Original Paper

Inhibition of microRNA-200a Upregulates the Expression of Striatal Dopamine Receptor D2 to Repress Apoptosis of Striatum via the cAMP/PKA Signaling Pathway in Rats with Parkinson's Disease

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Key Words

microRNA-200a • Parkinson's disease • Striatal dopamine receptor D2 • cAMP/PKA signaling pathway • Apoptosis

Abstract

Background/Aim: Parkinson's disease (PD) is a neurodegenerative movement disease with a high annual incidence. Accumulating evidence demonstrates that microRNAs play important roles in the pathogenesis of multiple neurological disorders, including PD. This study aims to investigate how microRNA-200a (miR-200a) regulates striatal dopamine receptor D2 (DRD2) to affect apoptosis of striatum in rats with PD and to explore the associated mechanism. **Methods:** After successfully establishing a PD model by 6-hydroxydopamine injections, PD rats were randomly treated with miR-200a mimics, inhibitors, Forskolin or a combination of miR-200a inhibitors and Forskolin. High-performance liquid chromatography-electrochemical detection (HPLC-ECD) was employed to detect the levels of dopamine, 3, 4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and chemistry colorimetric methods were applied to detect the levels of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). A TUNEL assay and immunocytochemical staining were performed to observe apoptosis and tyrosine hydroxylase (TH)-positive cells in the striatum. The expression of miR-200a, DRD2, Bad, Bax, Bcl-2, cAMP and PKA was determined by reverse transcription

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quantitative polymerase chain reaction (RT-qPCR) and western blot assays. **Results:** In the cellular experiments, after transfection with the inhibitor of miR-200a, decreased levels of Bax, GSH-Px, SOD, dopamine, DOPAC and HVA but increased levels of MDA and Bcl-2 were found along with a reduced apoptosis rate and increased TH-positive cell number. In addition, downregulating miR-200a resulted in lower expression of AKT, cAMP and PKA but higher expression of DRD2 and CREB, indicating that the downregulation of miR-200a increases DRD2 expression, which blocks the cAMP/PKA signaling pathway. **Conclusion:** This study provides evidence that the inhibition of miR-200a can repress apoptosis in the striatum by inhibition of the cAMP/PKA signaling pathway by upregulating DRD2 expression in PD rats.

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Introduction

As a representative neurodegenerative movement disease, Parkinson's disease (PD) can produce numerous motor and cognitive impairments [1, 2]. PD usually occurs at the age of 65 to 70 years and affects 1 to 2 per 1000 people with an annual incidence of 15 per 100000 [3]. This disease is characterized by the loss of dopaminergic neurons, which is caused by damage to dopamine in the striatum and neuronal apoptosis in the substantia nigra pars compacta [4]. PD patients generally have some symptoms such as tremor, rigidity, bradykinesia, impairment of the autonomic nervous system, micrographia, depression and freezing [5]. In addition, it is well known that PD is caused by inflammation, mitochondrial dysfunction, oxidative stress, genetic susceptibility, protein aggregation and aging [6]. Currently, oral levodopa is the major method used for the treatment of PD, but it still has numerous side effects, such as the end-of-dose phenomenon and switch effect [7]. Therefore, it is necessary to understand the molecular mechanism underlying PD to discover a better therapeutic method for PD.

MicroRNAs (miRNAs) constitute a class of endogenous (19~24 nucleotides), non-coding RNAs that silence gene expression by binding the 3'-untranslated region (3'-UTR) of their target mRNAs [8]. In addition, miRNAs play pivotal roles in the pathogenesis of various neurological disorders, such as Huntington's disease (HD) [9]. MiR-200a belongs to the miR-200a family, which is associated with numerous cellular processes, including proliferation, differentiation and cell cycle exit [10]. MiR-200a can inhibit the expression of transcriptional repressors, thus regulating the epithelial phenotype [11]. Cyclic adenosine-3',5'-monophosphate (cAMP) is a secondary messenger that plays a pivotal role in muscle contraction, neurotransmission, differentiation, cell growth and exocytosis [12]. In addition, altered cAMP regulates some normal brain functions, including neuronal memory, striatal plasticity and synapses, and is associated with various neurological diseases, such as HD and PD [13]. Protein kinase A (PKA), which is a key mediator of cAMP, is involved in synaptic plasticity and long-term memory formation [14, 15]. PKA contains the following four subunits: two regulatory subunits that bind cAMP and two catalytic subunits that initiate cAMP response element binding protein (CREB) [16]. Importantly, a prior study revealed that the cAMP/PKA signaling pathway can regulate complex I, whose defects are involved in several neurological diseases, including PD and primary mitochondrial diseases [17]. However, studies investigating the effect of miR-200a on PD are limited. The present study investigated the role of miR-200a in the regulation of the cAMP/PKA signaling pathway in PD to identify a new treatment method for PD patients.

Materials and Methods

Establishment of the animal model

In total, 90 specific pathogen-free male Sprague-Dawley (SD) rats (aged 6~8 weeks and weighing 250 ± 20 g) raised by Hunan Slac Jingda Laboratory Animal Co., Ltd. (Changsha, China) were purchased by our laboratory and included in this study. After 7 d of adoptive feeding (an important process of feeding experimental rats for a few days before they were moved from the supplier to a laboratory animal house to

albumin (BSA) sealing liquid for 20 min and cooled to room temperature. The sections were incubated with a rabbit anti-rat TH (ab68476, 1 : 1500, Abcam Inc., Cambridge, MA, USA) polyclonal antibody overnight at 4°C, and then, the sections were incubated with the secondary antibody immunoglobulin G (IgG) (ab6789, 1 : 1000, Abcam Inc., Cambridge, MA, USA) serum labeled with biotin at room temperature for 20 min. After the incubation at room temperature with an SABC kit for 20 min, the sections were developed by 3'-diaminobenzidine (DAB), stained with hematoxylin, dehydrated, cleared, mounted and observed under an optical microscope. One surface section of the interpeduncular nucleus was selected from one animal per group; three SNc fields of vision ($\times 400$) were randomly selected from each immunohistochemical section followed by positive cell counting and photographing. The cytoplasm and membranes of the positive cells were obviously brown.

