Research Article

P2X4 receptor participates in autophagy regulation in Parkinson's disease

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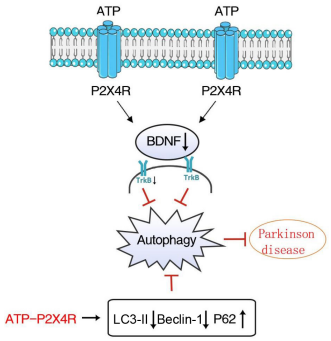
Graphical Abstract *Mechanism of P2X4R receptor (P2X4R)-mediated autophagy in Parkinson's disease*



Abstract

Dysfunctional autophagy often occurs during the development of neurodegenerative diseases, such as Parkinson's disease, Huntington's disease, and Alzheimer's disease. The purinergic P2X4 receptor is an ATP-gated ion channel that is widely expressed in the microglia, astrocytes, and neurons of the central and peripheral nervous systems. P2X4R is involved in the regulation of cellular excitability, synaptic transmission, and neuroinflammation. However, the role played by P2X4R in Parkinson's disease remains poorly understood. Rat models of Parkinson's disease were established by injecting 6-hydroxydopamine into the substantia nigra pars compacta. P2X4R-targeted small interfering RNA (siRNA) was injected into the same area 1 week before injury induction to inhibit the expression of the P2X4 receptor. The results showed that the inhibition of P2X4 receptor expression in Parkinson's disease model rats reduced the rotation behavior induced by apomorphine treatment, increased the latency on the rotarod test, and upregulated the expression of tyrosine hydroxylase, brain-derived neurotrophic factor, LC3-II/LC3-I, Beclin-1, and phosphorylated tropomyosin receptor kinase B (TrkB) in brain tissue, while simultaneously reducing p62 levels. These findings suggest that P2X4 receptor activation might inhibit neuronal autophagy through the regulation of the brain-derived neurotrophic factor/TrkB signaling pathway, leading to dopaminergic neuron damage in the substantia nigra and the further inhibition of P2X4 receptor-mediated autophagy. These results indicate that P2X4 receptor might serve as a potential novel target for the treatment of Parkinson's disease. This study was approved by the Animal Ethics Committee of Affiliated Hospital of Qingdao University (approval No. QYFYWZLL26119) on April 12, 2016.

Key Words: 6-hydroxydopamine; apomorphine; brain-derived neurotrophic factor; dopaminergic neurons; neuron degeneration; P2X4R; Parkinson's disease; TrkB; autophagy

Chinese Library Classification No. R453; R741; Q253

Introduction

The primary hallmark of Parkinson's disease (PD) is the degeneration of dopaminergic (DA) neurons located in the substantia nigra pars compacta (SNpc). PD is a common neurodegenerative disease with a prevalence of 1–2% in people aged 65 years and older (Poewe et al., 2017). PD primarily manifests as rigidity, static tremor, bradykinesia, and postural imbalance (Dickson, 2018). Levodopa is the currently established gold standard therapy for PD; however, the long-term usage of levodopa appears to be ineffective (Müller, 2012). Several factors contribute to the development of PD, such as heredity, environmental factors, and aging. These factors appear to induce cumulative effects on oxidative stress, apoptosis, neuroinflammation, and autophagy-mediated damage that ultimately result in the development and progression of PD (Nasrolahi et al., 2019; Pain et al., 2019). However, the mechanisms underlying autophagy-mediated DA neuron degeneration in PD remain incompletely understood.

Autophagy is an important process through which cells remove damaged organelles and misfolded and abnormally aggregated proteins to maintain cell homeostasis. Macroautophagy, the ubiquitin-proteasome system, and chaperone-mediated autophagy have all been shown to play roles in α-synuclein clearance (Gao et al., 2019) and are thought to result in extracellular α-synuclein release (Poehler et al., 2014). Although autophagic responses are part of the normal aging process, dysfunctional autophagy has been linked

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to neurodegenerative diseases, including PD, Huntington's disease, and Alzheimer's disease (Meng et al., 2019; Ye et al., 2020). Patients with PD demonstrate impaired autophagy, which can lead to reduced levels of lysosome-mediated α-synuclein multimer clearance, resulting in PD (Jin et al., 2018; Papagiannakis et al., 2019). Therefore, directly increasing autophagy levels could be used as a novel therapeutic strategy for PD.

