# NEUROPROTECTIVE EFFECTS OF P2X4 RECEPTOR ON 6-OHDA-INDUCED PARKINSON'S DISEASE IN RATS

Yonghong Yang[1,#], Liang Zhou[2,#], Baike Zhu[3], Yong Wang[4], Liming Chen[5], Chao Wang[2], Yingchun Meng[2], Ke Han[2,*]
[1]Department of Neurology, The fifith hospital of Jinan, Jinan, PR China - [2]Department of Rehabilitation Medicine, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, PR China - [3]Department of Neurology, Shandong Provincial Third Hospital, Jinan, PR China - [4]Department of Tuina, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, PR China - [5]Department of Cardiology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, PR China
[#]*These authors contributed equally to this work as co-first author*

***ABSTRACT***

***Objective:*** *To investigate the neuroprotective mechanism and effect of P2X4 receptor (P2X4R) inhibition on 6-hydroxydopamine (6-OHDA)-induced Parkinson's disease in rats.*

***Methods:*** *Seventy-two clean, healthy male Wistar rats were randomly selected, and 6-OHDA was used to establish a rat model of Parkinson's disease. The rats were divided into control group, model group and intervention group, with 24 rats in each group. The number of tyrosine hydroxylase-positive neurons was calculated by fluorescence microscopy. The mRNA and protein expression levels of P2X4R, interleukin-18 (IL-18), interleukin-1β (IL-1β), caspase-1 and NLRP3 in the substantia nigra of each group were determined by real-time fluorescence quantitative PCR.*

***Results:*** *The number of tyrosine hydroxylase-positive neurons in the model group was significantly decreased compared to the control group, and the mRNA and protein expression levels of P2X4R, IL-18, IL-1β, caspase-1 and NLRP3 in the substantia nigra were significantly increased (P<0.05). Compared with the model group, the number of tyrosine hydroxylase positive neurons in the intervention group was significantly increased, and the mRNA and protein expression levels of P2X4R, IL-18, IL-1β, caspase-1 and NLRP3 in the substantia nigra were significantly decreased (P<0.05).*

***Conclusion:*** *Inhibition of P2X4R has a neuroprotective effect on 6-OHDA-induced Parkinson's disease, which may be a result of inhibiting the inflammatory response and reducing the degeneration and death of tyrosine hydroxylase-positive dopaminergic neurons in the substantia nigra of Parkinson's rats.*

***Keywords:*** *P2X4 receptor, 6-OHDA, parkinson's disease, neuroprotective effect, mechanism.*

*DOI: 10.19193/0393-6384_2021_2_171*

***Received*** *March 15, 2020;* ***Accepted*** *October 20, 2020*

## Introduction

Parkinson's disease is the second-most-common neurodegenerative disease in the world, after Alzheimer's disease. A significant pathological change of Parkinson's disease is that the midbrain substantia nigra and striatum dopaminergic neurons progressively die. As the midbrain substantia nigra and striatum dopamine concentration decreases, basal ganglia function adjustment disorder, which takes the form of static tremor, slow movement, myotonia, abnormal gait posture, cognitive/mental disorder and sleep disorder symptoms[(1)]. At present, dopamine replacement therapy is used in the clinical treatment of Parkinson's disease. Dopamine replacement can make up for the lack of dopamine in the brain, thus alleviating the clinical symptoms.

However, it is not effective in the treatment of degeneration and apoptosis of dopaminergic neurons[(2)]. Some studies have found that a neuronal

inflammatory response in the brain is involved in the occurrence and development of Parkinson's disease, but the specific mechanism is still unclear[3]. The P2X4 receptor (P2X4R) is an ion channel-type purine receptor controlled by the ligand adenosine triphosphate, which is distributed in the respiratory, circulatory, digestive and endocrine systems, and is most widely distributed in the nervous system[4].

Damaged dopaminergic neurons can release adenosine triphosphate to the outside of the cell, which can bind P2X4R on the surface, accelerate the activation of NLRP3 inflammasome, and thus participate in the denatured death of dopaminergic neurons in the substantia nigra[5]. The purpose of this study was to investigate the neuroprotective effect of P2X4R inhibition on 6-hydroxydopamine (6-OHDA)-induced Parkinson's disease in rats and its mechanism.

## Materials and methods

### *Experimental animals*

Seventy-two healthy, male Wistar rats of clean grade were randomly selected (purchased from Shanghai Kai Student Material Technology Co., Ltd., production license SCXK (Shanghai) 2017-0001), with a weight of 214±25 g and a temperature of 22±3 °C.