Measurement of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px)

After the experiment, the rats were decapitated, and their brains were removed and placed on an ice plate. Then, the bilateral striata were rapidly separated, and their qualities were accurately measured. Normal saline was added to the striata according to the mass to volume ratio to generate 10% tissue homogenates for further use. The colorimetric method was applied to measure the levels of MDA, SOD and GSH-Px according to the instructions in the kit provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

HE staining

The paraffin sections were dewaxed, soaked in xylene and immersed in methylbenzene I, xylene II, and 100%, 95%, 85% and 80% alcohol (each for 5 min). Then, the sections were stained with hematoxylin for 8 min, differentiated with 1% hydrochloric alcohol and turned blue with 0.25% ammonia for 1 min. Subsequently, the sections were stained with 1% eosin for 1 min, dehydrated with 80% alcohol I, 80% alcohol II, 90% alcohol I, 90% alcohol II, 100% alcohol I, 100% alcohol II, xylene I and xylene II (each for 3 min). Finally, the sections were sealed with neutral gum, and the pathological changes in the striata were observed under an Olympus CX41 microscope (Olympus Corporation, Tokyo, Japan). The nuclei were bluish purple, the cytoplasm was pink and the red blood cells were jacinth.

TUNEL staining

The paraffin sections were baked in an oven at 50°C for 2 h, dewaxed with xylene, and then dehydrated with 100%, 95%, 90%, 80% and 70% of gradient ethanol. In the dark, the slides were incubated with 3% hydrogen peroxide for reaction at room temperature for 10 min and washed with Tris-buffered saline (TBS) 3 times for 2 min each wash. Then, the slides were incubated with proteinase K for reaction at 37°C for 10 min and washed with TBS 3 times for 2 min each wash. The sections were dried, marked with labeling buffer (20 μ L per section) for 2 min in a wet box at 37°C, and washed with TBS 3 times for 2 min each wash. Each slide was incubated with 10 μ L sealing solution for reaction at room temperature for 10 min. The sections were incubated with biotinylated anti-digoxin antibody (50 μ L each), placed into a wet box at 37°C, and reacted for 1 h. After washing with TBS 3 times (2 min each wash), the sections were incubated with streptavidin-biotin complex (SABC) (each for 50 μ L), placed in a wet box at 37°C, and reacted for 30 min. Subsequently, the sections were washed with TBS 4 times for 5 min each wash, colorized with DAB for 6 min, and washed with water. Then, the sections were lightly counterstained with hematoxylin and washed with water and distilled water. The sections were dehydrated with gradient ethanol, cleared with xylene, mounted with neutral gum and observed under an Olympus CX41 microscope (Olympus Corporation, Tokyo, Japan). The apoptotic nuclei were tan, and the normal nuclei were blue.

Immunohistochemical staining

The paraffin sections were baked in an incubator at 60°C for 30 min, conventionally dewaxed with toluene I, xylene II, and 100%, 95%, 85% and 80% alcohol (5 min each), and washed with water for 2 min. The sections underwent antigen retrieval by 1 mM tromethamine-ethylene-diamine tetra-acetic acid (pH = 8.0), cooled to room temperature and washed with phosphate buffer saline (PBS) 3 times for 5 min each wash. The sections were soaked in 3% H₂O₂-methanol at room temperature for 10 min to block endogenous peroxidase and then washed with PBS twice (5 min per wash). The sections were incubated with primary antibodies, including B-cell leukemia/lymphoma-2 (Bcl-2) associated X protein (Bax, ab32503, 1 : 50 - 1 :

100), Bcl-2 (ab32124, 1: 100) and dopamine D2 receptor (DRD2, ab21218, 1: 80), at 4°C overnight. On the following day, after washing with 0.1% PBST (PBS with Tween-20) 3 times (5 min per wash), the sections were incubated with polymer reinforcing agent for 20 min at room temperature. After washing with 0.1% PBST 3 times (5 min per wash), the sections were incubated with enzyme labeled anti-rat/rabbit polymer (PV-9000-D, ZSGB-Bio, Beijing, China) at room temperature for 30 min, followed by washing with 0.1% PBST 3 times (5 min per wash). DAB was applied to develop the sections for 5 min, and the sections were washed with distilled water to stop development. The sections were counterstained with hematoxylin, washed with water, differentiated and then washed with water to turn back to blue. Finally, the sections were conventionally dehydrated, cleared, and mounted with neutral gum. The sections were observed and pictures were taken under an Olympus CX41 microscope (Olympus Corporation, Tokyo, Japan). Within 0.1 mm², the cells with brown granules in the cytoplasm were regarded as positive cells and counted.