The purinergic P2X receptors are ligand-gated channels that are activated by extracellular ATP. P2X receptors consist of seven subunits, and the family includes both the homo- and heterotrimeric channels P2X1–P2X7 (North, 2002). A subtype of the P2X purinergic receptor, the P2X4 receptor (P2X4R), can trigger multiple cellular responses when stimulated by extracellular ATP, such as the release of cytokines and neurotrophic factors. P2X4R is widely expressed in microglial and astrocytic cells and neurons throughout the central and peripheral nervous systems and is involved in the regulation of cell excitability, synaptic transmission, and neuroinflammation (Qureshi et al., 2007; Shieh et al., 2014). An immunohistochemical study conducted by Amadio et al. (2007) confirmed that P2X4R was expressed by DA neurons in the SNpc and in γ-aminobutyric acid (GABA)ergic neurons found in the substantia nigra pars reticulata and the striatum. Wyatt et al. (2014) reported that P2X4R knockout rats exhibited alterations in sensorimotor functions, social behaviors, and alcohol-drinking behaviors. Brain-derived neurotrophic factor (BDNF) is expressed by both immune cells and neurons, and the ATP-induced activation of P2X4Rs appears to result in BDNF release from microglia (Malcangio, 2017). BDNF exerts physiological effects by forming bonds with the high-affinity tropomyosin receptor kinase B (TrkB) (Parkhurst et al., 2013). P2X4Rs regulate the BDNF/TrkB signaling pathway, resulting in the increased excitability of a chronic inflammatory pain model (Lalisse et al., 2018). A study examining P2X4 receptor participation in excitatory amino acid transporter 3 regulation in a trigeminal allodynia model found that P2X4R blockade resulted in a decrease in the levels of p38, BDNF, excitatory amino acid transporter 3, and calcitonin gene-related peptide (Liu et al., 2018).

In this study, we aimed to evaluate the role played by P2X4R in 6-hydroxydopamine (6-OHDA)-induced DA neuronal damage during the early stages of PD and further studied the effects of P2X4R activation on autophagy and DA neurons.

Materials and Methods

Animals and treatment

Animal protocols were designed in compliance with the National Institutes of Health Guide for the Care of Animals and were approved by the Animal Ethics Committee of Affiliated Hospital of Qingdao University (approval No. QYFYWZLL26119) on April 12, 2016. All experiments were designed and reported according to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. Specific-pathogen-free male Wistar rats (provide by Laboratory Animal Center of Animal Experiments of the Affiliated Hospital of Qingdao University, license No. SYXK20150003) weighing 300 ± 20 g and aged between 12 and 14 weeks were reared at room temperature (22 ± 2°C) and provided with tap water and food ad libitum under a 12-hour light-dark cycle. Rats were anesthetized with isoflurane (Macklin, Shanghai, China) (3% induction, 1.5% maintenance) and positioned on a stereotaxic apparatus (Reward, Shenzhen, China). The following stereotaxic coordinates relative to Bregma were used: anteroposterior, –5.0 mm; mediolateral, 2.1 mm; and dorsoventral, 7.7 mm (Paxinos and Watson, 1986).

Rats were randomly divided into the following six groups: 1) control group, which received a 2 μL injection of normal saline solution into the SNpc; 2) 6-OHDA group, which received a 6-OHDA (Sigma, St. Louis, MO, USA) (8 μg/2 μL) injection into the rostral SNpc; 3) P2X4R-NC group, which received an injection of 2 μL no-load negative lentivirus (LV-P2X4R-NC, Shanghai GeneChem, Shanghai, China) into the SNpc followed by a 2 μL saline injection in the same anatomical area after 1 week; 4) P2X4R-siRNA group, which received an injection of 2 μL P2X4R-targeted small interfering RNA (siRNA)-containing lentivirus (LV-siP2X4R, 8×10^8 TU/mL) into the SNpc, followed by a 2 μL saline injection into the same anatomical area after 1 week; 5) P2X4R-NC + 6-OHDA group, which received an injection of 2 μL LV-P2X4R-NC (4×10^8 TU/mL) into the SNpc, followed by a 6-OHDA (8 μg/2 μL) injection into the same anatomical area after 1 week, 6) P2X4R-siRNA + 6-OHDA group, which received an injection of 2 μL LV-siP2X4R (8×10^8 TU/mL) was injected into the SNpc followed by a 6-OHDA (8 μg/2 μL) injection into the same anatomical area after 1 week. The targeted lentivirus vector was GV248. The sequence of P2X4R-siRNA was 5′-CGG GAC ATA GCT TCC AGG AGA-3′. The P2X4R-NC sequence was 5′-TTC TCC GAA CGT GTC ACG T-3′. A heating pad and an overhead lamp were used to maintain the rats' body temperatures at 37°C during stereotaxic surgery and throughout the recovery from anesthesia.

Apomorphine-induced rotation

Rats were treated with apomorphine (Sigma, 0.5 mg/kg, intraperitoneally) containing 0.02% ascorbic acid (Sigma) 3 weeks after the 6-OHDA injection to validate PD model generation. Apomorphine is thought to act by directly stimulating supersensitive postsynaptic DA receptors to induce contralateral turning (Hefti et al., 1980). Rats that turned unilaterally at a frequency of at least 7 turns/min in each test after the apomorphine injection were considered to be successfully generated models (Sun et al., 2014). Rats that rotated continuously to the contralateral side and at a rate of more than 20 turns per 5 minutes were used for further studies (Pang et al., 2016). The number of rat rotations was counted over a period of 30 minutes.

Rotarod test

A rotarod test was performed based on previously established protocols (Rozas et al., 1997). The velocity of the rod (Med Associates Inc., Atlanta, GA, USA; diameter, 5 cm) ranged from 4 r/min to 40 r/min. The rats were trained before testing by placing them on a rod rotating at 5 r/min for 15 minutes per day for 3 consecutive days. Magnetic trip plates recorded the latency automatically. The rats were allowed a maximum cut-off latency of 300 seconds to prevent the induction of stress and fatigue. Each interval should be at least an hour. This experiment was performed three times for each animal, and average times were calculated.