### *Main instruments and reagents*

Inverted fluorescence microscope (Shanghai Wumo Optical Instrument Co., Ltd., model: WMF-3580); low-temperature high-speed centrifuge (Shanghai Hetian Scientific Instrument Co., Ltd., model: TG18G); ultra-low temperature refrigerator (Meiling Biomedical, model: YCDEL450); electronic balance (Shenyang Longteng Electronics Co., Ltd., model: JD-2); micro-injection pump (Shenzhen Nuoshen Technology Co., Ltd., model: NOTON-16); real-time fluorescence quantitative PCR instrument (Beijing Shengke Xinde Technology Co., Ltd., model: CFX96); brain stereotactic locator (Beijing Ji Nuotai technology development co. Ltd., model: jNT-DTY); 6-OHDA (Dalian Meilun Biotechnology Co., Ltd., specification: 200MG); tyrosine hydroxylase antibody (Shanghai Xinyu Biotechnology Co., Ltd.); goat P2X4R polyclonal antibody (Shenzhen Xinbosheng Biotechnology Co., Ltd.).

### *Establishment of Parkinson's rat model and experimental grouping*

First, the rats were anaesthetized. Then, after brain stereotaxic instrument, the skin, the skull was fully exposed, and the anterior fontanelle was used to mark the origin coordinates of the lateral ventricle anterior fontanelle.

A dental drill was used in the skull surface. drilling before they are buried pipe, Using a microsyringe, from 5μg/μL P2X4R inhibitors (5-BDBD) or 0.9% NaCl solution at 0.5μL/min to locate injection, injection speed of retreat at a rate of 1 mm/min after injection. The scalp of the rats was sutured for 7 d, and penicillin was injected continuously for 3 d to prevent infection.

The coordinates of the substantia nigra were defined, and the rats were divided into a control group, a model group and an intervention group, with 24 rats in each group. Control group rats were raised normally without treatment. Rats in the model group were pretreated with 0.9% NaCl solution for 7 d and injected with 8 g/kg 6-OHDA at the location of the substantia nigra. The rats in the intervention group were pretreated with P2X4R inhibitor 5-BDBD for 7 d and injected with 8 g/kg 6-OHDA at the location of the substantia nigra. All rats were killed after the experiment.

### *Observation indicators*

The number of tyrosine hydroxylase positive neurons: the rats were anaesthetized, and the brain tissues of the rats were put into 4% formaldehyde solution for fixation, then dehydrated, and frozen sections were made. Each slice was 10 μm thick. After rinsing, the anti-rabbit fluorescent secondary antibody was added and cultured for another 2 h. After rinsing, 60 g/L glycerophosphate buffer was added to seal the tablets, and the number of tyrosine hydroxylase positive neurons was calculated by fluorescence microscopy.

P2X4R, interleukin-18 (IL-18), interleukin-1 (IL-1β), caspase-1 and NLRP3 mRNA expression levels in the substantia nigra of rats in each group were detected by real-time fluorescent quantitative PCR. The expression levels of P2X4R, IL-18, IL-1β, caspase-1 and NLRP3 in the substantia nigra were determined by Western blot.

### *Statistical methods*

The SPSS20.0 software package was used for statistical data analysis, in which the measurement data were compared by independent sample t-test between the two groups, and the single factor and multiple data means were compared between the groups. The counting data were compared using test.

## Results

### *Comparison of the number of tyrosine hydroxylase positive neurons in each group*

Compared with the control group, the number of tyrosine hydroxylase positive neurons in the model group was significantly decreased (P<0.05). Compared with the model group, the number of tyrosine hydroxylase positive neurons in the intervention group was significantly increased (P<0.05). See Figure 1 and Table 1.