Pathological observation of rats by electron microscopy

The frozen brain tissues were removed, and the left and right brain tissues were carefully separated from the longitudinal fissure of the brain. The *Stereotaxic Atlas of the Rat Brain* by Paxinos and Watson was applied to locate the striatum, and then, 1 mm³ samples were carefully cut. The samples were fixed with glutaraldehyde fixative for 2 - 4 h. The samples were rinsed with 0.1 mol/L PBS at 4°C for 1 h, during which the fluid was changed 3 times. The samples were soaked in osmic acid at 0 - 4°C for 1 h, and then 30%, 70% and 90% acetone (each for 10 min) and then immersed in pure acetone 3 times (each for 10 min). The fully soaked samples were placed into the capsule of the embedding agent, polymerized at 60°C for 24 h, and then polymerized at 60°C for 24 h. The samples were cut into semithin sections by an ultramicrotome, and the location was examined under a light microscope. Finally, the samples were cut into ultrathin sections (40 - 50 nm) that were then observed under a transmission electron microscope (JEM-1011, JEOL Ltd., Akishima, Tokyo, Japan), and pictures were captured.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The brain tissues of the experimentally treated rats were dissected, and the striata were separated carefully. The tissues (50 mg) were extracted, and the total RNA was isolated by a miRNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). The total RNA (1 µL) was diluted 20 times with ultrapure water. The RNA concentration and purity were estimated using a UV-visible spectrophotometer (measured by absorbance at 260 nm and 280 nm). The ratio of OD₂₆₀/OD₂₈₀ was determined to be between 1.7 and 2.1, which met the needs of the follow-up experiment. The total RNA was reversely transcribed into a cDNA template using a PCR amplification instrument according to the instructions of the Reverse Transcription Kit (Beijing TransGen Biotech Co., Ltd., Beijing, China). The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), miR-200a, DRD2, Bcl-2, Bad, Bax, AKT, CREB, Bcl-2, cyclic adenosine 3',5'-monophosphate (cAMP) and protein kinase A (PKA) were designed and synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China) (Table 1). The reaction system was 20 µL. According to the instructions of Beijing TransGen First-Strand cDNA Synthesis SuperMix (AE301-02, Beijing TransGen Biotech Co., Ltd., Beijing, China), 10 µL mix reagent, 5 µL total RNA and 10 µL RNase Free H₂O were added into an Eppendorf tube, centrifuged, and reverse transcribed using the PCR instrument (9700, Beijing Dingguo Technology Co., Ltd, Beijing, China). The reactions were incubated at 37°C for 15 min, heated by 95°C for 5 s and 4°C to terminate the reaction. The obtained cDNA was stored at -20°C. RT-qPCR was conducted with the cDNA according to the instructions of the SYBR®Premix Ex Taq™ II Kit (TaKaRa Technology Co., Ltd, Dalian, China). The reaction system was 20 µL containing 10 µL of SYBR Premix, 2 µL of cDNA template, 0.6 µL of forward and reverse primers, and 6.8 µL of sterile water. RT-qPCR was conducted using an ABI7500 qPCR instrument (7500, Applied Biosystems, Carlsbad, CA, USA), and GAPDH was used as an internal control. The reaction conditions were as follows: pre-denaturation at 95°C for 30 s, 45 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 20 s, and extension at 72°C for 34 s. The 2^{-ΔΔCt} method [21] was employed to calculate the expression of miR-200a, DRD2, Bad, Bax, AKT, CREB, Bcl-2, cAMP and PKA. The 2^{-ΔΔCt} method indicated the ratio of the target gene expression between the experiment group and control group as follows: $\Delta\Delta C_t = \Delta C_t^{\text{the experiment group}} - \Delta C_t^{\text{the control group}}$. The cycle number at which the fluorescence reaches the threshold value is the threshold cycle (Ct), and the amplification is logarithmic growth at this point. The experiment was repeated 3 times.

Western blot assay

The rats were sacrificed by decapitation. The striata were removed according to the stereotaxic atlas of the rat brain, placed on ice and transferred into a homogenizer, and then, 5 times the cell homogenates were added for homogenization on ice for 10 min. The striata were transferred to a centrifuge tube, broken by an ultrasonic wave for 15 s (3 times), centrifuged at 12000 rpm for 20 min at 4°C, and then incubated in a boiling water bath for 10 min. The supernatant was used to determine the protein concentration using the bicinchoninic acid method and then stored at -20°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to prepare a 10% separation gel and 5% spacer gel. The protein was isolated by electrophoresis on a polyacrylamide gel, transferred to a nitrocellulose membrane by the wet transfer method, and then sealed with 5% BSA at room temperature for 1 h. The membrane was incubated with diluted rabbit polyclonal antibodies against cAMP (1 : 1000, ab26322), PKA (1 : 1000 - 1 : 5000, ab38949), Bad (1 : 500 - 1 : 1000, ab28840), phosphorylated Bad (p-Bad) (1 : 100 - 1 : 1000, ab216829), Bax (1 : 1000 - 1 : 10000, ab32503), Bcl-2 (1 : 500 - 1 : 2000, ab196495), DRD2 (1 µg/mL, ab85367), CREB (1 : 20, ab32515), AKT (1 : 500, ab106693) and GAPDH (1 µg/mL, ab37168) at 4°C overnight. All antibodies above were purchased from Abcam Inc. (Cambridge, MA, USA). After washing with TBST (10 min per wash), the membrane was washed with TBST three times (10 min per wash), and goat anti-rabbit polyclonal secondary antibodies (ab7312, Abcam Inc., Cambridge, MA, USA) were diluted with 5% BSA. The membrane was incubated with secondary antibodies corresponding to the primary antibodies, shaken for 1 h at room temperature, and then washed with PBST 3 times (15 min per wash). The membrane was developed with a developer and Bio-Rad gel imaging system (MG8600, Bio-Rad Laboratories, Richmond, CA, USA), and a quantitative analysis was performed by Image-Pro Plus 7.0 (IPP7.0, Media Cybernetics, Silver Spring, MD, USA). The relative protein expression levels of cAMP, PKA, Bcl-2 and DRD2 and the extent of p-Bad were calculated by the ratio between the gray value of the target band and that of the control band with GAPDH as the internal control. The experiment was repeated three times.