Immunofluorescence staining

Rats were deeply anesthetized by isoflurane and transcardially perfused with 4% paraformaldehyde. Coronal brain sections 20-μm-thick containing the SNpc were washed in 0.1 M phosphate-buffered solution (PBS) contained 0.3% Triton, placed in blocking buffer containing 3% goat serum for 2 hours, and then probed with rabbit anti-tyrosine hydroxylase (TH) antibody (1:1000; Cat# AB152; Millipore, Bedford, MA, USA) to label DA neurons. Sections were labeled with primary antibodies at 4°C overnight, rinsed three times with 0.3% Triton in 0.1 M PBS, and subsequently exposed for 2 hours to goat anti-rabbit IgG (H+L) Alexa Fluor 555 (1:500; Cat# A-21428; Invitrogen, Eugene, OR, USA) at room temperature. Sections were rinsed in PBS and fixed using 70% glycerol. The fluorescent signals were acquired using an Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany). Image Pro Plus software (Media Cybernetics Inc., Rockville, MD, USA) was used to count TH neurons in the SN from every section at 100× magnification. The substantia nigra of each rat was diricled into eight sets of parallel sections, and the total

number of neurons in each brain was calcalated by adding the neurons in the eight sets of sections. The number of total TH neurons in each brain was obtained by multiplying the counts by eight sections.

Western blot analysis

Radioimmunoprecipitation assay lysis buffer (15 mM NaCl, 50 mM Tris-HCl, 1% Nonidet-40, 0.5% sodium deoxycholate, 1 mM ethylenediaminetetraacetic acid, and 1 mM phenylmethylsulfonyl fluoride; NCM Biotech Co., Suzhou, China) containing protease inhibitors (1 g/mL each of pepstatin, aprotinin, and leupeptin; NCM Biotech Co.) was used to lyse brain samples for 30 minutes on ice. A Bradford protein assay kit (NCM Biotech Co.) was then used for total protein quantification. Samples containing 20 μg protein were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. A blocking buffer containing 10% non-fat milk was used to block endogenous reactions in the samples for 2 hours at room temperature. The samples were then subjected to an overnight incubation at 4°C with rabbit anti-P2X4R (1:1000; Cat# 70659; CST, Boston, MA, USA), rabbit anti-TH (1:2000; Cat# AB152; Millipore), rabbit anti-BDNF (1:1000; Cat# ab108319; Abcam, Cambridge, UK), rabbit anti-TrkB (1:500; Cat# 4603; CST), rabbit anti-phospho-TrkB (p-TrkB; 1:1000; Cat#4619; CST), rabbit anti-LC3-II/I (1:1000; Cat# ab192890; Abcam), rabbit anti-Beclin-1 (1:1000; Cat#3495; CST), rabbit anti-P62 (1:1000; Cat# 39749; CST), and rabbit anti-β-actin (1:10,000; Cat# ab115777; Abcam). The samples were then rinsed and further probed for 2 hours with mouse anti-rabbit IgG-horseradish peroxidase (1:10,000; Cat# sc-2357; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). An enhanced chemiluminescence kit (NCM Biotech Co.) was used to determine the degree of cross-reactivity. The optical densities of the target proteins were analyzed using a BioSpectrum 810 (UVP, Upland, CA, USA) imaging system.

Statistical analysis

Data analysis was performed using GraphPad Prism5 software (GraphPad Software Inc., San Diego, CA, USA). Comparisons between three or more groups were analyzed by one-way analysis of variance followed by the Bonferroni *post hoc* test. All data are presented as the mean ± standard error of the mean (SEM). $P < 0.05$ was considered significant.

Results

P2X4R protein levels increase in the SNpc of PD model rats

To investigate variations in P2X4R expression, we first detected the levels of P2X4R in 6-OHDA-treated rats. Western blot analysis revealed a significant increase in P2X4R levels after 6-OHDA treatment compared with the levels in normal controls ($P < 0.001$). In contrast to the P2X4R-NC group, the P2X4R protein expression level significantly decreased in the P2X4R-siRNA group ($P < 0.001$), which indicated that the lentivirus administration reduced P2X4R expression. Furthermore, compared with the P2X4R-NC + 6-OHDA group, the P2X4R-siRNA + 6-OHDA group showed lower P2X4R protein levels $P < 0.001$; **Figure 1**). Thus, 6-OHDA treatment led to an elevation in P2X4R levels, and P2X4R siRNA effectively interfered with P2X4R mRNA transcription, inhibiting protein expression.

