) main pathologic feature is degeneration of dopaminergic neurons in the nigral-striatum system, which leads to decreased dopaminergic secretion and a series of extravertebral system reactions (Olanow and Tatton, 2009), and the main etiology remains unclear. Main risk factors for PD include genetic factors, environmental factors, increased oxidative stress, mitochondrial dysfunction, accelerated aging, excitatory neurotoxicity, calcium cytotoxicity, infection, immunological abnormalities, etc. (Barnham and Bush, 2008; Brown *et al.*, 2006; Jones and Miller, 2008; Lai *et al.*, 2002; Peng *et al.*, 2007; Petersen *et al.*, 2008). Although genetic factors are inherent properties in the occurrence and development of PD, environmental factors also play a significant role (Andersen and Lim, 2017; Mathy and Chen, 2017; Muers, 2011; Rinn *et al.*, 2007). The

discovery of the α-synuclein (α-syn) is an important event in the history of PD research. It was found to be present in the Italian PD family and plays an important role in the pathogenesis of PD (Kebabian and Calne, 1979). However, the molecular and cellular mechanisms underlying the development of PD have not yet been fully clarified.

As for environmental factors, paraquat (PQ) is a herbicide widely used in agricultural production. Paraquat enters the body through inhalation from the respiratory tract, skin contact, or contaminated food. Numerous studies have shown that environmental chemicals, such as pesticides, metals, polychlorinated biphenyls, solvents, and suspended particles, are risk factors for PD (Bellou *et al.*, 2016; Pang *et al.*, 2019; Rai and Singh, 2020; Ponting *et al.*, 2009; Peng *et al.*, 2004). Paraquat reduces dopamine (DA) levels and the number of DA neurons in the substantia nigra (SN) (Drechsel and Patel, 2008; Rai and Singh, 2020; Richter *et al.*, 2017). Paraquat decreases the activity of DA, the number of DA neurons in the SN and increases the expression of α-syn protein that accumulates in the dopaminergic neurons in the ventral brain of mice (Dauer and Przedborski, 2003; Drechsel and Patel, 2008; Manning-Bog *et al.*, 2002; Peng *et al.*, 2004). These studies suggest that PQ may be one of the environmental factors leading to PD, but its specific molecular mechanism remains to be further elucidated.

Genetic factors are considered to be important factors in the development of PD, among which epigenetic factors may be an crucial node to reveal the pathogenesis of PD. Long noncoding RNAs (lncRNAs) as an important molecule of epigenetic regulation, generally refer to noncoding RNA transcripts greater than 200 nucleotides in length. LncRNAs could regulate gene expression at multiple levels, affecting numerous physiologic and pathologic processes in the body (Sun *et al.*, 2017; Wang *et al.*, 2017; Wu and Du, 2017). LncRNAs are closely related to species evolution, embryo development, metabolism, cell fate determination, tumorigenesis, and neurologic diseases (Fitzpatrick *et al.*, 2002; Szyliowicz, 2006). Genetic research of the nervous system found that the temporal and spatial expression of lncRNAs play an important role in the growth and development of the nervous system, and in neurotoxicity induced by neurotoxic substance (Ye *et al.*, 2018). LncRNAs are important components of noncoding RNAs involved in neuronal differentiation and brain development processes (Pizard *et al.*, 2001). In addition, lncRNAs are involved in the maintenance of neural morphology and characteristics at developmental stages and affect the development of PD by regulating mitochondrial homeostasis-related genes (Bates *et al.*, 1991; Grigoriadis *et al.*, 1988; Wu and Du, 2017). Therefore, lncRNA, an important element in epigenetic regulation, may play a crucial role in neurodegeneration induced by PQ (Logan *et al.*, 2018; Wang *et al.*, 2018).

To investigate the role of lncRNA in neurotoxicity induced by PQ, we have successfully established an animal model of PQ-induced neurodegeneration damage, and screened for differentially expressed lncRNA in the *SN* tissues of normal and PQ-induced PD mice (Lee *et al.*, 2000). Among numerous differentially expressed lncRNAs, NR_030777 attracted our attention because it is highly conserved among species. NR_030777 is 2208 bp long and overlaps with the Zfp326 (Zinc finger protein 326, also known as ZAN75) gene, with the same transcription direction as Zfp326 gene. Therefore, it is a sense lncRNA. NR_030777 is highly expressed in the *SN* tissues of mice, and is mainly located in the cytoplasm of nerve cells by fluorescence in situ hybridization (FISH) assays (Lee *et al.*, 2000). ZFP326 is a transcription activator with DNA-binding activity highly expressed in the brain and heart. It is involved in the regulation of cell cycle, cell growth, embryonic development, and neuronal differentiation (Luca *et al.*, 2013). Copine 5 (CPNE5) (also known as Copine5) is a member of calcium-dependent lipid-binding copine proteins that are evolutionarily conserved from *Arabidopsis* to humans (Ding *et al.*, 2008). We observed changes in Cpne5 mRNA expression through coding-noncoding (CNC) prediction and comparison with mouse SN samples from PQ-treated versus -untreated microarrays (Wang *et al.*, 2018). Therefore, NR_030777 may participate in the process of PQ-induced neurotoxicity by regulating Zfp326 and Cpne5.

Our study demonstrates that there is a specific protective role of lncRNA NR_030777 in PQ-induced neurotoxicity. We showed that NR_030777 regulated the expression and stability of Zfp326 and Cpne5, and exerted protections on motor function in PQ-induced neuronal damage. In addition, we evaluated the effects of NR_030777 in PQ-induced neurotoxicity and revealed the role of reactive oxygen species (ROS) in NR_030777 up-regulation. We also found that pre-RNA splicing factor splicing factor 3b subunit 3 (SF3B3) mediated the regulation of Zfp326 and NR_030777.

MATERIALS AND METHODS

Cell Culture

Mouse neuroblastoma cells (Neuro-2a, N2a) were purchased from cell bank of Chinese Academy of Sciences (Shanghai, China). Mouse dopaminergic cells (MN9D) were kindly presented by Dr Xiaoxu Mao, Southern Medical University. Cells were cultured in DMEM (HyClone) or F12 (HyClone) medium supplemented with 10% fetal bovine serum (FBS, HyClone), 100 U/ml penicillin, and 50 μg/ml streptomycin, and were kept in an incubator with humidified atmosphere of 5% CO_2 at 37°C. NR_030777 overexpression (OE) N2a cell line was kept in the medium supplemented with 0.6 μg/ml puromycin.

Primary Cortical Neuron Culture and Purity Identification

Postnatal P1 – 2 mouse pups were used for primary neuron isolation. The cortex was dissected and placed in digestion solution containing 1 mg/ml papain (Worthington), DMEM/HIGH GLUCOSE (HyClone), 50 U/ml DNase I (Worthington) for 30 min at room temperature. The activity of trypsin was stopped by adding 15% FBS (GIBCO) in DMEM/HIGH GLUCOSE. The tissue was dissociated using a 1 ml pipette by gentle trituration on the side of the tube 15 times, until no large chunks of tissue remained. The dissociated tissue was centrifuged at 1000 rpm for 5 min and the cell pellet was resuspended in Neurobasal medium (GIBCO) containing 2% B27 supplement (GIBCO) and 1% GlutaMAX (GIBCO). All cultures were maintained at 37°C in a humidified 5% CO_2-containing atmosphere. Subsequent experiments were performed on primary neurons on days 10 – 12 (DIV 10 – 12) *in vitro*.

Construction of NR_030777 overexpression N2a Cell Line

Recombinant lentiviral vector carrying lncRNA NR_030777 was constructed with standard molecular techniques which was provided by GenePharma Co., Ltd (Shanghai, China): (1) Acquire target gene sequence fragment of NR_030777; (2) Enzyme cleavage by *NotI* and *BamHI* was performed on lentivirus 5 (LV5) and the recombinant amplified target gene fragment was cloned into linear LV5; (3) Sequencing verification and mass extraction. N2a cells were infected with the recombinant lentivirus to generate stably transfected cells. Concentrated lentiviruses were

Table 1. The Sequence of siRNA-NR_030777, siRNA-258, siRNA-368, and siRNA-Sf3b3

	Sense5′−3′	Antisense5′−3′
siRNA-NR_030777	GGUCUGGCAUUCAAGGAUATT	UAUCCUUGAAUGCCAGACCTT
siRNA-258	CCAUGGAUUCCUACCUAAATT	UUUAGGUAGGAAUCCAUGGTT
siRNA-368	GGGCGAGAUCUGUACAGAUTT	AUCUGUACAGAUCUCGCCCTT
SiRNA- Sf3b3	GCUAACAGGUGGCACCAAATT	UUUGGUGCCACCUGUUAGCT

Table 2. The Primers for lncRNA NR_030777, zfp326, cpne5, and Sf3b3 mRNA

	Forward	Reverse
lncRNA NR_030777	TCTTACGGAGGTCTGGCATTCA	CTCTTGCAGTAGCAATCGTCACA
zfp326mRNA	TCGAGCATGGACTTCGAGGA	CCATGCCATAGGACTGGTTTAGGTA
cpne5 mRNA	GACGCACCGAGGTCATAGATAACA	TCCGGGCTCTTGGAGTCAAC
Sf3b3 mRNA	CAGTCCGTCGGCACATTGA	AGGTTGGAGTGGTGCCCAAG

transfected at a multiplicity of infection of DMEM with 5 μg/ml polybrene. The supernatant was replaced with complete culture medium after 24 h. The expression of lncRNA NR_030777 in infected cells was validated by quantitative real-time PCR (qRT-PCR).