Statistical analysis

SPSS21.0 software (IBM Corp. Armonk, NY, USA) was used for the data analysis. The measurement data are presented as the mean ± standard deviation. A *t*-test was applied for comparisons between two groups, and one-way analysis of variance (ANOVA) was applied for comparisons among multiple groups. *p* < 0.05 was considered to represent a statistically significant difference.

Results

PD rat model is confirmed to be successfully induced by APO

Seven weeks after modeling, the successfully established PD rat models were rotated for approximately 5 min after being injected intraperitoneally with APO. The rats were rotated to the right with the contralateral hind limb as the pivot point, and their bodies were laterally curved with a ring shape end-to-end. Simultaneously, the rats exhibited a slight tremor, warped-tail, gait instability, foraging and arching action. Ten minutes after the injection of

Table 1. Primer sequences for the RT-qPCR.

Notes: F, forward; R, reverse; Bcl-2, B-cell leukemia/lymphoma-2; Bax, Bcl-2 associated X; DRD2, dopamine D2 receptor; miR-200a, microRNA-200a; cAMP, cyclic adenosine 3',5'-monophosphate; PKA, protein kinase A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Bad, Bcl-x1/Bcl-2-associated death promoter; CREB, cyclic AMP response element binding protein; RT-PCR, reverse transcription quantitative polymerase chain reaction

Genes	Sequences
Bcl-2	F: 5'-GCTGAGCCCTTATG-3' R: 5'-GCCCCGCAAA-3'
PKA	F: 5'-AGTCACTTCCA-3' R: 5'-GGGCTCTGAGAGGGGTC-3'
Bax	F: 5'-AGGGTTCAGGATCGAGCAG-3' R: 5'-ATCTTCGCTGAGCGAG-3'
DRD2	F: 5'-GCCACACATGCGCTGG-3' R: 5'-ATGGCAACAGGTTTCAGG-3'
cAMP	F: 5'-ATCACTATCGGCAAT-3' R: 5'-TACGGATGTCAACGT-3'
miR-200a	F: 5'-CGGGCAGCAACACG-3' R: 5'-CCCTTTGCTCGTTCC-3'
Bad	F: 5'-AGGCCTATGCAAAAGAGGAT-3' R: 5'-CGCACCGGAAGGAATCT-3'
Bcl-2	F: 5'-CGTCTTCACCATGGAGA-3' R: 5'-CGGCCATCAGCCACAGCTT-3'
AKT	F: 5'-ATGAACGACGTAGCCATTGTGAAGG-3' R: 5'-TCAGATGATCCATGCGGGGC-3'
CREB	F: 5'-ATGACCATGGACTCTGGAGCAGACA-3' R: 5'-TTAATCTGACTTGTGGCAGTAAAGGTCC-3'

APO, the number of rotating circles within 30 min was recorded, and the average speed of each rat was above 7 RPM/min. The rats in the normal group did not show such rotational behavior after the injection of APO. As shown in Table 2, there were significant differences

in the rotation numbers 1, 2, 4, and 6 weeks after the operation ($p < 0.05$), and there was no significant difference between 4 and 6 weeks after the operation ($p > 0.05$).

Table 2. Experimental results of the rotational behavior induced by APO (rpm) demonstrate the successful establishment of the PD rat model. Notes: * $p < 0.05$ vs. 1 week. APO, apomorphine; PD, Parkinson's Disease

Groups	After 6-hydroxydopamine injection (weeks)			
	1	2	4	6
PD group	3.1 ± 0.2	$6.2 \pm 0.8^*$	$9.1 \pm 1.7^*$	$10.0 \pm 1.4^*$
Normal group	0	0	0	0

Inhibition of miR-200a or inactivation of cAMP increases the levels of GSH and SOD and decreases the level of MDA

The levels of GSH, SOD and MDA were subsequently determined. Compared with the normal group, the other six groups exhibited reduced levels of GSH and SOD but elevated MDA ($p < 0.05$). Compared with the blank and NC groups, the miR-200a mimic group exhibited reduced levels of GSH and SOD but elevated MDA, while the miR-200a inhibitor group exhibited elevated levels of GSH and SOD but reduced MDA ($p < 0.05$). There were no significant differences in the above indicators between the miR-200a inhibitor + Forskolin and blank groups and between the Forskolin and miR-200a mimic groups ($p > 0.05$) (Table 3). We concluded that the inhibition of miR-200a or the inactivation of cAMP could increase the levels of GSH and SOD and decrease the level of MDA.

Inhibition of miR-200a or inactivation of cAMP increases the levels of dopamine, HVA and DOPAC

HPLC-ECD was performed to determine the levels of dopamine, HVA and DOPAC. The levels of dopamine, HVA and DOPAC in the normal group were higher than those in the other six groups (all $p < 0.05$), and there were no significant differences among the blank, NC and miR-200a inhibitor + Forskolin groups (all $p > 0.05$). Compared with the blank group, the miR-200a mimic and Forskolin groups had significantly decreased levels of dopamine, HVA and DOPAC (all $p < 0.05$), but the miR-200a inhibitor group had significantly increased levels of dopamine, HVA and DOPAC (all $p < 0.05$). There was no significant difference in the levels of dopamine, HVA and DOPAC between the miR-200a mimic and Forskolin groups ($p > 0.05$) (Table 4). Taken together, the inhibition of miR-200a or the inactivation of cAMP could increase the levels of dopamine, HVA and DOPAC.