P2X4R suppression improves apomorphine-induced rotation in PD model rats

The apomorphine-induced rotation test was used to validate the PD animal model and to assess the influence of P2X4R. The rats selected for the test did not show any significant differences in rotational behavior during the initial screening process. Apomorphine did not induce any rotation in the control rats. The number of apomorphine-triggered rotations increased significantly in the 6-OHDA group when contrasted against the control group 21 days ($P < 0.001$) after lesion induction. The P2X4R-siRNA + 6-OHDA displayed a significant reduction in the number of apomorphine-induced rotations compared with the P2X4R-NC + 6-OHDA group 21 days after lesion induction ($P < 0.001$). No significant difference in apomorphine-induced rotations was observed when comparing the P2X4R-NC and P2X4R-siRNA groups ($P > 0.05$; **Figure 2A**).

P2X4R suppression decreases the severity of SN lesions in PD model rats

The rotarod test is thought to serve as a quantitative index for measuring the severity of SN lesions (Wang et al., 2020). **Figure 2B** depicts the rotarod behaviors for each group of rats. The duration spent on the beam was significantly decreased in the 6-OHDA group ($P < 0.001$) compared with the control group. Furthermore, no significant differences in latency were observed among the control, P2X4R-NC, and P2X4R-siRNA groups. Additional analyses suggested that the rotarod performances of rats were partially ameliorated in the P2X4R-siRNA + 6-OHDA group compared with those in the P2X4R-NC + 6-OHDA group ($P < 0.001$). These findings demonstrate that inhibiting P2X4R expression resulted in the alleviation of 6-OHDA-induced motor imbalances and coordination deficits.

The inhibition of P2X4R expression attenuates DA neuron degeneration in PD model rats

The DA neurons located in the SN were labeled with an anti-TH antibody and evaluated by immunofluorescence. The P2X4R-NC and P2X4R-siP2X4 lentivirus vectors also encoded the green fluorescent protein gene, allowing for the visualization of lentiviral expression. Through the use of double labeling, we were able to observe lentivirus expression in TH-expressing neurons and other neuronal cell types in the SN. We proceeded to quantify TH-positive cells and lentivirus-infected cells and found that the infection rate of TH-positive cells was over 90% (green fluorescent protein/red fluorescent protein, **Figure 3**).

The numbers of TH-positive neurons decreased by 49.5% after the injection of 6-OHDA. We additionally investigated whether P2X4R suppression encouraged DA neuron survival. Interestingly, treatment with P2X4R-siRNA led to a partial increase in TH neuron survival, and the survival ratio in P2X4R-siRNA-treated rats was 47.7% higher than that in P2X4R-NC + 6-OHDA-treated rats. Western blot results showed that TH protein levels were lower in the 6-OHDA group compared with those in the control group ($P < 0.01$). Similarly, compared with the P2X4R-NC + 6-OHDA group, the P2X4R-siRNA + 6-OHDA group showed higher TH protein levels ($P < 0.01$). The level of TH protein in the SN of PD model rats did not differ significantly between the P2X4R-NC, P2X4R-siRNA, and control groups ($P > 0.05$; **Figure 4**).

The inhibition of P2X4R expression further increases the BDNF/TrkB protein levels in PD model rats

Neurotrophins and their downstream signaling pathways can exert neuroprotective effects that might be regulated by P2X4R. The most common neurotrophic factor is BDNF, which is closely related to neuronal survival under various pathological conditions. TrkB, a membrane receptor, largely regulates BDNF function (Numakawa et al., 2010). We examined the expression levels phosphorylated (p-TrkB)/TrkB and BDNF protein in the SN of model rats, which significantly decreased compared with those in the control rats ($P < 0.001$ and $P < 0.001$, respectively). Compared with the P2X4R-NC + 6-OHDA group, the P2X4R-siRNA + 6-OHDA group showed increased p-TrkB/TrkB and BDNF expression levels in the SN (both $P < 0.01$), which suggested that the inhibition of P2X4R expression affected the BDNF/TrkB signaling pathway (**Figure 5**). On the basis of these results, 6-OHDA-induced

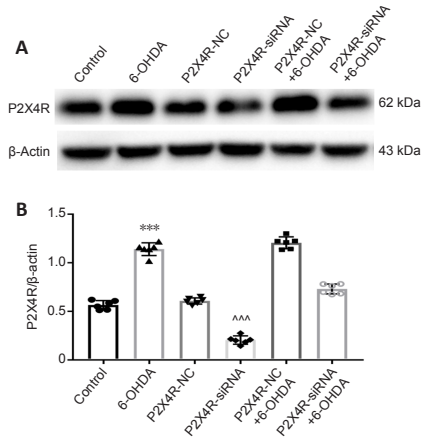

Figure 1 | **Level of P2X4R protein in the SN of Parkinson's disease model rats.**
(A) The P2X4R protein levels in the SN from different treatment groups were detected by western blot analysis. (B) Quantitative analysis of the changes in P2X4R protein levels in the SN. Data are expressed as the mean ± SEM (n = 6). ***P < 0.001, *vs*. control group; ^^^P < 0.001, *vs*. P2X4R-NC group; ###P < 0.001, *vs*. P2X4R-NC + 6-OHDA group (one-way analysis of variance followed by the Bonferroni *post hoc* test). Control group: Normal saline solution injected into the SNpc; 6-OHDA group: 6-OHDA injection into the rostral SNpc; P2X4R-NC group: no-load negative lentivirus was injected into the SNpc followed by a normal saline injection into the same anatomical area after 1 week; P2X4R-siRNA group: lentivirus P2X4R-siRNA was injected into the SNpc followed by a normal saline injection into the same anatomical area after 1 week; P2X4R-NC + 6-OHDA group: negative control lentiviral vector injected into the SNpc followed by a 6-OHDA injection into the same anatomical area after 1 week; P2X4R-siRNA + 6-OHDA group: lentivirus P2X4R-siRNA was injected into the SNpc followed by a 6-OHDA injection into the same anatomical area after 1 week. 6-OHDA: 6-Hydroxydopamine; P2X4R: P2X4 receptor; SN: substantia nigra; SNpc: substantia nigra pars compacta.

