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Original Paper

MicroRNA-128 Protects Dopamine Neurons from Apoptosis and Upregulates the Expression of Excitatory Amino Acid Transporter 4 in Parkinson's Disease by Binding to AXIN1

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Key Words
microRNA-128 • AXIN1 • Parkinson's disease • Dopamine neuron • Excitatory amino acid transporter 4

Abstract
Background/Aims: Parkinson's disease (PD) is a frequently occurring condition that resulted from the loss of midbrain neurons, which synthesize the neurotransmitter dopamine. In this study, we established mouse models of PD to investigate the expression of microRNA-128 (miR-128) and mechanism through which it affects apoptosis of dopamine (DA) neurons and the expression of excitatory amino acid transporter 4 (EAAT4) via binding to axis inhibition protein 1 (AXIN1). ***Methods:*** Gene expression microarray analysis was performed to screen differentially expressed miRNAs that are associated with PD. The targeting relationship between miR-128 and AXIN1 was verified via a bioinformatics prediction and dual-luciferase reporter gene assay. After separation, DA neurons were subjected to a series of inhibitors, activators and shRNAs to validate the mechanisms of miR-128 in controlling of AXIN1 in PD. Positive protein expression of AXIN1 and EAAT4 in DA neurons was determined using immunocytochemistry. miR-128 expression and the mRNA and protein levels of AXIN1 and EAAT4 were evaluated via RT-qPCR and Western blot analysis, respectively. DA neuron apoptosis was evaluated using TUNEL staining. ***Results:*** We identified AXIN1 as an upregulated gene in PD based on the microarray data of GSE7621. AXIN1 was targeted and negatively mediated

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by miR-128. In the DA neurons, upregulated miR-128 expression or sh-AXIN1 increased the positive expression rate of EAAT4 together with mRNA and protein levels, but decreased the mRNA and protein levels of AXIN1, apoptosis rate along with the positive expression rate of AXIN1; however, the opposite trend was found in response to transfection with miR-128 inhibitors. ***Conclusion:*** Evidence from experimental models revealed that miR-128 might reduce apoptosis of DA neurons while increasing the expression of EAAT4 which might be related to the downregulation of AXIN1. Thus, miR-128 may serve as a potential target for the treatment of PD.

Introduction

Parkinson's disease (PD) is the most prevalent neurodegenerative disorder and occurs more frequently among the elderly; PD has become the second largest killer of the elderly [1, 2]. PD is characterized by a large loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNc) of the midbrain [3]. The most common symptom of this disease is unilateral, typically resting tremors in body parts; these tremors are the most common in the upper extremities and, unfortunately, can spread to other parts of the body, such as the lips, chin, jaw and tongue [4]. PD is reported to be associated with various risk factors, such as aging, pesticide exposure, genetic inheritance and environmental chemicals (e. g., synthetic heroin use) [2]. Although drug treatment and surgery have exhibited certain effects on alleviating PD, controlling the development and progression of PD still remains a large challenge because of its complicated pathogenesis [5]. Recent evidence suggests that post-transcriptional regulation is an important process in PD pathophysiology [6]. Some microRNAs (miRs) are differentially expressed in the human brain and may regulate the expression of genes that are associated with PD [7, 8].

miR-128 is expressed in the brain at a high level and plays a crucial role in the development of the nervous system and the maintenance of normal physical functions [9]. The expression of miR-128 is notably increased in differentiating DA neurons [10]. miR-128 governs motor activity by suppressing the expression of various ion channels and signaling components of the extracellular signal-regulated kinase 2 (ERK2) network that regulate neuronal excitability [11]. Based on a bioinformatics prediction and a dual-luciferase reporter gene assay, miR-128 targets and negatively regulates axis inhibition protein 1 (AXIN1). AXIN1 is an inhibitor of the Wnt/beta-catenin signaling pathway that regulates the level of beta-catenin [12]. In the central nervous system (CNS), Wnt proteins and other components of the Wnt signaling cascade, that is, beta-catenin and AXIN1, exert critical roles in the developmental processes of normal CNS development [13]. Knockdown of AXIN1 shows that it may act as a tumor suppressor gene in medulloblastoma, which is the most frequent malignant brain tumor [14]. Excitatory amino acid transporter 4 (EAAT4) is a member of the high-affinity Na+/K+-dependent glutamate transporter family and is highly expressed in Purkinje cells of the cerebellum [15]. EAAT4 plays a vital role in the synaptic activity of cerebellar Purkinje cells by limiting extracellular glutamate concentrations and facilitating the induction of long-term depression [16]. Here, to identify a therapeutic target for the treatment of PD, we proposed a hypothesis that miR-128 could mediate DA neuron apoptosis and EAAT4 via the regulation of AXIN1.

Materials and Methods

GEO database screen and differential expression analysis

The GEO database (https://www.ncbi.nlm.nih.gov/geo/) was searched with "Parkinson" as the keyword, from which the GSE7621 dataset was obtained. GSE7621 included 25 samples, with 9 normal control samples and 16 PD samples. Substantia nigra tissues from the postmortem brain were sequenced via the GPL570 platform. The "Limma" package of the R language was used for the differential analysis, with

|logFoldChange (logFC)| > 2 and $p < 0.05$ as differential gene screening criteria. Finally, a heat map with differential gene expression was drawn using "pheatmap" of the R language.