Inhibition of miR-200a or inactivation of cAMP alleviates histopathological changes in the striatum of PD rats

H&E staining was performed to observe the histopathological changes in the PD rats, and the results (Fig. 1) demonstrated that the nucleus of the rat striatum was stained bluish violet, whereas most of the cytoplasm was pink. Compared with the normal group, the other six groups exhibited clearly reduced number of nerve cells, most cells in these groups were atrophic and loosely arranged and some cells exhibited cytoplasmic swelling and vacuolar degeneration. There were no differences in these histopathological changes among the blank, NC and miR-200a inhibitor + Forskolin groups (all $p > 0.05$). Compared with the blank group, the miR-200a inhibitor group had an elevated number of nerve cells with a slightly denser arrangement, but many cells still showed slight atrophy and vacuolar degeneration; the miR-200a mimic and Forskolin groups exhibited a drastically reduced number of nerve cells, and most cells were atrophic with cytoplasmic swelling and vacuolar degeneration. Therefore, the inhibition of miR-200a or the inactivation of cAMP could alleviate the histopathological changes in the striatum of PD rats.

Table 3. Inhibition of miR-200a or inactivation of cAMP increases the levels of GSH and SOD and decreases the level of MDA. Notes: * $p < 0.05$ vs. the normal group. #, $p < 0.05$ vs. the blank group. GSH-Px, glutathione peroxidase; MDA, malondialdehyde; SOD, superoxide dismutase; miR-220a, microRNA-220a; cAMP, cyclic adenosine 3',5'-monophosphate; NC, negative control

Groups	n	GSH-Px (U/mg)	MDA (mmol/mg)	SOD (U/mg)
Normal group	10	887.56 ± 306.25	7.45 ± 0.70	28.25 ± 3.98*
Blank group	10	442.23 ± 105.84*	12.02 ± 1.32*	18.16 ± 2.02*
NC group	10	443.31 ± 107.83*	11.98 ± 1.21*	18.23 ± 1.97*
miR-200a mimic group	10	221.96 ± 78.85*#	15.34 ± 1.62*#	13.24 ± 1.76*#
miR-200a inhibitor group	10	664.54 ± 204.76*#	9.70 ± 1.70*#	22.12 ± 1.21*#
Forskolin group	10	218.82 ± 86.29*#	15.36 ± 1.68*#	13.27 ± 1.66*#
miR-200a inhibitor + Forskolin group	10	443.79 ± 98.63*	12.00 ± 1.28*	18.23 ± 1.93*

Table 4. Inhibition of miR-200a or inactivation of cAMP increases the levels of dopamine and DOPAC (ng/g wet weight). Notes: * $p < 0.05$ vs. the normal group. #, $p < 0.05$ vs. the blank group. HVA, homovanillic acid; DOPAC, 3, 4-dihydroxyphenylacetic acid; miR-220a, microRNA-220a; cAMP, cyclic adenosine 3',5'-monophosphate; NC, negative control

Groups	n	Dopamine	HVA	DOPAC
Normal group	10	1024.50 ± 100.27	305.44 ± 38.18	546.43 ± 87.74
Blank group	10	605.76 ± 58.65*	305.44 ± 43.52*	387.05 ± 40.27*
NC group	10	598.11 ± 55.31*	310.11 ± 41.79*	383.04 ± 36.09*
miR-200a mimic group	10	412.22 ± 45.32*#	232.33 ± 32.26*#	305.26 ± 34.78*#
miR-200a inhibitor group	10	821.15 ± 98.76*#	385.98 ± 45.30*#	464.16 ± 75.87*#
Forskolin group	10	400.27 ± 49.89*#	240.16 ± 38.41*#	303.45 ± 39.13*#
miR-200a inhibitor + Forskolin group	10	613.51 ± 62.13*	305.21 ± 40.13*	380.85 ± 38.26*

Inhibition of miR-200a or inactivation of cAMP inhibits apoptosis of striatum

Subsequently, TUNEL staining was performed to investigate the potential effect of miR-200a and cAMP on apoptosis in the striatum. Compared with the normal group, the blank, miR-200a mimic and miR-200a inhibitor groups exhibited elevated apoptosis rate (all $p < 0.05$), there were no differences among the blank, miR-200a mimic and miR-200a inhibitor + Forskolin groups (all $p > 0.05$). Compared with the blank group, the miR-200a inhibitor group had a reduced apoptosis rate, whereas the miR-200a mimic and Forskolin groups had an elevated apoptosis rate ($p < 0.05$) (Fig. 2). These results suggest that the inhibition of miR-200a or the inactivation of cAMP could inhibit apoptosis of striatum.

Fig. 1. Inhibition of miR-200a or inactivation of cAMP alleviates histopathological changes in the striatum of PD rats as detected by HE staining ($\times 400$). Notes: The data are presented as the mean $\pm$ standard deviation and were analyzed by one-way ANOVA. The experiment was independently repeated three times. miR-220a, microRNA-220a; cAMP, cyclic adenosine 3',5'-monophosphate; HE, hematoxylin eosin; NC, negative control.

Inhibition of miR-200a or inactivation of cAMP increases TH-positive cells

Immunohistochemical staining was performed to investigate whether miR-200a or cAMP could affect TH-positive cells. The results showed that the TH-positive cells were yellow or tan, mainly concentrated in the cytoplasm, and cone-shaped or elliptical (Fig. 3A).