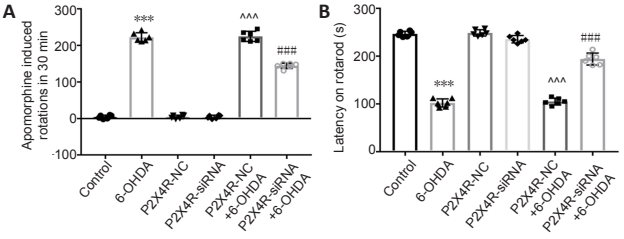

Figure 2 | **Effects of P2X4R inhibition on apomorphine-induced rotation (A) and rotarod latency (B) in Parkinson's disease model rats.**
(A) Apomorphine-induced rotation. The rotation was observed for 30 minutes and expressed as the net contralateral(difference between contralateral and ipsilateral) rotation. The test was conducted 21 days after surgery. (B) Rotarod test. The latency time in 6-OHDA treated rats was markedly decreased compared with control rats. After P2X4R siRNA administration in the P2X4R-siRNA + 6-OHDA group, the rotation behavior performance of rats partly improved compared with that in P2X4R-NC + 6-OHDA rats. Data are expressed as the mean ± SEM (n = 6). ***P < 0.001, *vs*. control group; ^^^P < 0.001, *vs*. P2X4R-NC group; ###P < 0.001, *vs*. P2X4R-NC + 6-OHDA group (one-way analysis of variance followed by the Bonferroni *post hoc* test). Control group: Normal saline solution injected into the SNpc; 6-OHDA group: 6-OHDA injection into the rostral SNpc; P2X4R-NC group: no-load negative lentivirus injected into the SNpc, followed by a normal saline injection into the same anatomical after 1 week; P2X4R-siRNA group: lentivirus P2X4R-siRNA was injected into the SNpc, followed by a normal saline injection into the same anatomical area after 1 week; P2X4R-NC + 6-OHDA group: negative control lentiviral vector injected into the SNpc, followed by a 6-OHDA injection into the same anatomical area after 1 week; P2X4R-siRNA + 6-OHDA group: lentivirus P2X4R-siRNA was injected into the SNpc, followed by a 6-OHDA injection into the same anatomical area after 1 week. 6-OHDA: 6-Hydroxydopamine; SNpc: substantia nigra pars compacta.

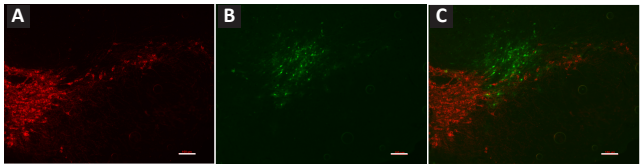

Figure 3 | **Successful lentivirus transfection in the left substantia nigra of rats.**
(A) Immunofluorescence of TH-positive neurons (red, stained by Alexa Fluor 555) in the substantia nigra. (B) Immunofluorescence of siP2X4R lentivirus vectors (green-labeled) in the substantia nigra. (C) Merged images showing siP2X4R lentivirus vectors (green-labeled) in TH neurons in the substantia nigra. Original magnification 100×; scale bars: 100 μm. TH: tyrosine hydroxylase.