Small Interfering RNA Transfection

Small interfering RNA (SiRNA) that targeted NR_030777 (siRNA-NR_030777), Zfp326 (siRNA-258 and siRNA-368), and a scrambled negative control (NC) (siRNA NC) were provided by GenePharma Co., Ltd (Shanghai, China). The sequences for all siRNAs were shown in Table 1. Because the siRNA-258 knockdown (KD) effect is better, it is used for subsequent experiments. N2a cells were transfected with either 80 nmol of siRNA-NR_030777, siRNA-Zfp326, or siRNA-NC using DharmaFECTTM1 transfection reagent (GE Healthcare Dharmacon, Inc.) according to the manufacturer's instruction. After incubation for 24 h, cells were harvested for qRT-PCR to determine the transfection efficiency and follow-up testing.

Chemicals Treatment *In Vitro*

Paraquat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treatment. Paraquat dichloride hydrate (99.2% w/w) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) hydrochloride were purchased from Sigma Aldrich Co. (St. Louis, Missouri). NR_030777 overexpressing or KD N2a cells were treated with 100, 300 μM PQ for 24 and 36 h refer to our previous series studies of PQ-induced neurotoxicity (Wang *et al.*, 2017, 2018). Then, the cells were harvested for follow-up experiments.

N-acetylcysteine and diphenyleneiodonium chloride treatment. N-acetylcysteine (NAC) and diphenyleneiodonium chloride (DPI) were purchased from Sigma Aldrich Co. After 24 h incubation, N2a cells were switched to medium free heat-inactivated FBS for treatment. N2a cells were incubated with or without 5 mM NAC or 10 mM DPI for 2 h, followed by exposure to 200 μM PQ for 24 h. NAC and DPI were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 0.1% (vol/vol) and had no effect on the parameters measured.

H_2O_2 treatment. H_2O_2 was purchased from Sigma Aldrich Co. After 24 h incubation, N2a cells were treated with 15, 30, 45 μM H_2O_2 for 24 h. H_2O_2 was dissolved in DMEM.

Quantitative Real-Time PCR

The expression of lncRNA NR_030777, zfp326, and cpne5 mRNA was analyzed by qRT-PCR. Total RNA was extracted using TRIzol (Invitrogen, California); 1 μg of each of the total RNA was subjected to cDNA synthesis. First-strand cDNA was generated using the PrimeScript RT reagent kit with gDNA Eraser (Takara, RR047A). qRT-PCR was processed by SYBR Premix Ex TaqTM II (Takara, DRR820A) according to the manufacturer's instructions. The PCR protocol consisted of 3 steps: 30 s at 95°C, 40 cycles of 5 s at 95°C and 20 s at 58°C, or 15 s at 95°C and 1 min at 60°C. The expression level of the target gene was normalized to the level of GAPDH in the same sample. The primers for lncRNAs and mRNAs were shown in Table 2.

Preparation of Total Cellular Protein

After treatment, cells were collected by scraping, and washed thrice with ice-cold phosphate buffered saline. Total cellular protein was extracted with radio immunoprecipitation assay lysis buffer including 10% proteinase inhibitor phenylmethylsulfonyl fluoride and protease inhibitor cocktail (PIC) (from Phygene, Fuzhou, China) according to the manufacturer's protocol. The protein concentrations were determined by a bicinchoninic acid (BCA) protein assay kit. The BCA protein assay kit.

Western Blotting

Briefly, protein extracts were resolved by 10%−12% (wt/vol) sodium dodecyl sulfate-polyacrylamide gel, transferred to nitrocellulose membranes, and blocked for 2 h in Tris-buffered saline (TBS), 0.1% (vol/vol) Tween-20 and 5% milk. The polyvinylidene fluoride membranes were incubated for 2 h with primary antibody diluted in the same buffer. Dilution for anti-ZFP326 antibody 1:800 (ProteinTech, Chicago), anti-CPNE5 antibody 1:2000 (Abcam, Cambridge, UK), anti-α-syn antibody 1:2000 (Abcam, Cambridge, UK), and anti-GAPDH antibody 1:10000 (Abcam, Cambridge, UK). After washing with 0.1% (vol/vol) Tween 20 in TBS, the membranes were incubated with HRP-labeled Goat Anti-Rabbit IgG (H + L) (Beyotime Biotechnology, Shanghai, China) secondary antibody 1:5000 for 1 h at room temperature. Western blot images were captured with the Tanon 5200 Automatic Chemiluminescence Image Analysis System (Shanghai, China). Data were analyzed using Tanon Image software (Shanghai, China).

mRNA Stability Assay

N2a cells were seeded in 6-well plates. A 5 μg/ml actinomycin D (ActD) (APExBIO, Huston) was added to inhibit *de novo* RNA synthesis. Total RNA was collected at indicated times, and mRNA expression was measured by qRT-PCR. mRNA half-life was determined by comparing to the mRNA level before adding ActD.

Cell Counting Kit-8 Assay

N2a cells were seeded in 96-well plates. After upstream operations, 10 μl Cell counting Kit-8 (CCK-8, Boster, Wuhan, China) was incubated with 100 μl DMEM at 37°C for an additional hour. The absorbance of the cell suspensions was determined at 450 nm.

Flow Cytometry

Cell cycle and apoptosis were detected by flow cytometry. After harvest, N2a cells were resuspended in binding buffer at a density of 1×10^6 cells/ml. After double-staining with fluorescein isothiocyanate (FITC)-Annexin V/propidium iodide (PI) or 7-aminoactinomycin D (7-AAD) and PE using FITC-Annexin V Apoptosis Detection Kit I and PI/RNase Staining Buffer (BD Biosciences, San Jose, CA). The cells were analyzed using an FACScan flow cytometer equipped with Cell Quest software (BD Biosciences) according to the manufacturer's instructions. Experiments were performed in triplicate.

Animals, PQ/MPTP Treatment, and Dissection of Brain Tissues

All mice were provided by Shanghai Model Organisms. At the time of experiment, male mice were 6 – 8 weeks of age with weight between 18 and 22 g, consistent with previous studies (Lee *et al.*, 2000; Vernice and Serge, 2007). The reason for selecting male mice is that MPTP fatality rate is higher in females. Mice were housed in cages at an ambient temperature of 20 – 25°C under a 12 h light/dark cycle and allowed free access to food and water. The experimental protocols were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were carried out with the approval of the Animal Use Committee, Fujian Medical University. Efforts were made to minimize animal suffering.

Sixty male mice were equally divided into 6 groups: NC-saline, NC-PQ, NC-MPTP, OE-saline, OE-PQ, OE-MPTP. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP) was purchased from Sigma Aldrich Co. NC-saline and OE-saline groups were intraperitoneally injected with saline at 2-day interval. The NC-PQ and OE-PQ were intraperitoneally injected with 10 mg/kg PQ dichloride hydrate (dissolved in saline) at 2-day interval. NC-MPTP and OE-MPTP groups were administrated with 30 mg/kg MPTP (dissolved in saline) by hypodermic injection every day for 5 days as classic PD animal models (Li *et al.*, 2012; Peng *et al.*, 2004). A 10 mg/kg was chosen for the following reasons in this study. Firstly, permissible concentration-time weighted average (PC-TWA) of PQ is 0.5 mg/m^3 proposed by National Health Commission of the People's Republic of China, and based on the daily respiratory volume per person (10 – 20 m^3) and the uncertainty coefficient (100), the dose for the animal could be 10 – 20 mg/kg/day after conversion. Secondly, in our previous researches, we found that the mice developed neurodegenerative lesions after treated with this dose for 4 weeks (Li *et al.*, 2012; Wang *et al.*, 2017). Therefore, this dose of neurotoxicity is suitable for the establishment of PD mouse model and the study of the pathological mechanism of PD (Li *et al.*, 2012; Mak *et al.*, 2010; Prasad *et al.*, 2007).

For neurobehavioral and histological analyses, mice were executed under anesthesia 7 days after the last administration with 0.5% sodium pentobarbital and perfused via a left ventricular puncture of the heart with cold 0.9% saline (4°C) followed by 4% paraformaldehyde. The brains were removed and stored overnight in10% formalin solution. The coronal section (5 μM thick) passing through the *SN* was cut and then embedded in paraffin. For immunoblot analysis, mice were executed under anesthesia 4 h after the last administration. The brain tissue was immediately removed, and *SN* tissue was obtained from the coronal slices. The dissected brain tissue was immediately frozen and stored at −80°C until further processing.

Behavioral Testing

Behavioral testing was carried out 2 days after poisoning. For climbing experiment, a 2.5 cm diameter foam ball was fixed on the top of a 60 cm long and 1 cm thick wooden pole. The wooden pole was wrapped with 2 layers of gauze to prevent slipping. The mice were placed on the top of the ball and the time required for the mouse to climb the pole was recorded. For suspension experiment, the mice were suspended on a horizontal wire with their forelimbs, and the suspension time was recorded.

Hematoxylin-Eosin and Hoechst Staining

Formalin-fixed, paraffin-embedded tissue samples were sectioned into 5 μM-thick slices using a microtome. The tissue sections were deparaffinized, rehydrated, and stained using Hematoxylin-Eosin (HE) staining Kit (Phygene, Fuzhou, China), and Hoechst staining Kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. The images were observed by fluorescence microscopy (OLYMPUS Co., Japan) using appropriate filters for blue fluorescence.

Immunohistochemical Staining

The entire brain was sliced using a sliding microtome into 5 μM consecutive sections. Every third section was immunohistochemically processed for tyrosine hydroxylase (TH). For TH staining, anti-mouse TH antibody (diluted at 1:1000) and a Servicebio HRP-polymer anti-rabbit IHC kit were used. Sections incubated in the absence of a primary antibody were used as NCs. Six views were selected randomly for each section and observed under a light microscope (200× and 400× magnification).