Gene retrieval and protein interaction analysis

DigSee (http://210.107.182.61/geneSearch/) is a text mining search engine that provides evidentiary sentences that describe that "genes" are involved in the development of "disease" through "biological events". We retrieved the database with "Parkinson" as the keyword and selected the first 20 genes for the follow-up analysis. STRING (https://string-db.org/cgi/input.pl?UserId = L4tX36b7ki01&sessionId = b4KDEkf0ZeVc&input_page_show_search = off) can predict interactions between proteins, through which, a protein interaction analysis between the 25 differentially expressed genes and 20 PD-related genes was performed, with the intersection of the related genes and 20 PD-related genes obtained. Finally, PD-related genes were extracted from the differentially expressed genes.

Regulation prediction

The regulatory miRNA of AXIN1 was predicted using an online database which included microRNA.org (http://34.236.212.39/microrna/home.do), miRWalk (http://129.206.7.150/) and TargetScan (http://www.targetscan.org/vert_71/). The procedure was as follows: we selected mice and then input AXIN1; next, Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/) were adopted to construct a Venn image of the prediction results obtained from the three databases. Subsequently, the intersection of the results from the three databases was prepared for the following study.

Model establishment

A total of 20 male C57BL/6J mice (6 ~ 7 weeks old) weighing 18 ± 2 g were included. Eight mice were randomly selected as control group, and the remaining 12 mice were treated with an intraperitoneal injection of 1-methyl,4-phenyl-1, 2,3, 6-tetra-hydropyridine (MPTP) (30 mg/kg, 23007-85-4, Sigma, St. Louis, MO, USA) once a day for 7 days to establish the PD model [17]. Mice were administered 0.9% normal saline (30 mg/kg) once a day for 7 days. After an intraperitoneal injection for 15 min every day, the general condition of the mice was observed. One hour later, pole-climbing and suspension experiments were conducted to identify whether the PD model was successfully established or not [18, 19]. The pole-climbing experiment was conducted as follows: a cork ball was fastened at the top of a wooden pole (50 cm in thickness and 1 m in length) that was coated with gauze to avoid slipping. Mice were placed onto the cork ball, and the following information was recorded: (1) the time to finish climbing 1/2 of the upper pole and (2) the time to finish climbing 1/2 of the lower pole. The scoring criteria were: (1) a time over 6 s was 1 point; (2) a time within 6 s was 2 points; and (3) a time within 3 s was 3 points. The procedure for the suspension experiment was as follows: mice were suspended on a horizontal wire and were given a score of 1 point when the two hind paws could not catch the wire, 2 points when one hind paw caught the wire and 3 points when the two hind paws caught the wire.

Dual-luciferase reporter gene assay

A bioinformatics prediction website was used to analyze the target gene of miR-128. In addition, a dual-luciferase reporter gene assay was performed to evaluate whether AXIN1 was the direct target gene of miR-128. A synthetic AXIN1 3'untranslated region (UTR) was inserted into the pMIR-reporter via *Hind III* and a *Spe I* restriction sites. The sequence in the pAXIN1-mutant (mut) was designed based on the pAXIN1 wild type (wt), which was then digested via restriction endonuclease and inserted into the pMIR-reporter plasmid using T4 DNA ligase. The miR-128 mimic plasmid and NC plasmid were co-transfected into HEK-293T cells (CRL-1415, Shanghai Xinyu Biotech Co., Ltd., Shanghai, China) using a luciferase reporter vector; 48 h later, the cells were collected and lysed. After centrifugation for 3 - 5 min, the supernatant was obtained. In accordance with the instructions of the firefly luciferase assay kit (RG005, Beyotime Biotechnology Co., Ltd., Shanghai, China), renilla luciferase detection buffer and firefly luciferase detection agent were dissolved. Next, a 100 μL aliquot per sample buffer was removed, and renilla luciferase detection working fluid was prepared with the addition of the substrate at a ratio of 1 : 100. Subsequently, 20 - 100 μL of sample from each group was extracted and evaluated using a fluorometer. Next, 100 μL of renilla luciferase detection working fluid was dropped and mixed evenly, with the report gene lysate as the blank control. Next, 100 μL of the luciferase detection working fluid was dropped and mixed evenly to detect renilla luciferase activity.

Renilla luciferase was treated as the internal reference. The ratio between the relative luciferase unit (RLU) of the firefly luciferase activity to the RLU of renilla luciferase activity was regarded as the relative luciferase activity [20].

Cell isolation and identification

Ten PD mice were prepared, disinfected, and decapitated, and brain tissues were obtained. The tissues were placed in an ice-bathed D1-Hanks solution. The substantia nigra pars compacta was dissected under a dissecting microscope (Jiangnan Optical Instrument Factory, Nanjing, Zhejiang, China), and the blood vessels and envelope of the selected brain tissues were removed. Next, the tissues were rinsed and cut into pieces. The tissues were treated with trypsin and incubated at 37°C for 10 min. A dropper with angle head was used to gently crush the tissue mass to form a cell suspension. After centrifugation at 178 × g for 6 min with the supernatant discarded, the cells were re-suspended in conditioned medium, filtered through a 200-mesh screen, adjusted to a density of 1×10^6 cells/cm^2, seeded into culture flasks and incubated in an incubator containing 5% CO_2 at 37°C. After an appropriate amount of time, the cells were passaged. Immunocytochemistry was performed to detect tyrosine hydroxylase (TH) to identify DA neurons.