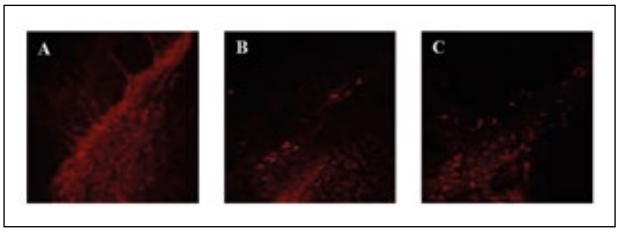


**Figure 1:** Comparison of the number of tyrosine hydroxylase positive neurons in each group.
*A: control group; B: model group; C: intervention group.*

| Group | Case (n) | Number of tyrosine hydroxylase-positive neurons |
|---|---|---|
| Control | 24 | 7269.37±67.65 |
| Model | 24 | 5095.32±39.95[a] |
| Intervention | 24 | 6386.71±49.65[b] |
| *F* | | 9965.59 |
| *P* | | < 0.001 |

**Table 1:** comparison of the number of tyrosine hydroxylase positive neurons in each group ($\bar{x}\pm s$).
*Note: a means compared with the control group, [a]P<0.05; b represents compared with the model group, [b]P<0.05.*

### *mRNA expression levels of P2X4R, IL-18, IL-1, caspase-1 and NLRP3 in each group*

Compared with the control group, the mRNA expression levels of P2X4R, IL-18, IL-1β, caspase-1 and NLRP3 in the model group were significantly increased (P<0.05). Compared with the model group, the mRNA expression levels of P2X4R, IL-18, IL-1β, caspase-1 and NLRP3 in the intervention group were significantly decreased (P<0.05). See Table 2 and Figure 2.

### *Expression levels of P2X4R, IL-18, IL-1β, caspase-1 and NLRP3 in the substantia nigra of rats in each group*

Western blot results showed that compared with the control group, the expression levels of P2X4R, IL-18, IL-1β, caspase-1 and NLRP3 in the model group were significantly increased (P<0.05). The expression levels of P2X4R, IL-18, IL-1β, caspase-1 and NLRP3 in the nigra of rats in the intervention group were significantly decreased (P<0.05), compared to the model group. See Figure 3 and Table 3.

| Group | Case (n) | P2X4R mRNA | IL-18 mRNA | IL-1β mRNA | Caspase-1 mRNA | NLRP3 mRNA |
|---|---|---|---|---|---|---|
| Control | 24 | 1.02±0.11 | 1.01±0.05 | 1.01±0.04 | 0.97±0.03 | 1.00±0.02 |
| Model | 24 | 3.36±0.26[a] | 3.44±0.12[a] | 3.94±0.12[a] | 4.01±0.11[a] | 3.97±0.10[a] |
| Intervention | 24 | 2.68±0.11[b] | 2.61±0.12[b] | 3.01±0.06[b] | 3.11±0.07[b] | 3.01±0.06[b] |
| *F* | | 1136.42 | 3509.44 | 8234.57 | 9808.63 | 11813.66 |
| *P* | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

**Table 2:** mRNA expression levels of P2X4R, IL-18, IL-1β, caspase-1 and NLRP3 in the substantia nigra of rats in each group ($\bar{x}\pm s$).
*Note: a means compared with the control group, [a]P<0.05; b represents compared with the model group, [b]P<0.05.*

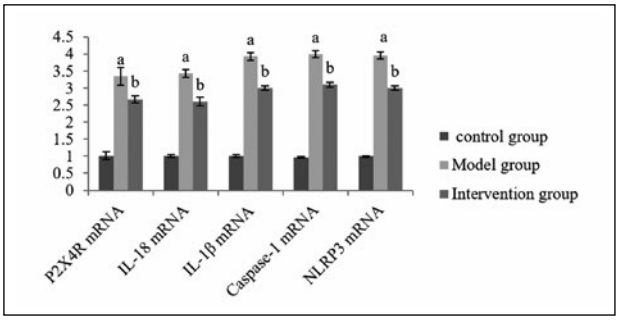


**Figure 2:** mRNA expression levels of P2X4R, IL-18, IL-1β, caspase-1 and NLRP3 in each group.

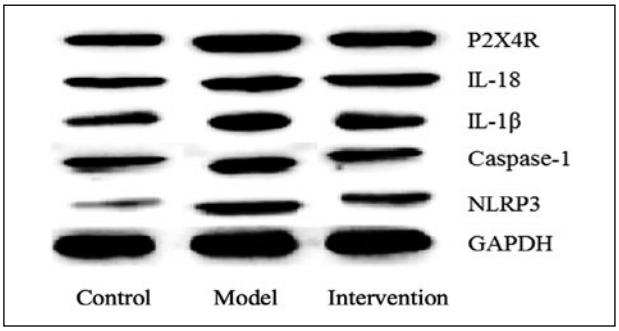


**Figure 3:** Expression levels of P2X4R, IL-18, IL-1β, caspase-1 and NLRP3 in the substantia nigra of rats in each group.