Tagged RNA Affinity Purification Assays

Glutathione S-transferase-MS2 (GST-MS2) fusion expression vector and the NR_030777-MS2 stem-loop structure tandem repeat vector were co-transfected into the cells to obtain GST-MS2 fusion protein and NR_030777-MS2 fusion RNA. Because the MS2 protein can specifically bind to the MS2 stem-loop structure, GST-MS2/NR_030777-MS2 complex is formed in the cell. The cells were lysed and extracted by glutathione affinity agarose beads to obtain a target protein-RNA complex. RNA-protein complexes were purified. RNA samples were analyzed by next generation sequencing and protein samples were analyzed by mass spectrometry.

Coimmunoprecipitation Mass Spectrometry

A 2.5 g of N2a cells was lysed with Triton X-100 lysis buffer (100 mM NaCl, 0.5% Triton X-100, 1 mM Dithiothreitol, 2 mM $MgCl_2$) supplemented with PIC. Rabbit anti-SF3B3 polyclonal antibody-coated beads were added to the cell lysate and incubated on a rotator for 30 min at 4°C. The beads were collected with a magnet. Purified SF3B3 and its interacting proteins were subjected to mass spectrometry. The partial quantitative

Table 3. The Partial Quantitative Intensity for CoIP-MS

Protein Names	Gene Names	Intensity
Replication initiator 1	Repin1	8.18143179374966
Enhancer of mRNA-decapping protein 4 decapping protein 4	Edc4	4.08225078065478
DBIRD complex subunit Zfp326	Zfp326	8.39323080423521
MICOS complex subunit Mic60	Immt	3.84971939463907

intensity for co-immunoprecipitation mass spectrometry (CoIP-MS) showed in Table 3.

Statistical Analysis

Each data bar represents the mean values ± standard error (SE) of at least 3 independent experiments in all cases. Results were analyzed using IBM PASW Statistics 18.0 software (Chicago, Illinois). Differences between groups were analyzed by analysis of variance (ANOVA). If the F values were significant, the least significant difference post hoc test was used to compare multiple groups. A p value of $<.05$ was considered statistically significant in all cases.

RESULTS

Paraquat Alters lncRNA NR_030777, Zfp326, and Cpne5 Expression *In Vitro*

According to NCBI annotation, the physical maps depicting lncRNA NR_030777 and Zfp326 mRNA are shown in Figure 1A. First, we examined the expression of NR_030777, Zfp326, and Cpne5 mRNA in PQ treated mouse neuroblastoma N2a cells. Compared with the control group, both 100 and 300 μM PQ treatment for either 24 or 36 h increased NR_030777 level and decreased Zfp326 mRNA level (Figure 1B). For Cpne5, low dose treatment for 24 or 36 h upregulated Cpne5 mRNA level. The effects were less obvious with high dose treatment. Then, we checked the protein level of ZFP326 and CPNE5 in N2a damage induced by PQ. In 24 h treatment group, the protein level of ZFP326 increased under both 100 and 300 μM PQ treatment, whereas the protein level of CPNE5 showed down regulation with 100 and 300 μM PQ treatment (Figure 1C). In 36 h treatment group, both ZFP326 and CPNE5 proteins were downregulated in 100 and 300 μM PQ treatment group (Figure 1C). We also verified the expression of NR_030777, Zfp326, and Cpne5 mRNA level in MN9D cells and primary cortical neuron. The fluorescence intensity of microtubule associated protein 2 (MAP2) prompted that the purity of the primary cortical neuron was quite high (Figure 1E). The results were similar to that of N2a cells (Figs. 1D and 1F). These results suggest that NR_030777, Zfp326, and Cpne5 might be involved in the neurodegeneration induced by PQ.

LncRNA NR_030777 Involves in Regulation of Zfp326 and Cpne5 Expression in Response to PQ in N2a Cells

To determine whether NR_030777 regulates its potential downstream target genes in PQ-induced damage, NR_030777 KD or overexpressing N2a cells were subjected to various amounts of PQ treatment (Figure 2A). The PQ treatment decreased Zfp326 mRNA and protein levels, and KD of NR_030777 caused further reduction in Zfp326 mRNA and protein expression (Figs. 2B and 2C). Surprisingly, OE of NR_030777 also led to a decrease in Zfp326 expression (Figs. 2B and 2E). This could be due to the complex regulatory mechanisms of lncRNAs and requires further investigation.

The PQ treatment caused a reduction in the protein level of CPNE5. However, KD of NR_030777 rescued that effect (Figure 2C). Overexpression of NR_030777 also caused an increase in Cpne5 mRNA and protein levels after PQ treatment (Figs. 2D and 2E).

Because there is a close relationship between mRNA stability and gene regulation (Maret *et al.*, 2004), we explored whether NR_030777 affected the stability of Zfp326 and Cpne5 mRNA. Knockdown of NR_030777 decreased the stability of Zfp326 mRNA, whereas OE of NR_030777 increased the stability of Zfp326 mRNA (Figure 2F). Neither KD nor OE of NR_030777 affected Cpne5 mRNA stability (Figure 2F).

LncRNA NR_030777 Regulates Cellular Activities and α-syn Expression in N2a Damage Induced by PQ

Our previous studies found that PQ inhibited cell proliferation, induced apoptosis, and caused cell cycle arrest (Wang *et al.*, 2017). Therefore, we further investigated the effect of NR_030777 on cell cycle progression, cell proliferation, and apoptosis. Indeed, NR_030777 KD further reduced cell proliferation rate on top of PQ exposure, whereas OE of NR_030777 alleviated PQ-induced inhibition of cell proliferation (Figure 3A). For apoptosis, NR_030777 KD increased apoptosis in the presence or absence of PQ treatment, whereas OE of NR_030777 alleviated PQ-induced apoptosis (Figure 3B).

We further analyzed the flow cytometry results. Paraquat treatment reduced the proportion of cells in G2/M phase (Figure 3C). Knockdown of NR_030777 caused an even greater reduction of cells in G2/M phase, whereas OE of NR_030777 reversed this trend and increased the proportion of cells in G2/M phase. Neither PQ treatment nor the KD of NR_030777 made a significant change the proportion of cells in G0/G1 phase. However, OE of NR_030777 reduced the proportion of cells in G0/G1 phase, presumably due to the increase of cells in G2/M and S phase.

Previous studies showed that α-syn played a key role in the pathogenesis of PD, and increased expression of α-syn could induce rapid formation of lewy corpuscle, which is closely related to neurodegenerative diseases (Cheng *et al.*, 2018a). We checked the expression of α-syn to see if NR_030777 could regulate α-syn expression in N2a damage induced by PQ. We found that PQ induced the expression of α-syn significantly. NR_030777 KD resulted in a reduction of α-syn protein, whereas OE of NR_030777 increased the amount of α-syn protein (Figure 3D).

Reactive Oxygen Species Induces the Expression of lncRNA NR_030777

It is generally believed that ROS and oxidative stress are the main mechanism of nerve poisons such as PQ (Dinis-Oliveira *et al.*, 2006). Paraquat produces a large number of ROS by intracellular REDOX cycle and induces direct or indirect damages to cellular structure, thereby producing cytotoxicity (Majidinia *et al.*, 2016; Schwarzenbach, 2016; Wang *et al.*, 2017). Hence, PQ may damage cells by producing excessive ROS and change the