Cell differentiation, grouping and transfection

After a five-day culture, second generation DA neurons were inoculated into 24-well plates with an aseptic slide treated with poly-L-lysine (PLL). The cells were differentiated with the addition of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco, 11330-032) containing 2% fetal bovine serum (FBS). Half of the solution was replaced every 2 - 3 days. Before the 24-h transfection, the DA neurons in the culture flasks were treated with trypsin, and the cells were seeded into 24-well plates until they reached approximately 50% confluence. Next, 20 μL of Lipofectamine2000 (11668019, Thermo Fisher Scientific, Inc., Waltham, MA, USA) was diluted in 500 μL of serum-free culture medium and incubated at 37°C for 5 min. Lipofectamine2000 and the sequences were mixed and incubated at 37°C for 20 min. After washing with serum-free medium 3 times, the cells were incubated with 5 mL of serum-free medium in each well. The mixture solution was added to the medium for incubation for 5 - 24 h. After 3 washes in serum-free medium, the cells were incubated with 20% DMEM without antibiotics for 48 h. The DA neurons were assigned into control (without any transfection), negative control (NC) (transfection with NC sequences), miR-128-mimic (transfection with miR-128 mimics), miR-128-inhibitor grop (transfection with miR-128 inhibitors), sh-AXIN1 (transfection with sh-AXIN1), and miR-128 inhibitor + sh-AXIN1 (transfection with miR-128 inhibitor + sh-AXIN1) groups. Sequence transfection was designed and synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China). The final concentration was 50 mol/L. After a 6-h transfection, the solution was replaced and cultured for 48 h.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

In accordance with the instructions of Trizol (16096020, Life Technologies, Grand Island, New York, USA), total RNA was extracted. The purity and concentration of RNA were detected using an ultraviolet spectrophotometer (DU640, Beckman Coulter, Fullerton, CA, USA). The mRNA was reversely transcribed into cDNA using a Primescript TMRT reagent Kit (Takara Bio, Otsu, Shiga, Japan). RT-qRCR was performed via the SYBR Green dye method using 9 μL of the SYBR Mix amplification system (Takara), 0.5 μL of forward primers, 0.5 μL of reverse primers, 2 μL of cDNA and 8 μL of RNase Free dH_2O. The reaction conditions consisted of pre-denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min. Next, SYBR Green fluorescence was detected. The primer sequences of miR-128, AXIN1, and EAAT4 are presented in Table 1. U6 [21] and β-actin were used as the internal controls of miR-128 and the genes, respectively. Relative expression of miR-128, AXIN1, and EAAT4 was determined using the $2^{-\triangle\triangle Ct}$ method. The experiment was repeated three times.

Immunocytochemistry

The collected cells were removed. Next, one drop of the culture solution was dropped onto the coated cover glass. Cells were cultured at 37°C with a volume fraction of 5% CO_2. When the cells adhered to the walls, the glass was washed with phosphate buffer saline (PBS). Later, the cells were fixed with 40 g/L paraformaldehyde and cultured with a volume fraction of 3% H_2O_2, kept at room temperature for 10 min and rinsed with PBS. After blocking with goat serum for 30 min, the cells were treated with primary antibody

rabbit anti-mouse AXIN1 (ab55906, 1 μg/mL) and rabbit anti-mouse EAAT4 (ab41650, 1 : 2000). All of the above antibodies were purchased from Abcam. (Cambridge, MA, USA). PBS was used as the negative control, and the cells were incubated at 4°C overnight. After three washes with PBS, the cells were incubated with a horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (ab6721, 1 : 2000, Abcam) secondary antibody at 4°C overnight. The cells were washed with PBS 3 times, treated with ABC complex, and incubated at room temperature for 30 min. After rinsing, diaminobenzidine (DBA) staining was performed. The cells were counterstained with hematoxylin for 5 min. Next, phosphoric acid buffer was added to terminate the reaction. Subsequently, the cells were conventionally patched, dehydrated, cleared, mounted, subjected to a microscopic examination and photographed. The microscopic images were measured using the Image ProPlus software (Media Cybernetics, Silver Spring, MD, USA).

Table 1. Primer sequences for RT-qPCR. Notes: miR-128, microRNA-128; RT-qPCR, reverse transcription quantitative polymerase chain reaction; AXIN1, axis inhibition protein 1; EAAT4, excitatory amino acid transporter 4

Genes	Primer sequences
miR-128	Forward: 5'-GTTGGATTCGGGGGCCGTAG-3'
	Reverse: 5'-GAGACCGGTTCACTGTGAGAA-3'
AXIN1	Forward: 5'-ACCGAAAGTACATTCTTGATAAC-3'
	Reverse: 5'-GGACCTCGGAGCAAGTTTCA-3'
EAAT4	Forward: 5'-AGCAGCCACGGCAATAGTC-3'
	Reverse: 5'-ATGCCAAGCTGACACCAATGA-3'
U6	Forward: 5'-GCAAGGATGACACGCAAT-3'
	Reverse: 5'-ATGGAACGCTTCACGAAT-3'
β-actin	Forward: 5'-GCTCTGGCTCCTAGCACCAT-3'
	Reverse: 5'-GCCACCGATCCACACAGAGT-3'