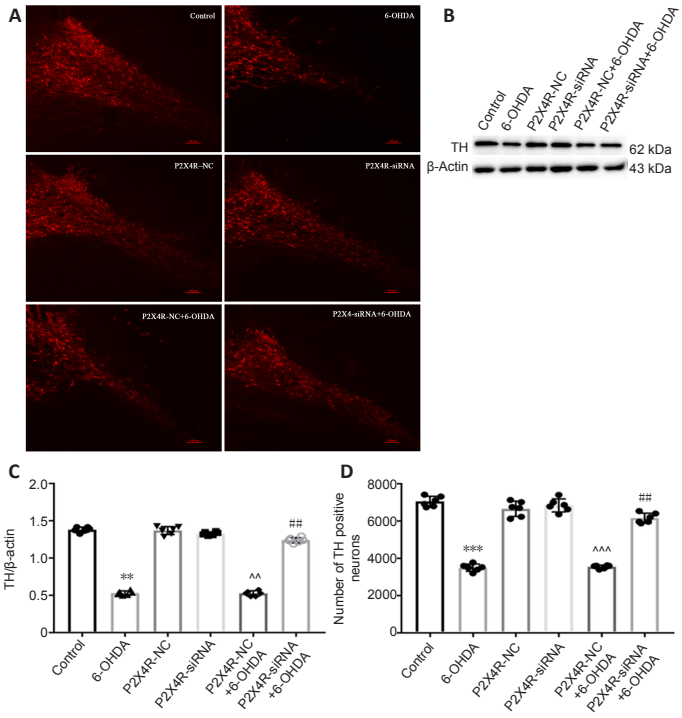

Figure 4 | **Effects of inhibiting P2X4 expression on the function of dopaminergic neurons in Parkinson's disease model rats.**
(A) TH-positive neurons (red, stained by Alexa Fluor 555) in the SN. Scale bars: 100 μm. (B) Image of a western blot demonstrating the TH expression level in the SN. (C) Quantitative analysis of the changes in TH protein levels in the SN. (D) Quantitative analysis of the changes in the number of TH-positive neurons in the SN. Data are presented as the mean ± SEM (n = 6). **P < 0.01, ***P < 0.001, *vs*. control group; ^^P < 0.01, ^^^P < 0.001, *vs*. P2X4R-NC group; ##P < 0.01, *vs*. P2X4R-NC + 6-OHDA group (one-way analysis of variance followed by the Bonferroni *post hoc* test). Control group: Normal saline solution injected into the SNpc; 6-OHDA group: 6-OHDA injection into the rostral SNpc; P2X4R-NC group: no-load negative lentivirus was injected into the SNpc, followed by a normal saline injection into the same anatomical area after 1 week; P2X4R-siRNA group: lentivirus P2X4R-siRNA was injected into the SNpc, followed by a normal saline injection into the same anatomical area after 1 week; P2X4R-NC + 6-OHDA group: negative control lentiviral vector injected into the SNpc, followed by a 6-OHDAinjection into the same anatomical area after 1 week; P2X4R-siRNA + 6-OHDA group: lentivirus P2X4R-siRNA was injected into the SNpc, followed by a 6-OHDA injection into the same anatomical area after 1 week. 6-OHDA: 6-Hydroxydopamine; SN: substantia nigra; SNpc: substantia nigra pars compacta; TH: tyrosine hydroxylase.

toxicity decreased neurotrophin levels and their downstream signaling regulators, and the inhibition of P2X4R expression restored BDNF expression, likely through the reactivation of the downstream signaling axis.

The inhibition of P2X4R expression further increases autophagy levels in PD model rats

To investigate whether P2X4R was involved in PD pathogenesis through the regulation of autophagic flux, we detected the expression of p62, Beclin-1, LC3-II, and LC3-I in SN tissue. As shown in **Figure 6**, animals treated with 6-OHDA demonstrated significant reductions in LC3-II/LC3-I levels, suggesting that 6-OHDA suppressed autophagy in the SN. LC3-II/LC3-I expression increased when rats were co-treated with P2X4R-siRNA (P < 0.01 *vs*. the P2X4R-NC + 6-OHDA group). The role of P2X4R was further explored by detecting the levels of Beclin-1, an autophagy-related protein. The results showed that 6-OHDA injections suppressed Beclin-1 protein expression, whereas the level of Beclin-1 in rats treated with P2X4R-siRNA combined with 6-OHDA significantly increased (P < 0.01). Furthermore, we measured the protein expression of the autophagy substrate p62 to verify the occurrence of autophagic flux. Treatment with 6-OHDA caused p62 accumulation compared with the level in the control group, whereas P2X4R-siRNA treatment significantly decreased p62 levels (P < 0.01; **Figure 6**), indicating that the inhibition of P2X4R expression promoted autophagy.