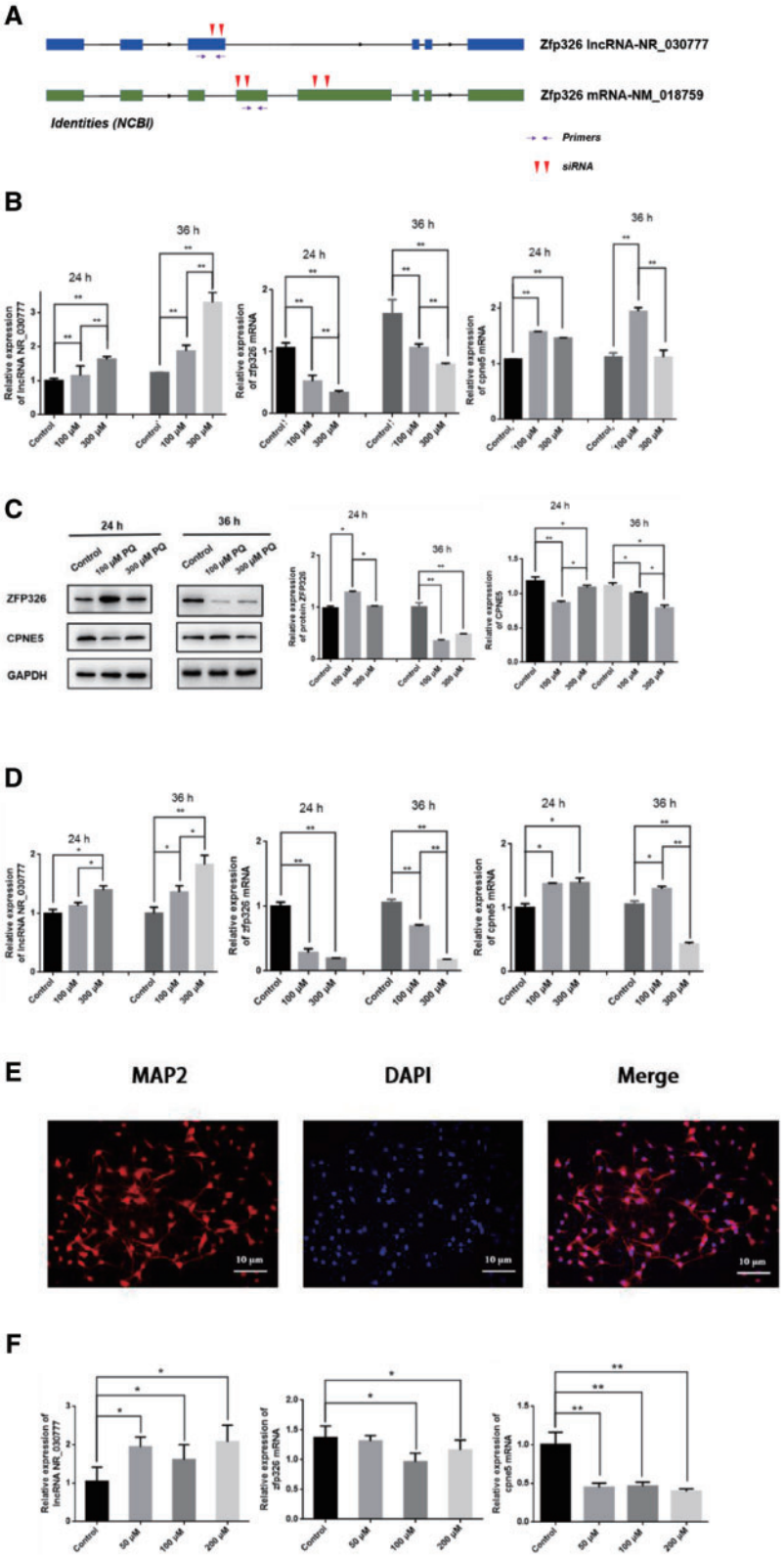

Figure 1. Paraquat alters lncRNA NR_030777, Zfp326 and Cpne5 expression *in vitro*. A, Physical maps depicting NR_030777 and Zfp326 mRNA-NM_018759. B, The level of lncRNA NR_030777, Zfp326, and Cpne5 mRNA in N2a cells. C, The expression of ZFP326 and CPNE5 protein in N2a cells. D, The level of lncRNA NR_030777, Zfp326, and Cpne5 mRNA in MN9D cells. E, The expression of MAP2 in primary cortical neuron. F, The level of lncRNA NR_030777, Zfp326, and Cpne5 mRNA in primary cortical neuron. Data represent the mean values ± standard error (SE) ($n = 3$). $^{*}p < .05$; $^{**}p < .01$.

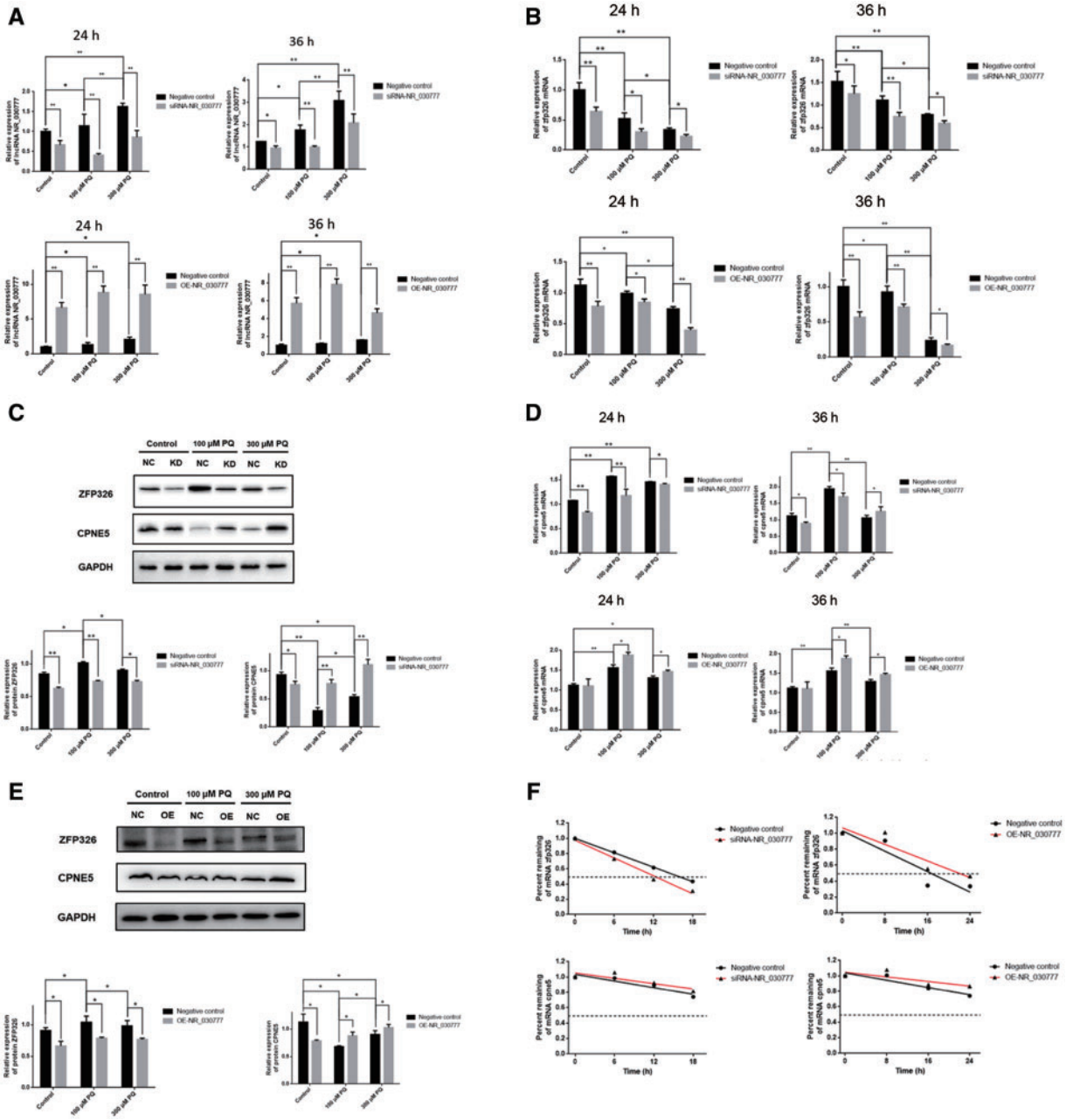

Figure 2. LncRNA NR_030777 involves in regulation of Zfp326 and Cpne5 expression in response to PQ in N2a cells. A, Knockdown (KD) or overexpression (OE) of NR_030777 in N2a cells. B, Effects of KD or OE of NR_030777 on Zfp326 mRNA expression. C, Knockdown effects of NR_030777 on CPNE5 and ZFP326 protein levels. D, Effects of KD or OE of NR_030777 on Cpne5 mRNA expression. E, Overexpression effects of NR_030777 on CPNE5 and ZFP326 protein levels. F, mRNA levels of Zfp326 and Cpne5 were measured at the indicated times with the addition of Actinomycin D (ActD, 5 μg/ml) in NR_030777 KD or overexpressing N2a cells. Data represent mean values ± SE ($n = 3$). $^{*}p < .05$; $^{**}p < .01$.

lncRNA expression state. Indeed, we found that PQ induced ROS production and NR_030777 expression in a dose-dependent manner (Figs. 4A, 4B, and 4E). When ROS was antagonized by NAC or DPI, the expression of NR_030777 was inhibited (Figs. 4C–4F). Similarly, another ROS-inducing agent, H_2O_2, also increased the expression of NR_030777 in a dose-dependent manner (Figure 4G).

Overexpression of NR_030777 in Nervous System of C57/BL6 Mice Alleviates Neurodegeneration Induced by PQ and MPTP

To further examine the role of NR_030777 *in vivo*, we constructed genetically modified mice overexpressing NR_030777 (Figure 5a; Supplementary Figure 1). Mice overexpressing NR_030777 were significantly lighter in weight (Figs. 5B and 5C). When treated with PQ, mice overexpressing NR_030777 were also significantly underweight. Treatment of MPTP, a prodrug known to cause PD by destroying dopaminergic neurons in the *SN*, had no clear effect (Figure 5C).

To explore the effects of NR_030777 on motor function in mice, we did behavioral tests including climbing pole experiment and suspension experiment. Compared with the control group, PQ and MPTP treated mice showed an increase in climbing pole time, suggesting a potential role in motor function damage (Figure 5D). Interestingly, mice overexpressing