Western blot analysis

The collected cells were removed. Next, a homogenate was prepared using a glass homogenizer after lysing with the lysate (50 mmol/L Tris-HCl, pH = 8.0, 15 mmol/L NaCl, 1% Triton X, 100 μg/mL PMSF). Then, the homogenate was treated with an equal volume of 2 × sample buffer that contained 0.125 mol/L Tris-HCl, 4% sodium dodecyl sulfate (SDS), 2% mercaptoalcohol, 20% glycerol, and 0.2% bromo- phenol blue (pH = 6.8). The mixture was boiled for 3 min and centrifuged at 10000 × g for 10 min with supernatant discarded. The protein concentration was detected using an ultraviolet spectrophotometer to identify the sample quantity of 50 μg of total protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Bio-Rad, Hercules, CA, USA) was performed with each well containing 15 μg of protein. Next, the samples were transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with rabbit anti-mouse AXIN1 (ab55906, 1 μg/mL), rabbit anti-mouse caspase-3 (ab13847, 5 μg/mL), and rabbit anti-mouse EAAT4 (ab41650, 1 : 2000). All of the above antibodies were purchased from Abcam. Next, the membrane was incubated with HRP-labeled goat anti-rabbit (A0208, Beyotime Biotechnology Co., Ltd.). Chemiluminescent two-component substrates were mixed at a ratio of 1 : 2 based on the protein Detector LumiGLO Western blot kit, added to the membrane, kept for 1 min, and developed for 10 min. The membrane was washed, and the remaining solution was discarded. β-actin (1 : 5000, ab32572, Abcam) was used as the internal reference. The Quantity One software was used for gray value analysis of the protein bands.

Immunofluorescence assay

The cells growing on the slides were rinsed 3 times in culture plates (3 min per rinsing) and fixed in 4% paraformaldehyde for 15 min. The slides were washed with PBS 3 times for 3 min each, and the excess PBS was discarded. After the addition of normal goat serum, the cells were blocked at 37°C for 30 min. Next, the excess sealing fluid was removed. Each slide was treated with an adequate amount of diluted caspase-3 antibody (ab13847, 5 μg/mL, Abcam), placed into humidity chambers and incubated at 4°C overnight. After washing three times with phosphate-buffered saline with/containing 0.5% Tween 20 (PBST) (3 min per washing), the excess solution on the slide was dipped off. After treatment with a CF350-labeled rabbit anti-mouse secondary antibody (ab150117, 1: 1000, Abcam), the cells growing on the slides were incubated at 20°C - 37°C for 1 h, followed by 3 washes with PBST (3 min per washing). Next, the sections were mounted in antifade mounting medium and observed under a fluorescence microscope, and images were acquired.

Terminal deoxyribonucleotidyl transferase (TDT)-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay

DA neuron apoptosis was evaluated in accordance with the instructions for the Apoptosis Detection kit (C1088, Beyotime Biotechnology Co., Ltd.). In this procedure, 50 μL of the cell suspension was aspirated, washed once with PBS, and fixed with 4% paraformaldehyde at 4°C for 30 min. After washing once with PBS, the excess fluid was discarded. Next, the cells were re-suspended with strong permeability liquid (P0097, Beyotime Biotechnology Co., Ltd.) and incubated at room temperature for 5 min. After washing in PBS twice, the excess fluid was removed. Subsequently, the cells were treated with 50 μL of TUNEL detection solution and incubated at 37°C without light exposure for 60 min. After washing with PBS 2 times, the cells were suspended with 250 μL of PBS. Subsequently, the cells were smeared and observed under a fluorescence microscope at an excitation light wavelength of 450 - 500 nm and detection wavelength of 515 - 565 nm (green fluorescence).

Statistical analysis

The SPSS 21.0 software (IBM Corp, Armonk, NY, USA) was employed for statistical analysis. The measurement data are displayed as the mean ± standard deviation (SD). Comparisons between two groups were analyzed using the *t* test, and comparisons among multiple groups were performed using one-way analysis of variance (ANOVA). Count data are expressed as the percentage, and comparisons were analyzed using the Chi square test. The level of significance was $p < 0.05$.

Results

Microarray analysis of PD-related genes and bioinformatics prediction of regulating miRNA

Initially, we used the GEO database to conduct the difference analysis for the GSE7621 profile data and found 25 differentiated expressed genes (Fig. 1A), among which 7 genes were found to be upregulated and the remaining 18 genes were found to be downregulated in the PD samples. To select genes that were correlated with PD, we used an online database, and the former 20 genes were enrolled in this analysis. Next, we analyzed the interaction between the 25 differentiated expressed genes and 20 PD correlated genes (Fig. 1B). The results showed that LRRK2, PARK2, and SNCA were at the core of the protein-protein interaction (PPI) network and that the number of genes that could interact with each other was 25. Next, we conducted a further analysis of these genes and found that in these 25 interacted genes, only 5 genes did not come from the genes retrieved from the online database correlated with PD (Fig. 1C). The 5 genes included DDX3Y, KDM5D, AXIN1, KCNJ6 and SLC18A2. Among these genes, KDM5D was not directly correlated with PD-related genes, DDX3Y and SLC6A3 showed a slight interaction with low reliability, and the difference multiplier of DDX3Y was much lower. Therefore, the two genes were not used in the following experiment. The interaction between KCNJ6 and DRD2 exhibited much higher reliability, whereas the difference multiplier was the lowest in PD; thus, these genes were not included in the following experiment. The difference multipliers of AXIN1 and SLC18A2 were relatively higher among the five differentially expressed genes. Furthermore, SLC18A2 was at the core site of the PPI network. Multiple reports have shown that SLC18A2 is involved in the regulation of PD progression [22-25]. Moreover, there was the higher reliability of the interaction between AXIN1 and LRRK2, and the expression multiplier of AXIN1 in PD was much higher. In addition, the relationship between AXIN1 and PD has seldom been reported; AXIN1 was prepared for the following treatment. We performed an online database to search for potential miRNA that can regulate AXIN1. Twenty-two miRNAs were obtained from the miRNA.org database, 192 miRNAs were obtained from the TargetScan database, 10 miRNAs were obtained from conserved sites, and 182 miRNAs were obtained from poorly conserved sites. Ten miRNAs obtained from conserved sites were selected for the following analysis. Next, 1426 unrepeated miRNAs were searched with miRWalk, and the previously defined 30 miRNAs were analyzed. We collected the intersection results obtained from the three