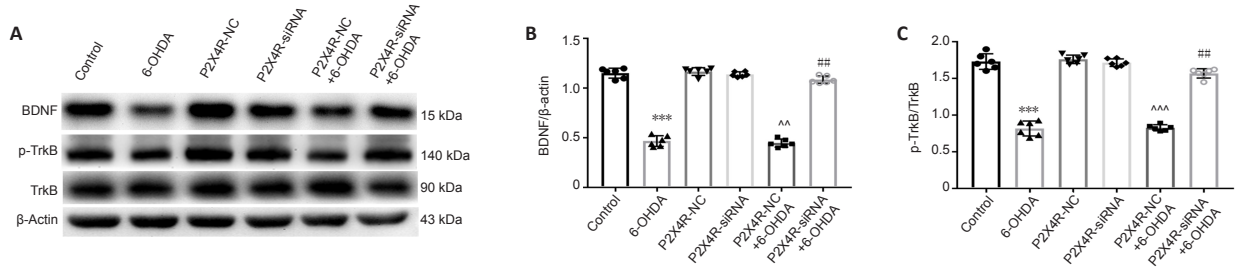

Figure 5 | **Effects of inhibiting P2X4R expression on the BDNF and TrkB protein levels in the SN in Parkinson's disease rats.**
(A) Western blot showing the protein levels of BDNF, p-TrkB, and TrkB in the SN. (B, C) Quantitative analysis of the changes in BDNF (B) and p-TrkB/TrkB (C) in the SN. Data are presented as the mean ± SEM (n = 6). ***P < 0.001, *vs*. control group; ^^P < 0.01, ^^^P < 0.001, *vs*. control group; ##P < 0.01, *vs*. P2X4-NC + 6-OHDA group (one-way analysis of variance followed by the Bonferroni *post hoc* test). Control group: Normal saline solution injected into the SNpc; 6-OHDA group: 6-OHDA injection into the rostral SNpc; P2X4R-NC group: no-load negative lentivirus was injected into the SNpc, followed by a normal saline injection into the same anatomical area after 1 week; P2X4R-siRNA group: lentivirus P2X4-siRNA was injected into the SNpc, followed by a normal saline injection into the same anatomical area after 1 week; P2X4R-NC + 6-OHDA group: negative control lentiviral vector injected into the SNpc, followed by a 6-OHDA injection into the same anatomical area after 1 week; P2X4R-siRNA + 6-OHDA group: lentivirus P2X4R-siRNA was injected into the SNpc followed by a 6-OHDA injection into the same anatomical area after 1 week. 6-OHDA: 6-Hydroxydopamine; BDNF: brain-derived neurotrophic factor; p-TrkB: phospho-TrkB; SN: substantia nigra; SNpc: substantia nigra pars compacta; TrkB: tyrosine kinase B.

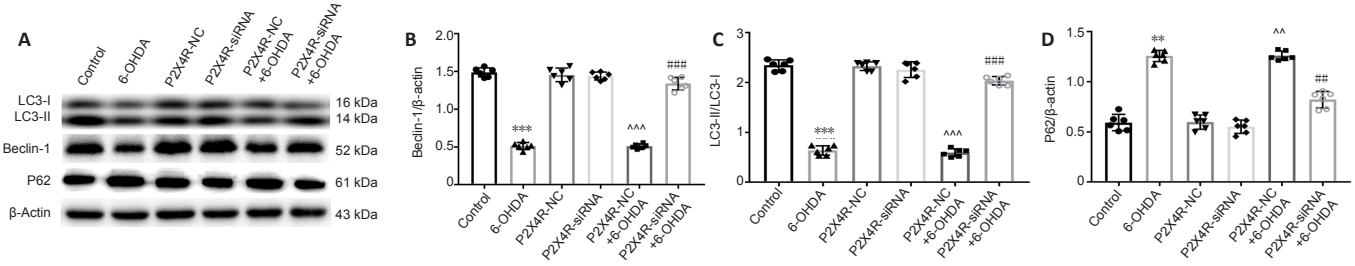

Figure 6 | **Effects of inhibiting P2X4R expression on the levels of autophagy-related proteins in Parkinson's disease model rats.**
(A) The LC3-II, LC3-I, Beclin-1, and P62 protein levels in the SN of different groups were detected by western blot analysis. (B–D) Quantitative analysis of the changes in LC3-II/LC3-I (B), Beclin-1 (C), and P62 (D) levels in the SN. Data are presented as the mean ± SEM (n = 6). **P < 0.01, ***P < 0.001, *vs*. control group; ^^P < 0.01, ^^^P < 0.001, *vs*. control group; ##P < 0.01, ###P < 0.001, *vs*. P2X4R-NC + 6-OHDA group (one-way analysis of variance followed by the Bonferroni *post hoc* test). Control group: Normal saline solution injected into the SNpc; 6-OHDA group: 6-OHDA injection into the rostral SNpc; P2X4R-NC group: no-load negative lentivirus was injected into the SNpc, followed by a normal saline injection into the same anatomical area after 1 week; P2X4R-siRNA group: lentivirus P2X4R-siRNA was injected into the SNpc, followed by a normal saline injection into the same anatomical area after 1 week; P2X4R-NC + 6-OHDA group: negative control lentiviral vector was injected into the SNpc, followed by a 6-OHDA injection into the same anatomical area after 1 week; P2X4R-siRNA + 6-OHDA group: lentivirus P2X4R-siRNA was injected into the SNpc followed by a 6-OHDA injection into the same anatomical area after 1 week. 6-OHDA: 6-Hydroxydopamine; LC3: microtubule-associated protein 1A/1B-light chain 3; SN: substantia nigra; SNpc: substantia nigra pars compacta.

Discussion

This investigation highlights the crucial function played by P2X4R in 6-OHDA-mediated PD pathology, suggesting that P2X4R may represent a potential therapeutic candidate target for PD. PD rat models were observed to express high levels of P2X4R in the SN, and P2X4R activation was associated with DA neuron degeneration, whereas DA neuron degeneration decreased following the inhibition of P2X4R expression, a phenomenon that we attribute to an increased autophagic response. Furthermore, the activation of P2X4R led to a decrease in BDNF/TrkB pathway activity, whereas the siRNA-mediated reduction of P2X4R expression partially activated the BDNF/TrkB pathway.