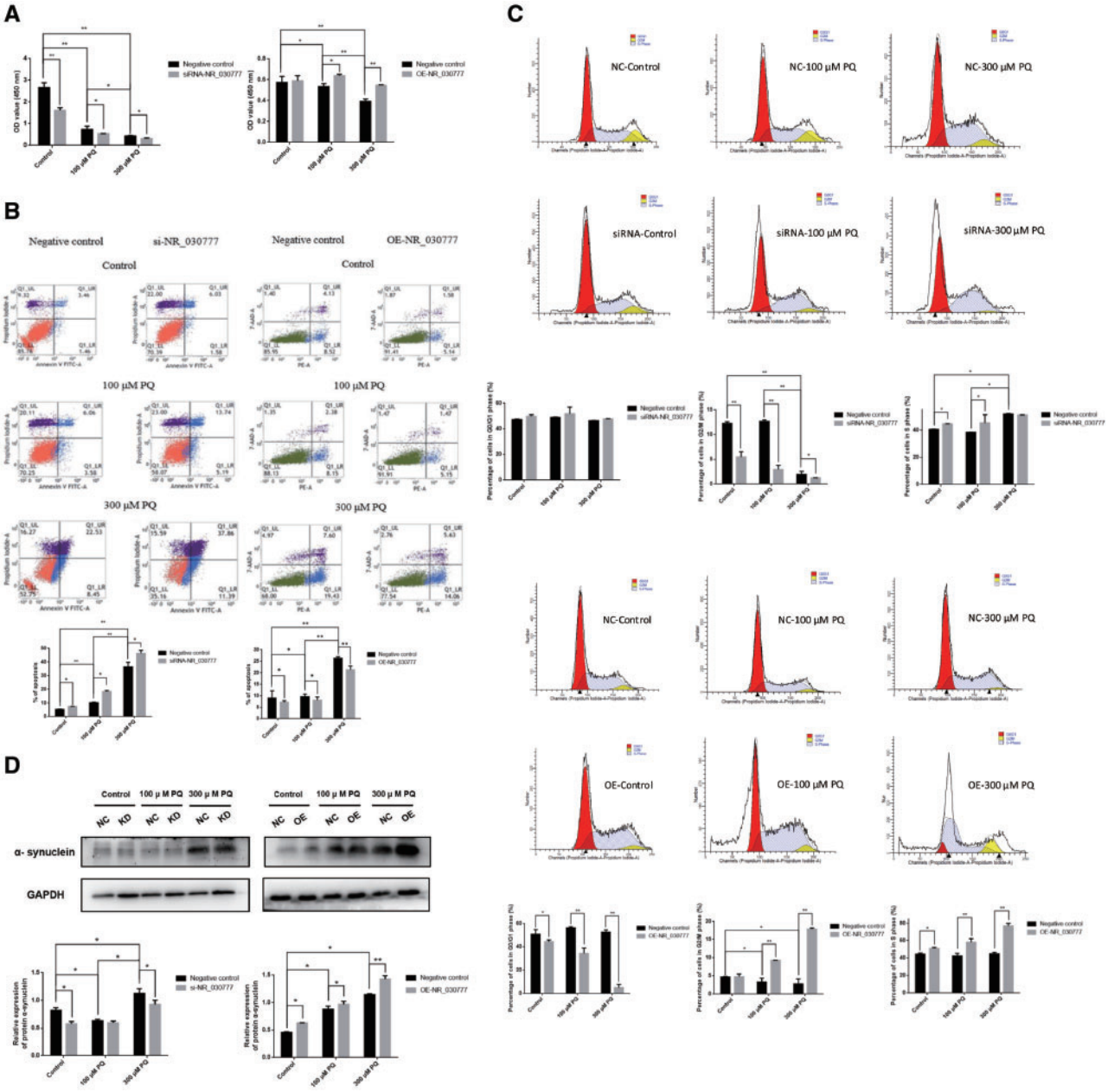

Figure 3. LncRNA NR_030777 regulates cellular activities and α-synuclein (α-syn) expression in N2a damage induced by PQ. N2a cell proliferation rate was measured by CCK-8 assays. Cell cycle and apoptosis was measured by flow cytometry. A, Effects of NR_030777 Knockdown (KD) or overexpression (OE) on cell proliferation ($n = 6$). B, Effects of NR_030777 KD or OE on apoptosis ($n = 3$). C, Effects of NR_030777 KD or OE on cell cycle ($n = 3$). D, Effects of NR_030777 KD or OE on the expression of α-syn ($n = 3$). Data represent mean values ± SE. $^{*}p < .05$; $^{**}p < .01$.

NR_030777 shortened the climbing pole time compared with all respective control groups. Likewise, mice overexpressing NR_030777 also showed an increase in suspension time compared with respective controls (Figure 5E).

The HE staining was used to check cell morphology, and Hoechst staining was used to check nuclear state and apoptosis of mouse *SN* affected by PQ and MPTP. Compared with control, apoptosis and nuclear pyknosis increased significantly in PQ and MPTP treated groups. These effects were alleviated with the OE of NR_030777 (Figs. 5F and 5G). We then detected the expression of TH in SN by Immunohistochemical staining. It is obvious that PQ and MPTP caused a significant reduction of TH in SN, yet the OE of NR_030777 rescued the effects induced by PQ and MPTP (Figure 5H). Taken together, these results suggest a protective role of NR_030777 in PQ/MPTP induced neurodegeneration damage.

Overexpression of NR_030777 Regulates the Expression of Zfp326 and Cpne5 in Neurodegenerative Damage Induced by PQ and MPTP *In Vivo*

Next, we examine the expression of NR_030777, Zfp326, and Cpne5 in the *SN* of mice. Paraquat and MPTP treatment increased the expression of NR_030777 in mice (Figure 6A, left panel). The expression of Zfp326 mRNA also increased with PQ and MPTP treatment. However, NR_030777 OE reduced the amount of Zfp326 transcripts (Figure 6A, middle panel). The

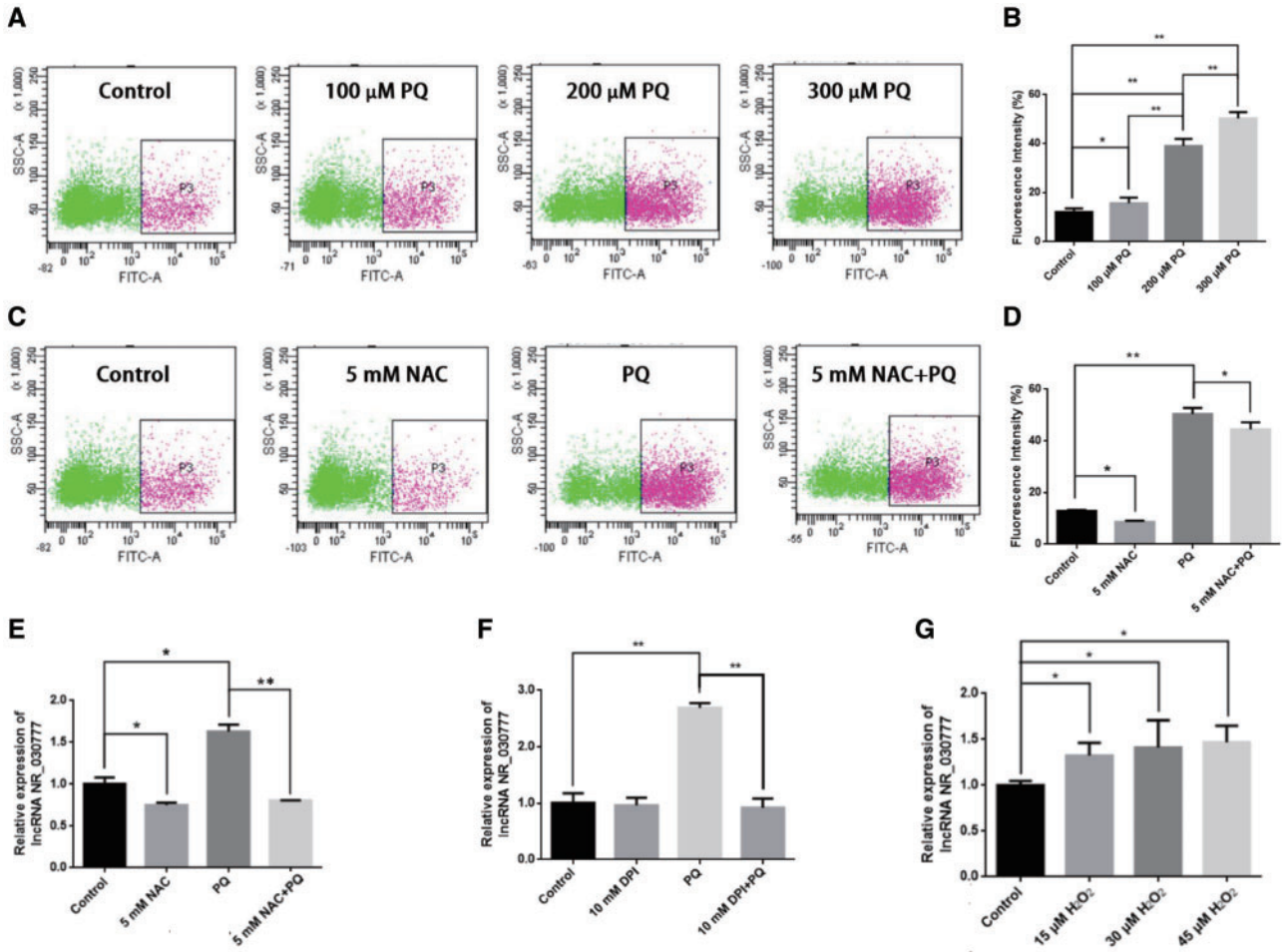

Figure 4. The ROS regulates the expression of NR_030777. A, Paraquat influences the production of ROS in N2a cells. B, Quantitative figure of (A). C, ROS inhibitor NAC reduces cellular ROS amount. D, Quantitative figure of (C). Relative expression of NR_030777 with (E) PQ and NAC; (F) PQ and DPI; (G) H_2O_2. Data represent mean values ± SE ($n = 3$). $^*p < .05$; $^{**}p < .01$.

amount of Cpne5 mRNA increased with PQ and MPTP treatment. Overexpression of NR_030777 further increased the expression of Cpne5 (Figure 6A, right panel). We also checked the expression profiles for these proteins in SN. ZPF326 protein level increased with PQ/MPTP treatment, and OE of NR_030777 further increased the amount of ZFP326 (Figure 6B). For CPNE5, OE of NR_030777 also increased the amount of CPNE5 protein, although PQ and MPTP treatment caused a reduction in CPNE5 protein level. We obtained similar results in hippocampus and cortex (Supplementary Figure 2). NR_030777 was not overexpressed in heart, liver, and kidney (Supplementary Figure 3).

Zfp326 Regulates Apoptosis, Cell Proliferation, and the Expression of NR_030777 and Cpne5 in Neurotoxicity Induced by PQ in N2a

Because NR_030777 regulates Zfp326 gene expression and has high sequence homology to Zfp326 mRNA, we wonder if Zfp326 and NR_030777 are mutually regulated. Indeed, KD of Zfp326 caused a decrease in NR_030777 and Cpne5 mRNA expression in N2a (Figure 7A). Paraquat treatment increased NR_030777 expression, but KD of Zfp326 reduced its expression. The effects on Cpne5 expression were less obvious (Figs. 7B and 7C).