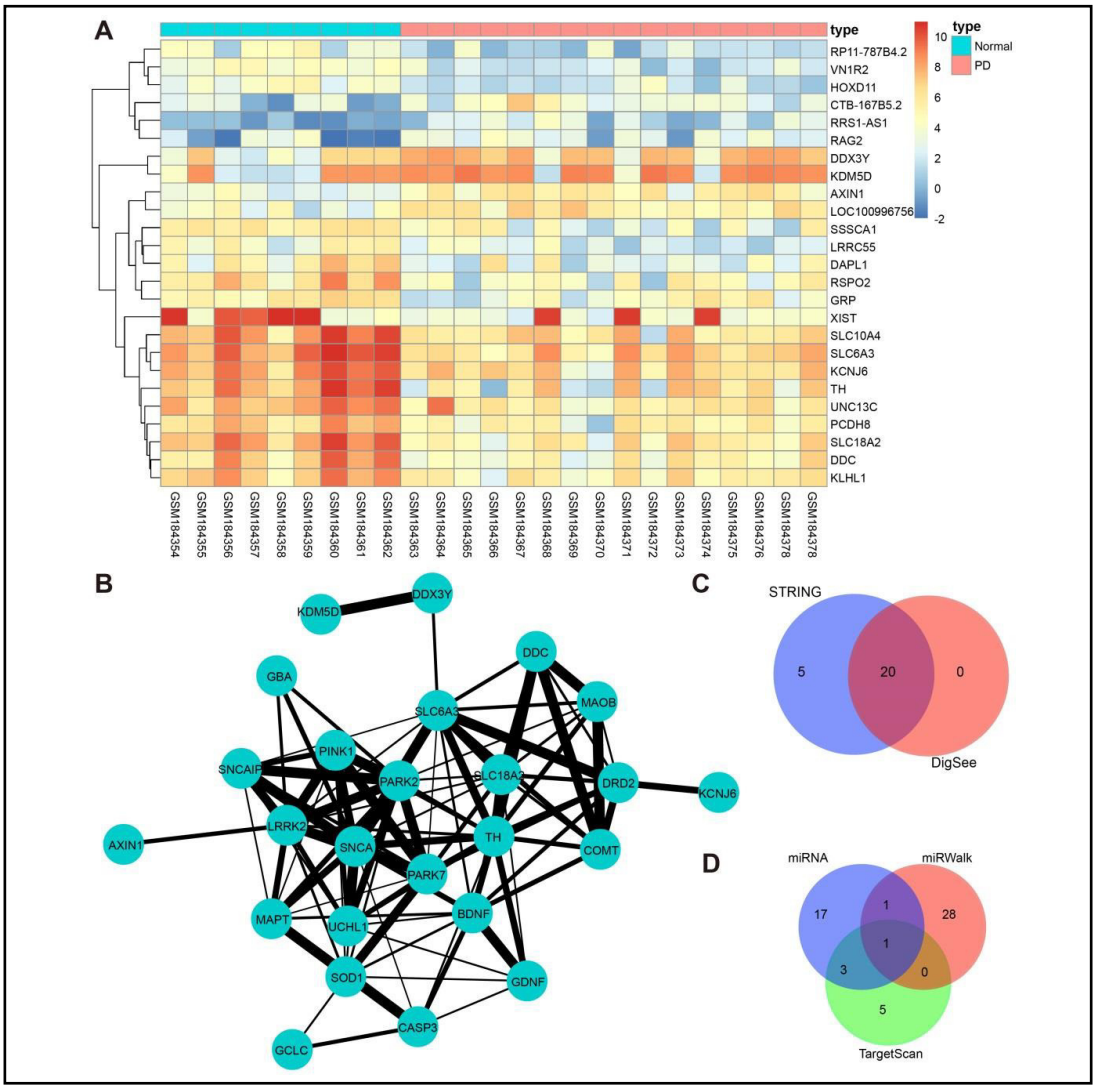

Fig. 1. PD profile data analysis and miRNA prediction indicated that miR-128 regulated AXIN1 in PD. A, GSE7621 data difference analysis; the abscissa represents the sample number, and ordinate represents differentially expressed genes; the diagram in the upper right represents the expression of different colors, and from top to bottom, the expression gradually decreases, during which the rectangle corresponds to the expression of one sample; each line represents the expressions of all differentially expressed gene samples; the dendrogram on the left represents cluster analysis of differentially expressed genes of different samples; the color bar in the upper represents the type of sample, where red refers to PD samples and blue refers to normal samples. B, The results of the STRING analysis, in which each blue circle represents a gene and the line connecting the circles represents an interaction between two genes, where the size of the line reflects the reliability of the interaction, that is, the thicker the line, the more reliable the interaction. C, The results of Venn analysis after STRING analysis and DigSee retrieval; the blue part represents the number of genes that can interact with each other, the red part on the right represents the results of the DigSee retrieval, and the middle part represents the intersection of the two results. The Fig. suggests that 20 genes interacted with each other through DigSee retrieval, and the difference analysis proved that only 5 genes did not meet the results of the DigSee retrieval. D, The intersection of miRNA regulation with AXIN1; the blue part in the upper left represents the results obtained through miRNA.org, the red part in the upper right represents the results predicted through the miRWalk database, and the green part in the bottom represents the results predicted through the TargetScan database; the intersection of the circles represents the intersection of the results obtained from different databases, and the middle part represents the prediction results by the above three databases. miR-128, microRNA-128; AXIN1, axis inhibition protein 1; PD, Parkinson's disease.

databases (Fig. 1D) and found that miR-128 was predicted by all three databases. From the above findings, we speculate that miR-128 is able to regulate AXIN1, thus, exerting a role in the progression of PD.