P2X receptors have been associated with CNS injury, inflammation, metabolic disorders, and neurodegeneration (Roberts et al., 2006; Tsuda and Inoue, 2006). Accumulating evidence has indicated that neurotransmission homeostasis is the central function for P2X4 (Pankratov et al., 2002; Kessels and Malinow, 2009; Franklin et al., 2014). Purinergic signaling has previously been shown to be an important component of several neurodegenerative diseases (Burnstock, 2015). Additionally, after metabolic stress, brain ischemia, and trauma, the levels of extracellular ATP and adenosine considerably exceed those observed under physiological conditions (Dale and Frenguelli, 2009; Rodrigues et al., 2015). Few studies have explored the role played by P2X in PD. P2X4R has been implicated in the damage caused to the nigrostriatal neural circuitry in 6-OHDA-induced PD rat models, based on the increased expression of GABAergic neurons in the SNpc and the decreased expression of ipsilateral striatal GABAergic neurons (Amadio et al., 2007). Our investigation revealed that PD rat models were characterized by elevated P2X4R expression. The 6-OHDA neurotoxin is transferred in a retrograde fashion after injection into the left medial forebrain bundle, leading to the degradation of approximately 49.5% of DA neurons in the SN, along with reduced TH levels at the site of the unilateral 6-OHDA-injection. The inhibition of P2X4R expression attenuated DA neuron degeneration and the associated deterioration of movement and balance. We did not administer bilateral 6-OHDA injections due to the development of a compensatory phenomenon that results in inconsistent levels of DA neuron damage, which can skew the experimental results. Unilateral SNpc injections of 6-OHDA also allowed us to simulate early-stage PD in our rat models, facilitating the delineate of P2X4R function in this disease.

PD is one of many neurodegenerative diseases that feature autophagic dysfunction (Moors et al., 2017). Anglade et al. (1997) first showed that nigral DA neuron loss was associated with apoptosis and autophagy in PD patients, and studies focused on PD pathology have revealed the reduced expression of autophagy–lysosome pathway-related proteins and reduced lysosomal enzyme activity in PD patients (Przedborski, 2017; Lu et al., 2020). Furthermore, autophagic dysfunction has been identified in various PD animal models and samples obtained from PD patients (Lynch-Day et al., 2012). Beclin-1, a mammalian orthologue of the yeast

autophagy-related gene ATG6, plays roles in the formation of autophagic vesicles, the localization of autophagic proteins, and the regulation of autophagy and cell death. Furthermore, mammalian target of rapamycin (mTOR) is a critical negative regulator of cellular autophagy, interacting with LC3-I and LC3-II (Yeh et al., 2016). The age-related downregulation of Beclin-1 expression has been observed in the brain, associated with a decrease in autophagy during neurodegenerative disease progression (Pickford et al., 2008). Haanes et al. (2012) reported that P2X7 might negatively mediate autophagy through the impairment of lysosomal function in mouse microglia and human epithelial cells. In the present study, P2X4R deficiency significantly upregulated the autophagic markers Beclin-1 and LC3-II and downregulated p62 in 6-OHDA-treated rats, indicating the activation of cellular autophagy and implicating P2X4R in the progression of PD.

To further explore the molecular mechanisms that underlie P2X4R involvement in PD pathogenesis, we investigated the impact of P2X4R on the BDNF/TrkB signaling pathway. The neurotrophin BDNF and its receptor TrkB are located upstream of the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mTOR signaling axis, which regulates neural survival, development, and differentiation. These factors can also augment neurotransmitter release between synapses, such as the release of acetylcholine from presynaptic nerve terminals, strengthening synaptic connections, and affecting neuronal plasticity. The downregulation of BDNF and the dysregulation of the BDNF-TrkB signaling axis is frequently observed in patients with AD, PD, and even chronic stress-induced depression (Lu et al., 2013). Michael et al. (1999) reported that BDNF expression is downregulated post-injury in peripheral nociceptive neurons. Bak et al. (2015) demonstrated that BDNF-stimulated autophagy might protect cortical neurons under hypoxic conditions. Lalisse et al. (2018) found that the P2X4R can regulate the BDNF/TrkB signaling pathway, resulting in increased excitability in a chronic inflammatory pain model. In this study, P2X4R deficiency upregulated the expression levels of BDNF and TrkB, which suggested that P2X4R further affects autophagy by impacting the BDNF signaling pathway. These findings indicated that the elimination of P2X4R might improve DA neuron activity in the SN, consequently hindering PD development and progression through BDNF/TrkB signaling pathway activation. However, additional research remains necessary to further examine the association between P2X4R and the BDNF/TrkB signaling pathway and determine the molecular mechanisms that underlie their effects on PD pathogenesis.

The present study had several limitations. First, we only examined the effects of P2X4R in a rat model of PD induced by 6-OHDA without performing *in vitro* evaluations. Second, the upstream molecular mechanisms through which P2X4R affects BDNF and p-TrkB/TrkB in PD remain unclear. Furthermore, several studies have confirmed that neuroinflammation plays an important role in the pathogenesis of PD (Lang et al., 2018; Harms et al., 2021), and the relationship between autophagy and inflammation requires further investigation.

In conclusion, our results indicated that P2X4R activation might inhibit neuronal autophagy by regulating the BDNF/TrkB intracellular signaling pathway, leading to DA neuron damage in the midbrain, particularly the SN, whereas the inhibition of P2X4R expression activated autophagy. The findings from this study reveal potential new targets for PD treatment.

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