As shown in Fig. 3B, the TH-positive cells in the other groups were significantly reduced ($p < 0.05$). Compared with the blank group, there was no significant difference in the number of TH-positive cells in the NC and miR-200a inhibitor + Forskolin groups ($p > 0.05$), while the TH-positive cells in the Forskolin and miR-200a inhibitor groups were decreased ($p < 0.05$). The above results suggested that the inhibition of miR-200a or the inactivation of cAMP could increase the number of TH-positive cells.

Inhibition of miR-200a or inactivation of cAMP downregulates Bax and upregulates Bcl-2 and DRD2

Subsequently, additional immunohistochemical staining was performed to explore the effect of miR-200a or cAMP on Bax, Bcl-2 and DRD2. The results of the immunohistochemical staining (Fig. 4) showed that the cells with a brown cytoplasm were positive. Compared with the normal group, the other six groups had increased levels of Bax (pro-apoptotic protein) and decreased levels of Bcl-2 (anti-apoptotic protein) (all $p < 0.05$). There were no differences in the levels of Bax and Bcl-2 among the blank, NC and miR-200a inhibitor + Forskolin groups.

Fig. 2. Inhibition of miR-200a or inactivation of cAMP inhibits apoptosis in the striatum as detected by TUNEL staining. Notes: A, Apoptotic staining map of each group. B, Apoptotic cell number in each group. *, $p < 0.05$ vs. the normal group. #, $p < 0.05$ vs. the blank group. The data are presented as the mean $\pm$ standard deviation and were analyzed by one-way ANOVA. The experiment was independently repeated three times. miR-220a, microRNA-220a; cAMP, cyclic adenosine 3',5'-monophosphate; TUNEL, TdT-mediated dUTP-biotin nick end-labeling; NC, negative control.

Fig. 3. Inhibition of miR-200a or inactivation of cAMP increases TH-positive cells as detected by immunohistochemical staining ($\times 200$). Notes: A, Immunohistochemical staining map of striatum tissues from each group ($\times 200$); Panel B, statistical chart of TH-positive cells. *, $p < 0.05$ vs. the normal group. #, $p < 0.05$ vs. the blank group. The data are presented as the mean $\pm$ standard deviation and were analyzed by one-way ANOVA. The experiment was independently repeated three times. miR-220a, microRNA-220a; cAMP, cyclic adenosine 3',5'-monophosphate; NC, negative control.

Fig. 4. Inhibition of miR-200a or inactivation of cAMP downregulates Bax and upregulates Bcl-2 and DRD2 as detected by immunohistochemical staining. Notes: A, Immunohistochemical staining result of Bax in each group. B, Immunohistochemical staining result of Bcl-2 in each group. C, Immunohistochemical staining result of DRD2 in each group. D, Positive cell number of Bax, Bcl-2 and DRD2 in each group. *, $p < 0.05$ vs. the normal group. #, $p < 0.05$ vs. the blank group. The data are presented as the mean $\pm$ standard deviation and were analyzed by one-way ANOVA. The experiment was independently repeated three times. miR-220a, microRNA-220a; cAMP, cyclic adenosine 3',5'-cyclic phosphate; Bcl-2, B-cell leukemia/lymphoma-2; Bax, Bcl-2 associated X; DRD2, dopamine D2 receptor; NC, negative control.

(all $p > 0.05$). Compared with the blank group, the miR-200a inhibitor group had reduced levels of Bax but elevated levels of Bcl-2, whereas the miR-200a mimic and Forskolin groups had elevated levels of Bax but reduced levels of Bcl-2 ($p < 0.05$). Compared with the normal group, the other six groups had decreased levels of DRD2 (all $p < 0.05$). Compared with the blank group, the miR-200a inhibitor and miR-200a inhibitor + Forskolin groups had increased levels of DRD2, whereas the miR-200a mimic group had decreased levels of DRD2 ($p < 0.05$); there was no difference among the blank, NC and Forskolin groups (all $p > 0.05$). These results suggest that the overexpression of miR-200a or the activation of cAMP can upregulate Bax and downregulate Bcl-2 and DRD2.

Inhibition of miR-200a or inactivation of cAMP improves the striatum structure in PD rats

The effects of miR-200a and cAMP on the striatum structure in the PD rats were explored by observations under an electron microscope. In the normal rats, the neuromuscular junctions in the striatum were arranged neatly, the structures of the presynaptic and postsynaptic membranes were intact, and the structures of the vesicles and mitochondria were complete. Compared with the normal group, the other six groups had different degrees of changes, such as dense muscle fibers, disordered arrangement, increased presynaptic membrane vesicles and mitochondria, and postsynaptic membrane folds. There were no differences in the above indicators among the blank, NC and miR-200a inhibitor + Forskolin groups (all $p > 0.05$). Compared with the blank group, the miR-200a inhibitor group had a slightly decreased muscle fiber density, slightly intact arrangement, unremarkably increased presynaptic vesicles and mitochondria and decreased postsynaptic membrane folds, whereas the miR-200a mimic and Forskolin groups exhibited more muscle fibers, more disordered arrangement, more presynaptic membrane vesicles and mitochondria and more wrinkled postsynaptic membranes (Fig. 5). Altogether, the inhibition of miR-200a or the inactivation of cAMP could improve the striatum structure in PD rats.

Fig. 5. Inhibition of miR-200a or inactivation of cAMP improves the striatal structure in PD rats as detected by electron microscopy ($\times 15K$). Notes: The data are presented as mean $\pm$ standard deviation and were analyzed by one-way ANOVA. The experiment was independently repeated three times. miR-220a, microRNA-220a; cAMP, cyclic adenosine 3',5'-monophosphate; Parkinson's disease; NC, negative control.