Previous results showed that NR_030777 regulated the expression of Zfp326 by changing the stability of Zfp326 mRNA (Figure 2F). Therefore, we checked if Zfp326 could also affect the stability of NR_030777 and Cpne5 mRNA. Knockdown of Zfp326 decreased the stability of NR_030777, but it did not affect the stability of Cpne5 mRNA (Figure 7D). We also checked the effects of Zfp326 on cell proliferation and apoptosis. Knockdown of Zfp326 increased cell proliferation even in the presence of PQ (Figure 7E) and reduced cell apoptosis (Figure 7F).

Interplay Between ZFP326, NR_030777, and Pre-mRNA Splicing Factor SF3B3

To identify potential NR_030777-interacting molecules, we employed tagged RNA affinity purification (TRAP) *in vitro* pull-down assays, and analyzed the associated proteins by next-generation sequencing and RNA by mass spectrometry in N2a. Through bioinformatics analysis, we discovered that the molecules bound to NR_030777 were widely involved in various cellular activities, with a majority focusing on neurodegenerative diseases such as Huntington's disease and PD, as well as oxidative stress processes (Supplementary Figs. 4A and 4B).

According to bioinformatics analyses, SF3B3 caught our attention. Mass spectrometry revealed that SF3B3 was pulled down by NR_030777 (Figure 8A). This is consistent with previous reports showing that SF3B3 could bind to DBC-1, a pre-mRNA splicing factor, which was shown to interact with ZFP326 (Jean-Philippe *et al.*, 2008; Mcllwain *et al.*, 2010). Indeed, CoIP experiment coupled with mass spectrometry showed that ZFP326 interacted with SF3B3 (Figure 8B). In addition, KD of Zfp326

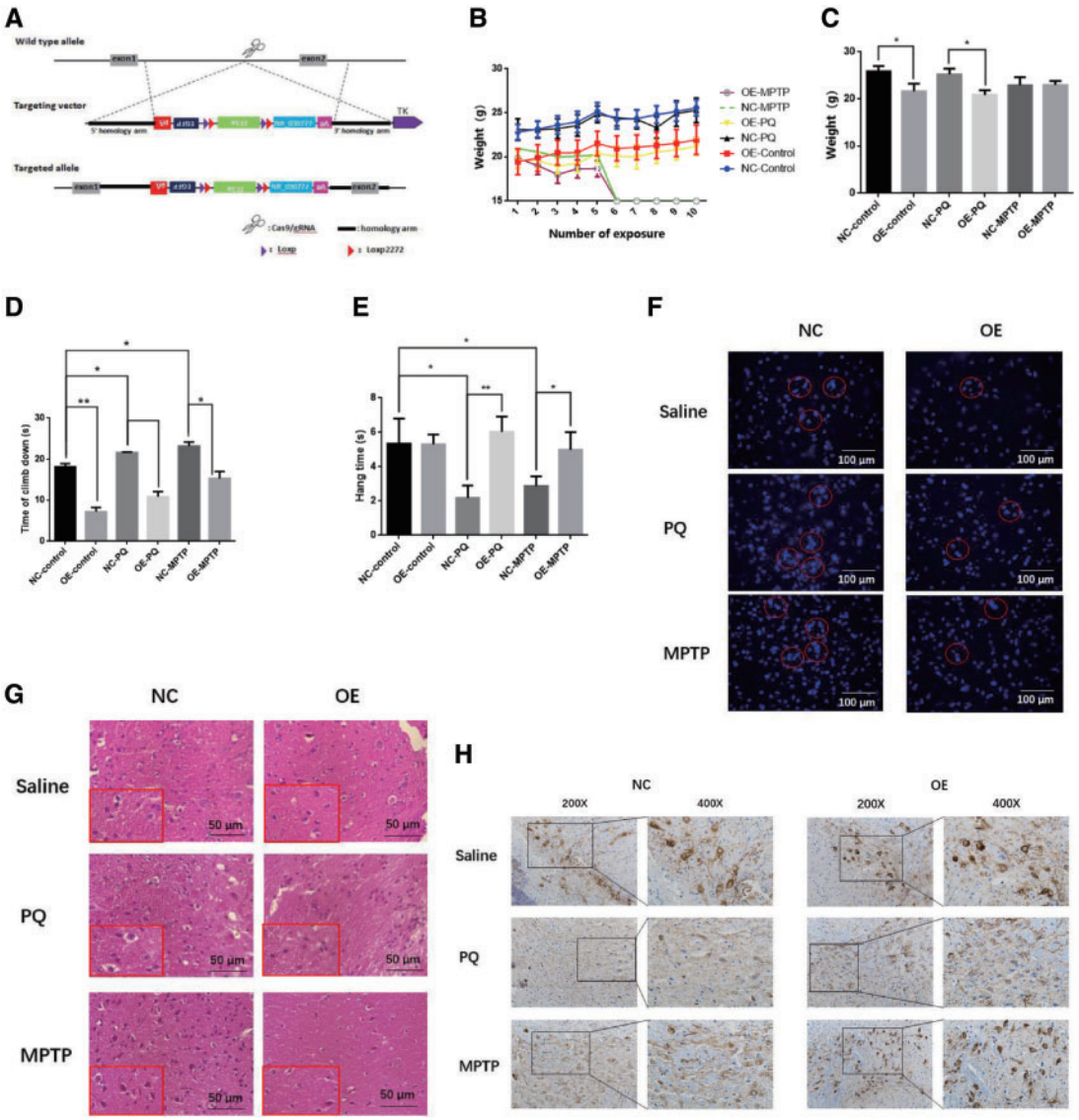

Figure 5. Overexpression (OE) of NR_030777 in nervous system of C57/BL6 mice alleviates neurodegeneration induced by PQ and MPTP. A, Construction strategy for a NR_030777 OE system *in vivo*. B, Weight change trend line of C57/BL6 mice during PQ exposure. C, Weight at the end of PQ/MPTP exposure in C57/BL6 mice. D, Climbing pole experiment. E, Suspension experiment. F, Hoechst staining showed cell apoptosis induced by PQ and MPTP in SN. G, The HE staining showed cell morphology destruction induced by PQ and MPTP in SN. H, Immunohistochemical staining showed the expression of tyrosine hydroxylase (TH) in SN. Data represent mean values ± SE ($n = 10$). $^{*}p < .05$; $^{**}p < .01$.

caused a reduction in SF3B3 protein level (Figure 8C), and KD of Sf3b3 caused a reduction in NR_030777 expression (Figure 8D). In all, these results suggest an interplay between ZFP326, NR_030777, and SF3B3.

DISCUSSION

Parkinson's disease is a common central nervous system degenerative disease. The molecular mechanisms underlying and initiating PD are still not clear. It is assumed that initiation of neurodegenerative diseases is promoted by a combination of genetic predisposition and environmental influences (Maele-Fabry *et al.*, 2012; Noyce *et al.*, 2012). Paraquat, a widely used herbicide, has neurotoxicity and may be involved in the development of PD (Ponting *et al.*, 2009; Wu and Du, 2017). A study showed that PQ burden in brain accumulated gradually with increments in age with a lifetime PQ exposure scenario. The PQ burden of the brain is estimated to be greater than 400 μM in a life-time exposure scenario for people older than 50 years (Cheng *et al.*, 2018a,b). Although chronic PQ exposure to humans is difficult to quantify, numerous epidemiological studies have revealed the relationship between PQ and PD. A systematic review and meta-analysis provide a quantitative estimate of the risk of PD associated with PQ exposure (OR = 1.64) (Tangamornsuksan *et al.*, 2019). An epidemiological cohort study has shown that daily exposure to PQ could increase the risk of PD and the risk of PD is related to the cumulative number of days of PQ using (Kamel *et al.*, 2007). Besides, a study performed with 120 patients in Taiwan, where the herbicide PQ is commonly sprayed over rice fields, showed a strong association between PQ exposure and PD risk. The hazard increased by more than 6 times in individuals who had been exposed to PQ for more than 20 years (Liou *et al.*, 1997). Occupational PQ exposure in other 57 cases also showed association with Parkinsonism in