General condition of mice following the MPTP injection

There were no marked changes in the control group. After receiving an injection of MPTP for 15 min, PD mice showed reduced autonomous activity, slower action, static tiny trembles, decreased activities, hindlimb stiffness, dyskinesia and a lower response to external stimuli. The above-mentioned symptoms reached a peak at 2 h, and all of these symptoms disappeared after 24 h. The observation was conducted on the following day after the injection.

Successful establishment of a mouse model of PD

Pole-climbing and tail-suspension experiments were performed to evaluate the establishment of the PD model. From the 1st day to the 7th day, no changes were observed in the control group; all of the mice could climb to the upper half and lower half of the pole within 3 s ($p > 0.05$). From the 1st day to the 7th day after PD mice were injected with MPTP, the time to finish climbing the upper half and lower half of the pole showed no notable differences ($p > 0.05$); the difference in time was significant compared with that of the control group ($p < 0.01$). No changes were observed in the control group, in which all of the mice were able to catch the wire with two paws ($p > 0.05$). No differences were identified in the ability to catch the wire before and after PD mice were injected with MPTP ($p > 0.05$), and the ability of the PD mice injected with MPTP was significantly weakened compared with that of mice from the control group ($p < 0.01$) (Table 2). These results implied that the PD model was successfully established.

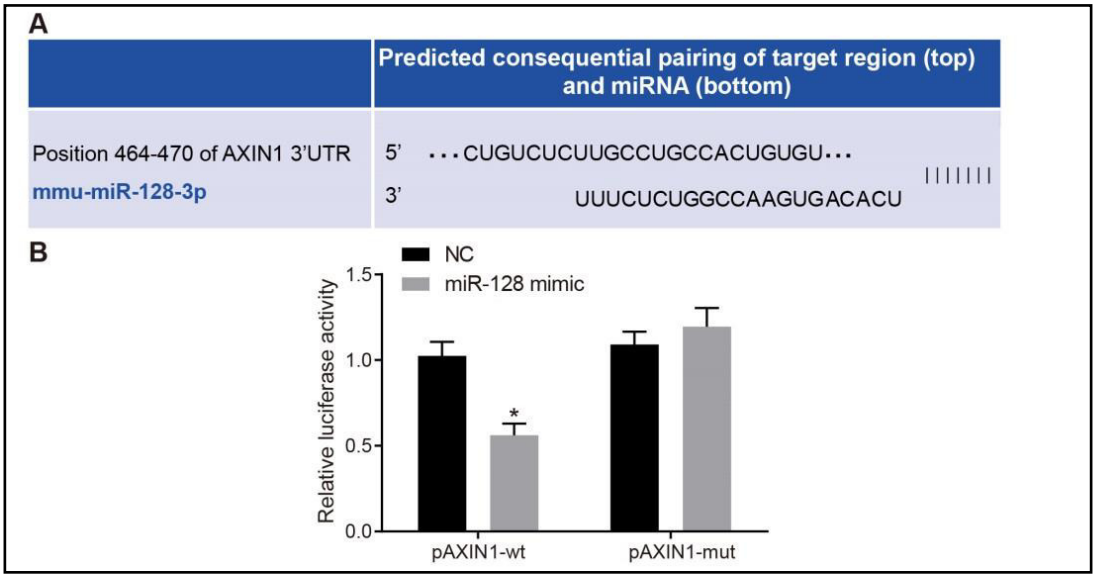

Fig. 2. Bioinformatics prediction and luciferase activity for determining the targeting relationship of miR-128 and AXIN1. A, The binding sites of miR-128 and AXIN1 3'UTR predicted by the website Target Scan. B, The luciferase activity was significantly reduced in cells co-transfected with miR-128 mimic and pAXIN-Wt in the miR-128 mimic group; *, P<0.05 vs. the NC group; the measured luciferase activity was measurement data, and expressed as the mean ± standard deviation; the experiment was repeated three times; Wt, wild-type; Mut, mutant; 3'UTR, 3'untranslated region; miR-128, microRNA-128; AXIN1, axis inhibition protein 1.