Inhibition of miR-200a decreases the mRNA expression of Bax, AKT, CREB, cAMP and PKA and increases the mRNA expression of Bcl-2 and DRD2

To determine the mRNA expression of miR-200a, Bax, AKT, CREB, cAMP, PKA, Bcl-2 and DRD2, RT-qPCR was conducted. There was no significant change in the mRNA expression of Bad among all groups (all $p > 0.05$). Compared with the normal group, the other six groups had increased miR-200a expression (all $p < 0.05$). Compared with the blank group, the miR-200a mimic group had elevated miR-200a expression, whereas the miR-200a inhibitor and miR-200a inhibitor + Forskolin groups had reduced miR-200a expression ($p < 0.05$). There was no difference in miR-200a expression among the blank, NC and Forskolin groups ($p > 0.05$). Compared with the normal group, the other six groups had increased mRNA expression of Bax, cAMP, AKT, PKA and decreased mRNA expression of Bcl-2 and DRD2 (all $p < 0.05$). Compared with the blank group, the mRNA expression of Bax, cAMP, AKT, CREB, PKA, DRD2 and Bcl-2 in the NC group did not significantly differ (all $p > 0.05$); the miR-200a inhibitor group had decreased mRNA expression of Bax, AKT, cAMP and PKA and increased mRNA expression of Bcl-2, CREB, and DRD2, whereas the miR-200a mimic group exhibited increased mRNA expression of Bax, cAMP, AKT and PKA and decreased mRNA expression of Bcl-2, CREB and DRD2 ($p < 0.05$); the mRNA expression of Bax, cAMP, AKT and PKA was increased ($p < 0.05$), the mRNA expression of Bcl-2 and CREB was decreased ($p < 0.05$) and the mRNA expression of DRD2 did not significantly change ($p > 0.05$) in the Forskolin group; the mRNA expression of DRD2 was increased ($p < 0.05$) and the mRNA expression of the other genes was not significantly changed ($p > 0.05$) in the miR-200a inhibitor + Forskolin group (Fig. 6). These results suggest that the inhibition of miR-200a could decrease the mRNA expression of Bax, AKT, CREB, cAMP and PKA and increase the mRNA expression of Bcl-2 and DRD2.

Inhibition of miR-200a decreases the protein expression of Bax, AKT, CREB, cAMP and PKA and increases the protein expression of Bcl-2 and DRD2 and the extent of p-Bad

To measure the protein expression of miR-200a, Bax, AKT, CREB, cAMP, PKA, Bcl-2 and DRD2 and the extent of p-Bad, a western blot assay was conducted. The results of the western blot assay (Fig. 7) revealed that there was no significant change in the protein expression of Bad among all groups (all $p > 0.05$). Compared with the normal group, the other six groups had an increased protein expression of Bax, AKT, cAMP and PKA and decreased protein

expression of Bcl-2, CREB, and DRD2 in addition to a reduced extent of p-Bad (all $p < 0.05$). Compared with the blank group, the protein expression of Bax, AKT, cAMP, PKA, DRD2 and Bcl-2 and the extent of p-Bad in the NC group did not significantly differ (all $p > 0.05$); the miR-200a inhibitor group had a decreased protein expression of Bax, AKT, cAMP and PKA, increased protein expression of Bcl-2, CREB, and DRD2 and an elevated extent of p-Bad, whereas the miR-200a mimic group had an increased protein expression of Bax, AKT, cAMP and PKA, decreased protein expression of Bcl-2, CREB and DRD2 and reduced extent of p-Bad ($p < 0.05$); the protein expression of Bax, AKT, cAMP and PKA was increased ($p < 0.05$), the protein expression of Bcl-2 and the extent of p-Bad was decreased ($p < 0.05$) and the protein expression of DRD2 did not significantly change ($p > 0.05$) in the Forskolin group; the protein expression of DRD2 was increased ($p < 0.05$) and the protein expression of the other genes did not significantly change ($p > 0.05$) in the miR-200a inhibitor + Forskolin group. These results suggest that the inhibition of miR-200a could decrease the protein expression of Bax, AKT, CREB, cAMP and PKA and increases the protein expression of Bcl-2 and DRD2 and the extent of p-Bad.

Fig. 6. Inhibition of miR-200a decreases the mRNA expression of Bax, AKT, CREB, cAMP and PKA and increases the mRNA expression of Bcl-2 and DRD2 as measured by RT-qPCR. Notes: The data are presented as the mean $\pm$ standard deviation and were analyzed by one-way ANOVA. The experiment was independently repeated three times. *, $p < 0.05$ vs. the normal group. #, $p < 0.05$ vs. the blank group. Bcl-2, B-cell leukemia/lymphoma-2; Bax, Bcl-2 associated X; DRD2, dopamine D2 receptor; miR-200a, microRNA-200a; Bad, Bcl-2 associated death promoter; cAMP, cyclic adenosine 3',5'-monophosphate; PKA, protein kinase A; RT-qPCR, reverse transcription quantitative polymerase chain reaction; NC, negative

Fig. 7. Inhibition of miR-200a decreases the protein expression of Bax, AKT, CREB, cAMP and PKA and decreases the protein expression of Bcl-2 and DRD2 and the extent of p-Bad as determined by a western blot assay. Notes: A, Gray value of Bad, Bax, Bcl-2, p-Bad, DRD2, cAMP and PKA protein bands. B, Protein expression of Bad, Bax, Bcl-2, p-Bad, DRD2, cAMP and PKA in each group. *, $p < 0.05$ vs. the normal group. #, $p < 0.05$ vs. the blank group. The data are presented as the mean $\pm$ standard deviation and were analyzed by one-way ANOVA. The experiment was independently repeated three times. miR-200a, microRNA-200a; Bcl-2, B-cell leukemia/lymphoma-2; Bax, Bcl-2 associated X; DRD2, dopamine D2 receptor; cAMP, cyclic adenosine 3',5'-monophosphate; Bad, Bcl-2 associated death promoter; PKA, protein kinase A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NC, negative control.