| Group | Case (n) | P2X4R | IL-18 | IL-1β | Caspase-1 | NLRP3 |
|---|---|---|---|---|---|---|
| Control | 24 | 0.95±0.01 | 0.96±0.01 | 0.93±0.01 | 0.93±0.01 | 0.92±0.02 |
| Model | 24 | 1.18±0.01[a] | 1.31±0.02[a] | 1.18±0.02[a] | 1.23±0.01[a] | 1.26±0.02[a] |
| Intervention | 24 | 1.07±0.01[b] | 1.10±0.01b | 1.06±0.01[b] | 1.09±0.02[b] | 1.05±0.01[b] |
| *F* | | 3176.00 | 3724.00 | 1876.00 | 2704.00 | 2354.67 |
| *P* | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

**Table 3:** Expression levels of P2X4R, IL-18, IL-1β, caspase-1 and NLRP3 in the substantia nigra of rats in each group ($\bar{x}\pm s$).
*Note: a means compared with the control group, [a]P<0.05; b represents compared with the model group, [b]P<0.05.*

## Discussion

Parkinson's disease is a common, chronic, progressive neurodegenerative disease characterized by slow movement, abnormal posture gait, static tremor and increased muscle tone[6]. The pathogenesis of Parkinson's disease is still unclear, and it is thought that the disease is related to oxidative stress, age-dependent mitochondrial dysfunction, elevated neurotoxicants, reduced neurotrophic factors, calcium overload, excitatory poisoning, immune disorders, inflammation and apoptosis. The affected neurons are able to form a chain reaction to cause abnormal or even death of cell function[7]. 6-OHDA is a drug used to induce Parkinson's disease in animal models that causes dopamine neuronal cells to die rapidly in a relatively short time[8]. This study used 6-OHDA to induce Parkinson's in a rat model to explore whether inhibition of P2X4R had neuroprotective effects.

P2X4R was first cloned in the brain and expressed specifically on microglia and neurons[9]. Microglia are the primary immune cells in the brain, found mainly in the substantia nigra. The results of this study showed that the number of tyrosine hydroxylase-positive neurons in the model group was significantly decreased. The number of tyrosine hydroxylase-positive neurons in the intervention group was significantly higher than that in the model group. It was suggested that inhibition of P2X4R significantly inhibited denaturation and death of tyrosine hydroxylase-positive neurons. P2X4R plays an important role in the pathogenesis of 6-OHDA-induced Parkinson's disease. This role may be related to the activation of microglia when stimulated, the release of a variety of inflammatory cytokines such as interleukins, mediation of blood-brain barrier damage or amplification of inflammatory responses leading to the degeneration of dopaminergic neurons[10-11].

IL-1β, IL-18 and so on are members of the interleukin family, and some studies have found that interleukin has a crucial role in the pathogenesis of Parkinson's disease[12]. IL-1β in the brain is produced by a variety of cells, including glial cells, which combine with IL-1 I receptors to play a variety of roles such as enhancing the oxidative stress response, exacerbating the inflammatory response, promoting calcium overload and promoting the formation of Lewy bodies[13]. IL-18 can induce the formation of interferon, promote the proliferation and differentiation of $CD_4^+$T cells, and increase the formation of inflammatory factors. Some scholars have found that microglia-mediated neuroinflammatory response plays a role in the pathogenesis of Parkinson's disease. IL-18-607C/A locus polymorphism is a risk factor for sporadic Parkinson's disease in the Chinese Han nationality[14]. NLRP3 inflammasome is a polyprotein complex. S et al. found that when the body has an immune activator, NLRP3 can be oligomerized and activated, while the downstream apoptosis-associated spotted protein can bind to the caspase-1 precursor to form a NLRP3 inflammasome, thus activating caspase-1 and promoting the mature release of precursor molecules such as IL-18 and IL-1β[15]. The results of this study found that the expression levels of P2X4R, IL-18, IL-1β, caspase-1, NLRP3 mRNA and protein in the model group were significantly increased, while the expression levels in the intervention group were significantly decreased. P2X4R, IL-18, IL-1β, caspase-1, NLRP3 may be involved in the pathogenesis of Parkinson's disease. The P2X4R inhibitor 5-BDBD can alleviate the damage to neuronal cells by inhibiting the expression of P2X4R, IL-18, IL-1β, caspase-1 and NLRLRP3.