Table 2. Results of the pole-climbing and tail-suspension experiments confirming the successful establishment of the mouse model of PD. Notes: PD, Parkinson's disease; **, p < 0.01 vs. the control group

Experiment	Group	The 1st day	The 2nd day	The 3rd day	The 4th day	The 5th day	The 6th day	The 7th day
Pole climbing	Control (upper 1/2)	2.57 ± 0.11	2.66 ± 0.18	2.63 ± 0.21	2.54 ± 0.16	2.61 ± 0.13	2.55 ± 0.12	2.68 ± 0.15
	Control (lower 1/2)	2.41 ± 0.23	2.78 ± 0.14	2.51 ± 0.22	2.65 ± 0.18	2.73 ± 0.25	2.64 ± 0.26	2.59 ± 0.27
	PD (upper 1/2)	5.02 ± 0.36**	4.93 ± 0.42**	4.99 ± 0.26**	5.03 ± 0.23**	4.88 ± 0.21**	5.07 ± 0.28**	4.94 ± 0.34**
	PD (lower 1/2)	5.11 ± 0.29**	5.06 ± 0.39**	4.87 ± 0.18**	4.93 ± 0.15**	4.99 ± 0.33**	5.04 ± 0.36**	5.09 ± 0.27**
Tail suspension	Control group	2.93 ± 0.15	2.98 ± 0.22	2.85 ± 0.24	2.92 ± 0.19	3.02 ± 0.26	2.88 ± 0.13	2.91 ± 0.18
	PD group	1.48 ± 0.15**	1.52 ± 0.16**	1.59 ± 0.17**	1.49 ± 0.13**	1.51 ± 0.12**	1.56 ± 0.11**	1.44 ± 0.09**

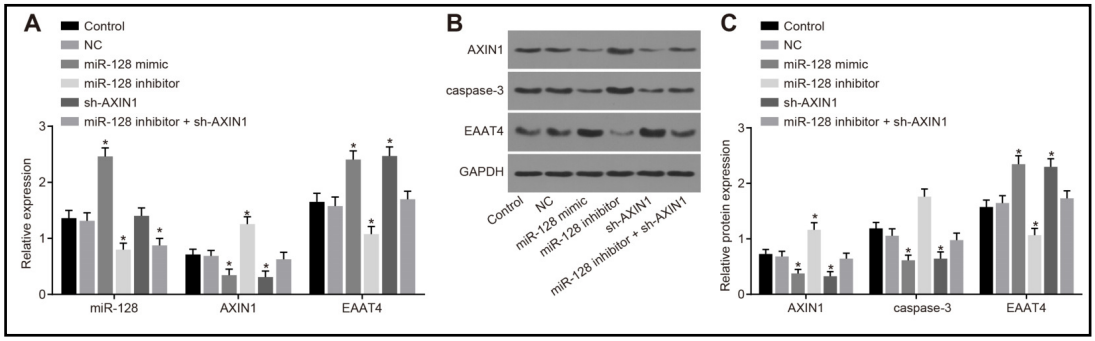

Fig. 3. Overexpression of miR-128 inhibited AXIN1 to promote EAAT4 expression in dopamine neurons. A, RT-qPCR analysis of the miR-128 expression and mRNA level of AXIN1 and EAAT4; B and C, Western blot analysis of the protein level of AXIN1 and EAAT4 *, P<0.05 vs. the control group; the expression of miR-128, AXIN1 and EAAT4 was expressed as the mean ± standard deviation, and analyzed via one-way analysis of variance; the experiment was repeated three times; miR-128, microRNA-128; AXIN1, axis inhibition protein 1; EAAT4, excitatory amino acid transporter 4; NC, negative control; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

AXIN1 is confirmed to be a target gene of miR-128

A bioinformatics prediction website and a dual-luciferase reporter gene assay were used to verify the targeting relationship between miR-128 and AXIN1. AXIN1 was verified to be a target gene of miR-128 via the online prediction website TargetScan (Fig. 2A). The results of the dual-luciferase reporter gene assay are shown in Fig. 2B. Compared with the NC group, the luciferase activity of the cells co-transfected with a miR-128 mimic and pAXIN-Wt in the miR-128 mimic group was significantly reduced ($p < 0.05$), while the luciferase activity of the cells co-transfected with a miR-128 mimic and pCTGF-Mut in the miR-128 mimic group showed no notable differences ($p > 0.05$). These findings provided evidence that miR-128 specifically binds to AXIN1.

DA neurons are successfully extracted

Immunocytochemistry was used to evaluate TH expression in cells extracted from the substantia nigra. The results shown in Supplementary Fig. 1 (For all supplemental material see www.karger.com/10.1159/000495872/) suggest that brown-stained cells with positive expression are DA neurons with a positive expression rate of 76.34%, indicating that the extracted cells are DA neurons.

Overexpressed miR-128 downregulates the expression of AXIN1 but upregulates that of EAAT4 in DA neurons of PD mice

RT-qPCR and Western blot analysis were conducted to measure the expression of miR-128 expression and mRNA level of AXIN1 and EAAT4 in DA neurons (Fig. 3A-C). Compared with the control group, the expression of AXIN1 and EAAT4 were not significantly different ($p > 0.05$); the expression of miR-128 was significantly decreased in the miR-128 inhibitor + sh-AXIN1 group ($p < 0.05$); the mRNA and protein levels of EAAT4 were significantly