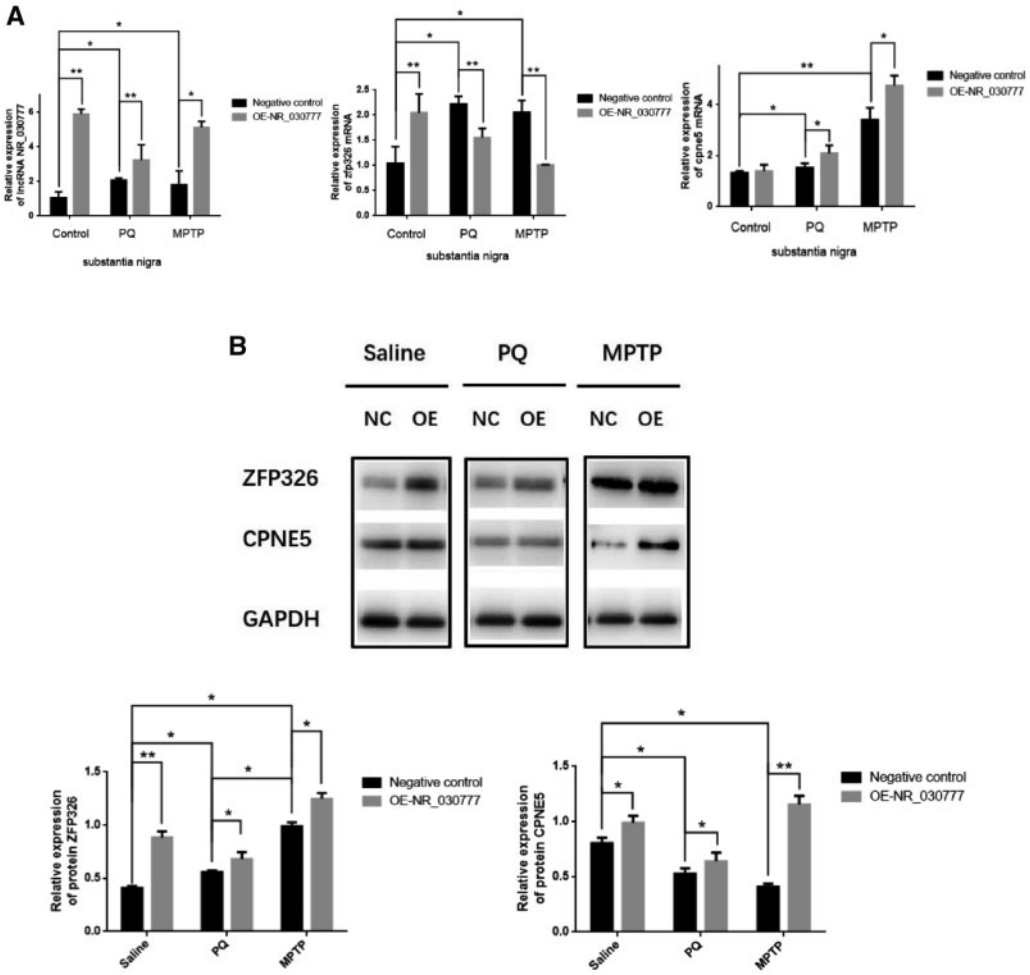

Figure 6. Overexpression of NR_030777 in nervous system of C57/BL6 mice regulates the expression of Zfp326 and Cpne5 in neurodegenerative damage induced by PQ and MPTP. A, The expression of NR_030777, Zfp326, and Cpne5 mRNA. B, The expression of ZFP326 and CPNE5 protein. Data represent mean values ± SE ($n = 3$). $^{*}p < .05$; $^{**}p < .01$.

British Columbia (Hertzman *et al.*, 1990). This indicates that chronic toxicity of PQ cannot be ignored and is closely related to PD. This indicates that chronic toxicity of PQ cannot be ignored and is closely related to PD. However, little is known on whether PQ causes neurodegeneration by disrupting lncRNA expression. Previously, we described changes in lncRNA expression in PQ-induced PD mouse model and verified the expression and localization of lncRNA NR_030777 (Wang *et al.*, 2018). Here we unveiled the role of lncRNA NR_030777 in PQ-induced neuronal damage. We used *in vitro* experiments to determine the regulation of NR_030777 on the homologous Zfp326 transcripts and its potential downstream target gene Cpne5 during PQ-induced neuronal damage. We further established an NR_030777 OE system in mice and revealed the functions of NR_030777 *in vivo*.

NR_030777 is a lncRNA with high sequence homology to Zfp326 mRNA. Zfp326 gene is located on chromosome 5 and encodes a conserved DNA binding protein with 2 zinc finger motifs. Zfp326 is a transcriptional activator with roles in neuronal differentiation during embryonic development (Liou *et al.*, 2012). We hypothesized a possible regulation between NR_030777 and Zfp326 in PQ-induced neuronal damage. Indeed, KD of NR_030777 decreased the expression of Zfp326 and vice versa, suggesting that their expressions are mutually affected. Studies have shown that lncRNA regulates homologous mRNA expression by homologous binding (Ng *et al.*, 2013). Whether this regulation pattern exists between NR_030777 and its homologous mRNA remains to be studied. We found that PQ inhibited the expression of Zfp326 mRNA and protein, and both KD and OE of NR_030777 caused further reduction in Zfp326 mRNA and protein expression. This is an interesting phenomenon. NR_030777 is highly homologous to zfp326 mRNA, and there is mutual regulation, and the regulation mode is extremely complex. Firstly, NR_030777 and zfp326 mRNA may competitively bind with miRNA. Therefore, KD of NR_030777 results in increased binding of miRNA to zfp326 mRNA, and causes decreased expression of zfp326 mRNA. Likewise, NR_030777 OE could cause increased expression of zfp326 mRNA. On the other hand, the high homology of this 2 nucleic acids results in the possibility of homologous binding degradation. Because the OE multiple of NR_030777 is much higher than that of the KD multiple, the homologous binding degradation effect caused by NR_030777 OE is much stronger than competitive combination effect. So, we observed that zfp326 mRNA was suppressed by the OE of NR_030777. The specific regulatory mechanism between NR_030777and zfp326 mRNA still needs to be further verified.

Previous studies showed that Cpne5 expression was abnormal in SN upon PQ treatment and CNC analysis showed a relationship between NR_030777 and Cpne5 (Lee *et al.*, 2000). We suspect that

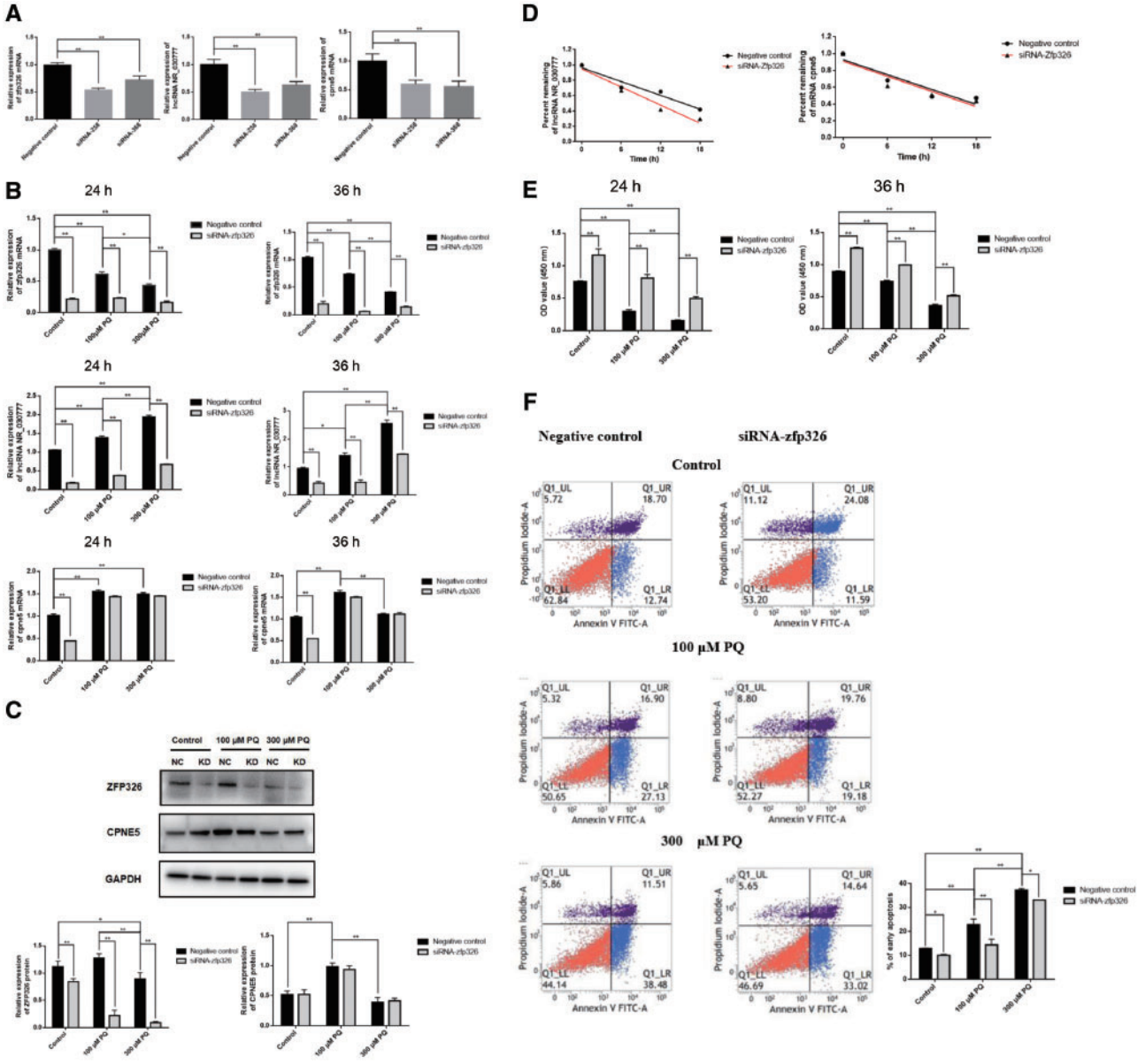

Figure 7. Zfp326 regulates apoptosis, cell proliferation, and the expression of NR_030777 and Cpne5 in neurotoxicity induced by PQ in N2a. A, Relative expression of Zfp326, NR_030777, and Cpne5 mRNA after Zfp326 knockdown (KD) in N2a cells. B, Relative expression of Zfp326, NR_030777, and Cpne5 mRNA after Zfp326 KD in the presence or absence of PQ. C, Relative protein levels of (B). D, mRNA levels of NR_030777 and Cpne5 were measured at the indicated times with the addition of actinomycin D (ActD, 5 μg/ml) in control or Zfp326 KD N2a cells. E, Cell proliferation analysis of N2a cells after Zfp326 KD in the presence or absence of PQ ($n = 6$). F, Cell apoptosis analysis of N2a cells after Zfp326 KD in the presence or absence of PQ ($n = 3$). Data represent mean values ± SE ($n = 3$). $^{*}p < .05$; $^{**}p < .01$.

NR_030777 could affect cellular processes by regulating the expression of Cpne5 in neuronal damage caused by PQ. Apoptosis is one of the main outcomes when cells are exposed to toxic exogenous chemicals such as PQ. Research showed that the PQ increased apoptosis rate in N2a cells. Indeed, Zfp326 and Cpne5 are key genes mediating apoptosis in nerve cells. Zfp326 can regulate cell cycle, cell growth, and differentiation of neurons during embryonic development (Jin *et al.*, 2014; Luca *et al.*, 2013), and report has shown that CPNE5 is involved in apoptosis in neural development (Ng *et al.*, 2002). Here we further demonstrate that lncRNA NR_030777 has a significant role in this process.