Discussion

As a disease that occurs later in the patients' lives, PD has a high incidence and produces great burden on patients, families and society [22, 23]. Moreover, the mechanism underlying the cognitive impairment in PD remains poorly understood [6], but recently a study indicated that miRNAs play a significant role in PD [24]. The present study aimed to investigate the role of miR-200a in PD. Our results provided evidence that the inhibition of miR-200a can upregulate DRD2 via the cAMP/PKA signaling pathway, thus suppressing apoptosis in striatal neuron cells.

Our results showed that PD rats exhibited decreased levels of GSH-Px, SOD, dopamine, DOPAC and HVA but increased levels of MDA. The miR-200a inhibitor group exhibited increased levels of GSH-Px, SOD, dopamine, DOPAC and HVA but decreased levels of MDA. PD has been reported to be caused by oxidative stress [6]. As the primary and important enzymes in the human antioxidant system, GSH-Px and SOD can resist oxidative stress [25]. In addition, MDA is an endogenous genotoxic substance produced from the rapid peroxidation of unsaturated fatty acids in phospholipids [26]. Moreover, increasing MDA is accompanied by oxidative stress [27]. Interestingly, a previous study noted that the levels of GSH-Px and SOD were increased and the MDA level was decreased in PD patients [28]. PD is mainly characterized by the loss of dopamine [28]. Furthermore, HVA and DOPAC are neuronal metabolites of dopamine; thus, PD patients have low levels of DOPAC and HVA [29, 30]. Furthermore, dopamine degradation is associated with oxidative stress [31]. Importantly, miR-200a can be upregulated by oxidative stress [32]. Therefore, the inhibition of miR-200a can increase the levels of GSH-Px, SOD, dopamine, DOPAC and HVA but decrease the levels of MDA.

According to the results of the western blot assay and RT-qPCR, the expression of miR-200a, Bax, AKT, cAMP and PKA was significantly increased, but the expression of DRD2, CREB and Bcl-2 was decreased in the PD rat striatum. In addition, the miR-200a inhibitor group exhibited a lower expression of miR-200a, Bax, AKT, cAMP and PKA but higher expression of DRD2, CREB and Bcl-2. DRD2 is located at chromosomes 11p12-23 and is generally expressed in striatal regions [33]. It is well known that DRD2 is negatively associated with PD because it affects the dopamine response [33]. PD has been reported to be associated with oxidative stress, which can increase the expression of the miR-200 family and DRD2 [32, 35, 36], suggesting that miR-200a is responsible for the upregulation of DRD2. As a pro-apoptotic member of the Bcl-2 family, Bax can induce caspase activation and cell apoptosis by promoting the release of cytochrome c from the mitochondrial intermembrane space into the cytosol [37]. Bcl-2 is a protein that inhibits apoptosis and is normally expressed in neuronal cells, such as pigmented forebrain nigral neurons [38]. Importantly, as shown in a previous study, inhibiting miR-200a can reduce the Bax/Bcl-2 ratio and then suppress apoptosis [10]. In addition, the increase in striatal cAMP has been shown to play a pivotal role in the loss of dopaminergic neurons in PD rats [39]. Furthermore, cAMP can activate PKA by binding to regulatory subunits of PKA [40]. Consistently, miR-200c has been shown to inactivate the PI3K/Akt signaling pathway [41]. In addition, miR-200b could negatively regulate CREB1 in human malignant glioma [42]. As mentioned above, miR-200a inhibition can increase the expression of DRD2, CREB and Bcl-2 and decrease the expression of Bax, AKT, cAMP and PKA in the cAMP/PKA signaling pathway in PD rats.

Furthermore, in the present study, we observed that rats transfected with the miR-200a inhibitor displayed decreased apoptosis. It is well known that the miR-200a family has antitumor effects, such as increasing tumor cell apoptosis [43]. Importantly, a prior study revealed that the inhibition of miR-200a mitigated apoptosis resulting from oxygen-glucose deprivation/reperfusion by repressing p53 and cytochrome c and reducing the Bax/Bcl-2 ratio [10]. cAMP can induce the activation of PKA, while PKA can repress nuclear factor- κ -gene binding, which can mediate apoptotic target genes, including Bcl-2, Bax and survivin, thereby promoting lymphocyte apoptosis [44]. Consistent with our results, mice treated with bucladesine, which is a cAMP agonist, have been shown to exhibit significantly

increased neuronal apoptosis in Alzheimer's disease compared with controls [45]. Therefore, inhibiting miR-200a can suppress neuronal apoptosis via the inhibition of the cAMP/PKA signaling pathway.

In summary, the present study found that the suppression of miR-200a can inhibit apoptosis in striatal neuron cells through the repression of the cAMP/PKA signaling pathway by upregulating DRD2. Therefore, miR-200a can be a potential therapeutic target for PD. Further studies are needed to be performed in the future to further confirm our conclusion.

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No conflict of interest exists.

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