To sum, inhibition of P2X4R has a certain neuroprotective effect on 6-OHDA-induced Parkinson's disease, which may be achieved by suppressing the inflammatory response to reduce the degeneration death of tyrosine hydroxylase-positive dopaminergic neurons in the substantia nigra of Parkinson's rats.

## References

1) Redondo N, García-González N, Diaz-Prieto LE, Olmedilla-Alonso B, Martín-Diana AB, et al. Effects of ewe's milk yogurt (whole and semi-skimmed) and cow's milk yogurt on inflammation markers and gut microbiota of subjects with borderline-high plasma cholesterol levels: a crossover study. Eur J Nutr 2019; 58: 1113-1124.

2) Li J, Yuan Y, Wang M, Zhang J, Zhang L, et al. Alterations in regional homogeneity of resting-state brain activity in fatigue of Parkinson's disease. J Neural Transm (Vienna) 2017; 124: 1187-1195.

3) Liu C, Zhang CW, Lo SQ, Ang ST, Chew KCM, et al. S-Nitrosylation of Divalent Metal Transporter 1 Enhances Iron Uptake to Mediate Loss of Dopaminergic Neurons and Motoric Deficit. J Neurosci 2018; 38: 8364-8377.

4) Zhao WS, Sun MY, Sun LF. A Highly Conserved Salt Bridge Stabilizes the Kinked Conformation of 2, 3-Sheet Essential for Channel Function of P2X4 Receptors. J Biol Chem 2016; 291: 7990-8003.

5) Mackay L, Zemkova H, Stojilkovic SS, Sherman A, Khadra A. Deciphering the regulation of P2X4 receptor channel gating by ivermectin using Markov models. PLoS Comput Biol 2017; 13: 1005643.
6) Stuendl A, Kunadt M, Kruse N, Bartels C, Moebius W, et al. Induction of α-synuclein aggregate formation by CSF exosomes from patients with Parkinson's disease and dementia with Lewy bodies. Brain 2016; 139: 481-194.
7) Beilina A, Cookson MR. Genes associated with Parkinson's disease: Regulation of autophagy and beyond. J Neurochem 2015; 139: 91-107.
8) Guha D, Lorenz DR, Misra V, Chettimada S, Morgello S, et al. Proteomic analysis of cerebrospinal fluid extracellular vesicles reveals synaptic injury, inflammation, and stress response markers in HIV patients with cognitive impairment. J Neuroinflammation 2019; 16: 254.
9) Varaschin RK, Osterstock G, Ducrot C, Leino S, Bourque MJ, et al. Histamine H3Receptors Decrease Dopamine Release in the Ventral Striatum by Reducing the Activity of Striatal Cholinergic Interneurons. Neuroscience 2018; 376: 188-203.
10) Asif A, Khalid M, Manzoor S, Ahmad H, Rehman AU. Role of purinergic receptors in hepatobiliary carcinoma in Pakistani population: an approach towards proinflammatory role of P2X4 and P2X7 receptors. Purinergic Signal 2019; 15: 367-374.
11) Guo LH, Trautmann K, Schluesener HJ. Expression of P2X4 receptor by lesional activated microglia during formalin-induced inflammatory pain. J Neuroimmunol 2015; 163: 120-127.
12) Kringel D, Kaunisto MA, Lippmann C, Kalso E, Lötsch J. Development of an AmpliSeqTM Panel for Next-Generation Sequencing of a Set of Genetic Predictors of Persisting Pain. Front Pharmacol 2018; 9: 1008.
13) Cai M, Liu Z, Li W, Wang Y, Xie A. Association between rs823128 polymorphism and the risk of Parkinson's disease: A meta-analysis. Neurosci Lett 2018; 665: 110-116.
14) Liu Z, Guo J, Wang Y, Li K, Kang J, et al. Lack of association between IL-10 and IL-18 gene promoter polymorphisms and Parkinson's disease with cognitive impairment in a Chinese population. Sci Rep 2016; 6: 19021.
15) Zeng QZ, Yang F, Li CG, Xu LH, He XH, et al. Paclitaxel Enhances the Innate Immunity by Promoting NLRP3 Inflammasome Activation in Macrophages. Front Immunol 2019; 10: 72.

*Acknowledgement:*
*This paper was supported by Shandong Medical and Health Technology Development Plan (No.2017WS468); Science and Technology Development Plans of Traditional Chinese Medicine in Shandong Province (No.2019_0304).*

*Corresponding Author:*
Ke Han
Email: sj90xw@163.com
*(China)*