Why did PQ-induced stress cause an increase in the expression of NR_030777 and what is the mechanism underlying this process? It is generally believed that ROS and oxidative stress are the main neurotoxic mechanisms of nerve poisoning such as PQ (Dinis-Oliveira *et al.*, 2006). Paraquat can influence intracellular REDOX cycle and NADPH oxidase activation. This can cause oxidative stress and produce a large amount of ROS, producing cytotoxicity (Dawson and Dawson, 2003; Dinis-Oliveira *et al.*, 2006; Pang *et al.*, 2019). Therefore, it is plausible that PQ-induced excessive ROS leads to a change in lncRNA expression state. Importantly, ROS scavengers such as NAC and DPI can effectively interfere with the generation of free radicals and reduce cellular ROS (Li *et al.*, 2007). It is interesting to note that the expression of NR_030777 was significantly reduced after the removal of ROS by NAC, indicating a stimulatory role of ROS in the expression of NR_030777. We also observed the same effect with H_2O_2, a chemical commonly used in oxidative stress.

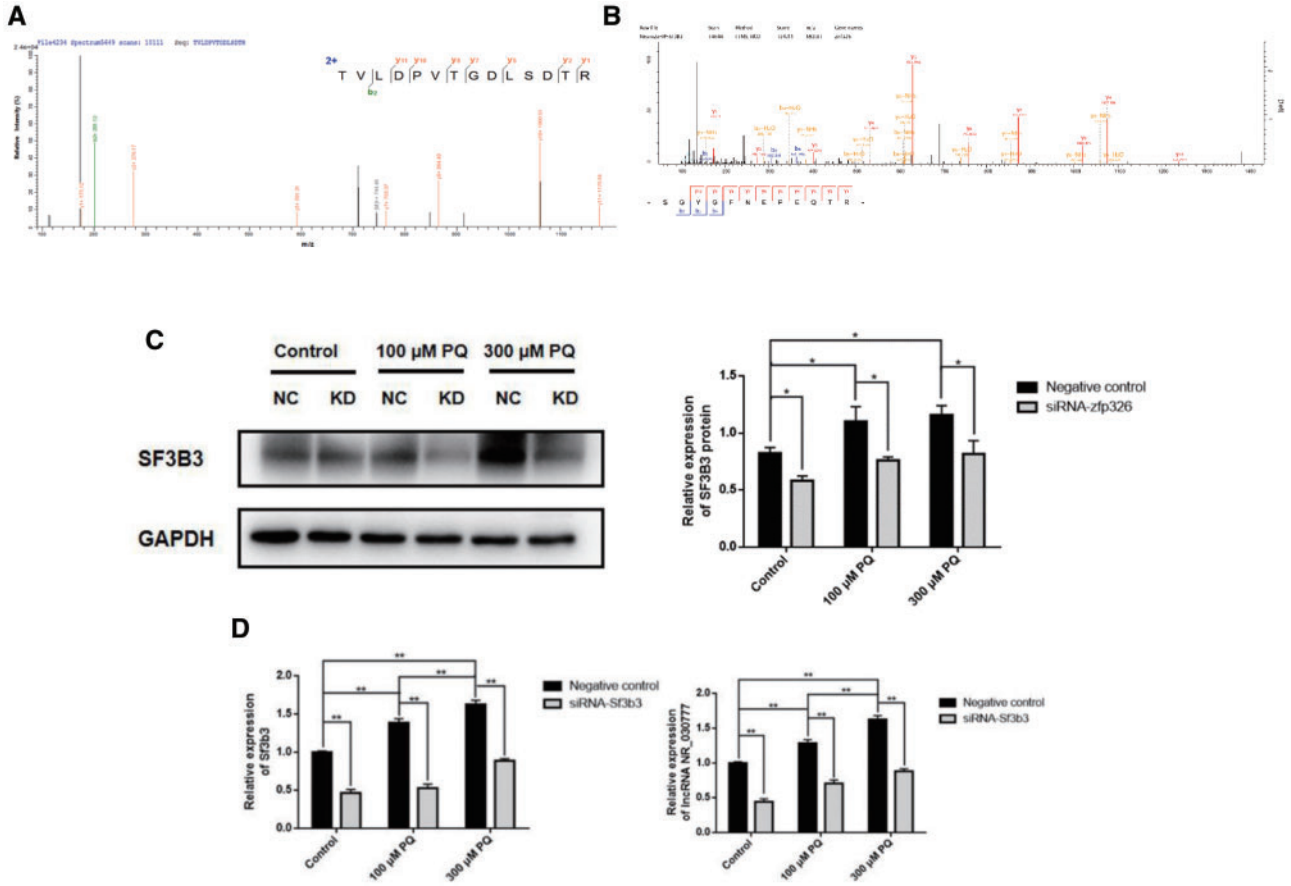

Figure 8. Zfp326 regulates NR_030777 expression by regulating SF3B3 expression in N2a damage induced by PQ. A, Mass spectrum of SF3B3. B, Mass spectrum of ZFP326. C, Knocking down of Zfp326 regulates SF3B3 protein expression in N2a damage induced by PQ. D, Knocking down of Sf3b3 regulates NR_030777 expression in N2a damage induced by PQ. Data represent mean values ± SE ($n = 3$). $^{*}p < .05$; $^{**}p < .01$.

Paraquat and MPTP selectively damage dopaminergic neurons, impair motor function, and cause PD (Min *et al.*, 2010; Peng *et al.*, 2004). For PQ/MPTP-induced cell damage, apoptosis of nerve cells is one of the major outcomes in the SN (Dinis-Oliveira *et al.*, 2006). Paraquat/MPTP increased neuron apoptosis significantly, resulting in nucleus pyknosis. Overexpression of NR_030777 significantly improved this state and reduced apoptosis. Additionally, PQ and MPTP significantly extended the climbing pole time and shortened the suspension time in mice. When NR_030777 was overexpressed in mice nervous system, climbing pole time was decreased and suspension time was increased. These results suggest that NR_030777 could alleviate the PQ damages to the motor function. Therefore, NR_030777 might have a protective role against PQ-induced neurodegeneration.

To explore the potential molecules binding to NR_030777, we employed MS2-TRAP assay and identified factors that are mainly involved in neurodegenerative diseases such as Huntington's disease and PD, as well as oxidative stress in cells. Therefore, it can be speculated that NR_030777 might have a crucial role in PQ-induced neuronal damage and neurodegenerative diseases. Interestingly, TRAP pulldown assay identified a Pre-mRNA splicing factor, SF3B3. Pre-mRNA splicing is a basic process in mammalian gene expression, and the alteration of RNA splicing is also key to the development of many diseases (Salton and Misteli, 2016). SF3B3 is a component of the splicing factor SF3B complex. The SF3B complex is required for the "A" complex in which U2 snRNP stably binds to the branch point sequence in the mRNA precursor. It belongs to a small U12-dependent splicing entity involved in splicing of the Pre-mRNA and CircRNA (Chen *et al.*, 2016; Gökmen-Polar *et al.*, 2015). We hypothesize that ZFP326 could regulate the expression of SF3B3, which in turn affects the total amount of functional lncRNA NR_030777 by splicing event.

CONCLUSION

In conclusion, our research shows that lncRNA NR_030777 plays an important role in PQ-induced neurotoxicity. It provides a new avenue to treat neurodegenerative diseases caused by common environmental toxicants such as PQ. We suggest that lncRNA could serve as a valuable alternative for the prevention and treatment of environmental neurodegeneration and related diseases such as PD (Figure 9).

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

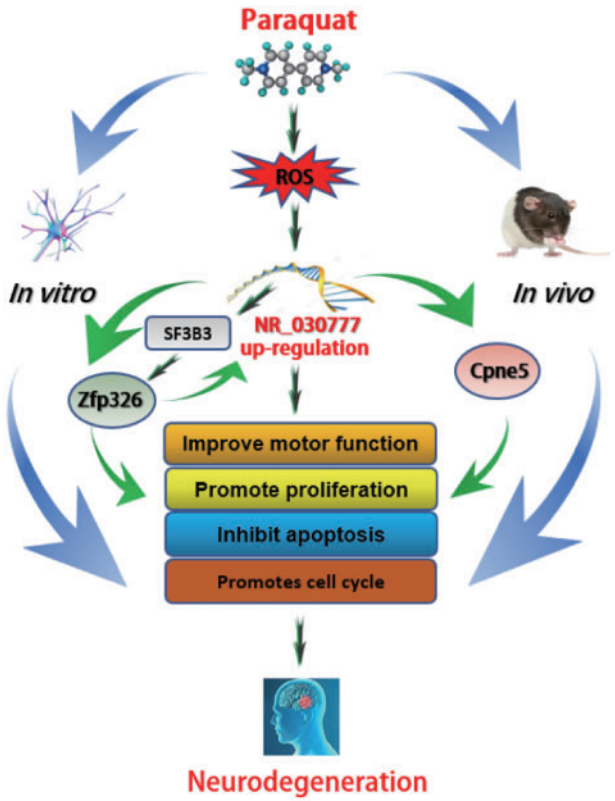

Figure 9. Protective role of LncRNA NR_030777 in neurotoxicity induced by PQ. Schematic diagram for the role of NR_030777 in neurotoxicity induced by PQ. Upon exposure to PQ, a surge of ROS induces the expression of NR_030777, which in turn affects the expression of Sf3b3, Zfp326, and Cpne5 to regulate downstream cellular activities and protect cells from neurodegenerative damage.

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