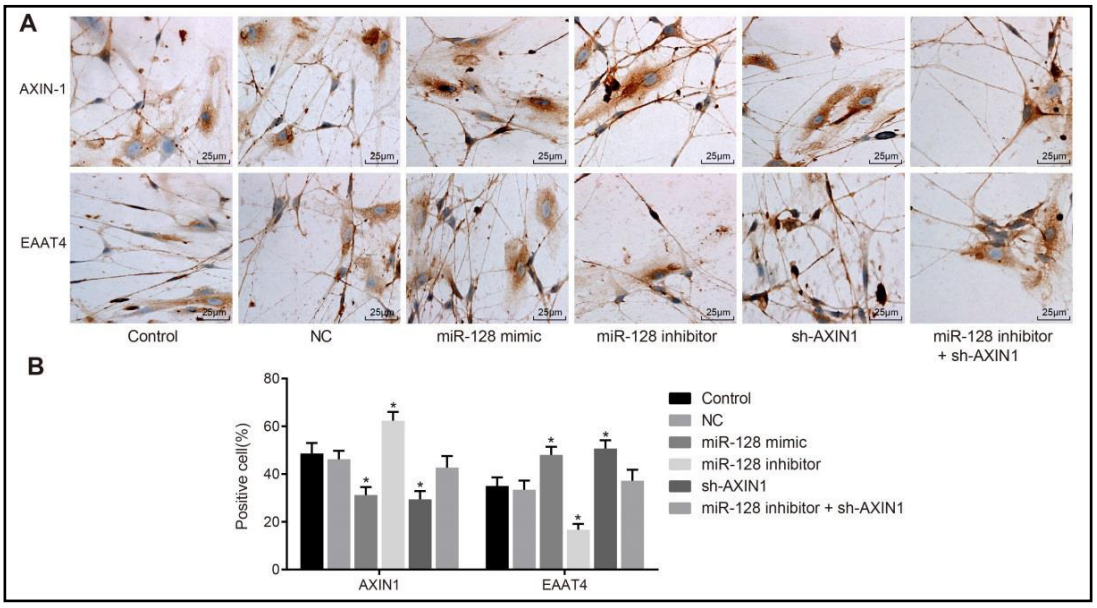

Fig. 4. Immunocytochemistry demonstrating the effect of miR-128 mimic or inhibitor and sh-AXIN1 on the positive expression of the EAAT4 protein in dopamine neurons. A, Images of positive expression of the AXIN1 and EAAT4 proteins in each group. B, Histogram showing the positive expression rate of AXIN1 and EAAT4 in each group; *, P<0.05 vs. the control group; the positive expression of AXIN1 and EAAT4 was expressed as the mean ± standard deviation, and analyzed via one-way analysis of variance; the experiment was repeated three times; miR-128, microRNA-128; AXIN1, axis inhibition protein 1; EAAT4, excitatory amino acid transporter 4; NC, negative control.

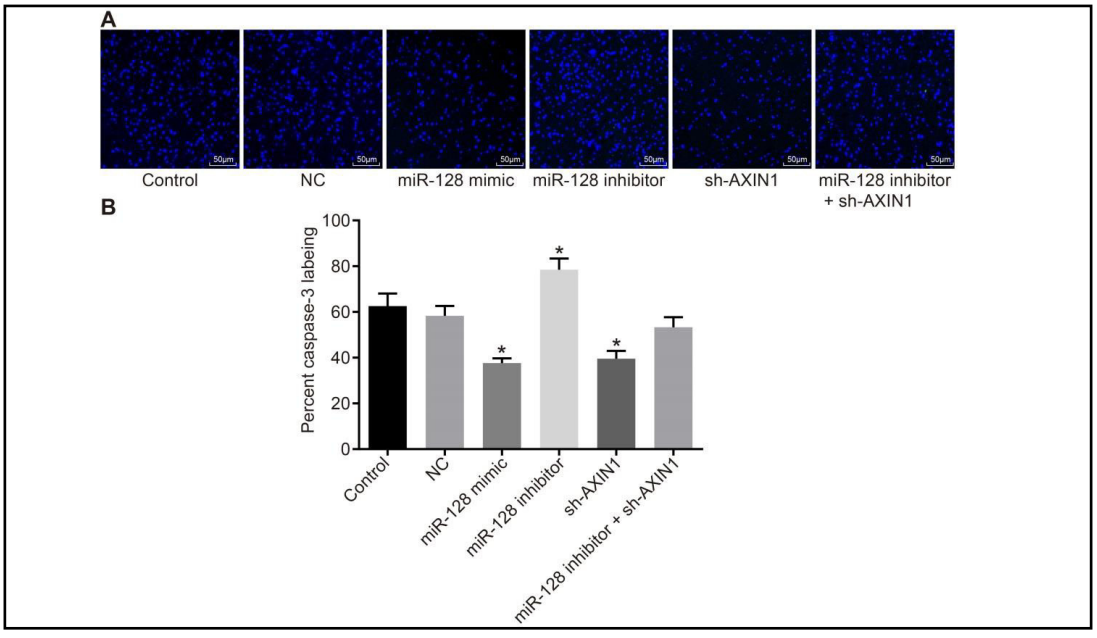

Fig. 5. Immunofluorescence assay (× 400) demonstrating suppressed caspase-3 expression in dopamine neurons in PD mice. A, Immunofluorescence image of caspase-3 expression in dopamine neurons after transfection in each group. B, Histogram of the percentage of caspase-3 labeling in each group; *, P<0.05 vs. the control group; the caspase-3 expression in dopamine neurons was expressed as the mean ± standard deviation, and analyzed via one-way analysis of variance; the experiment was repeated three times; miR-128, microRNA-128; AXIN1, axis inhibition protein 1; EAAT4, excitatory amino acid transporter 4; NC, negative control; PD, Parkinson's disease.

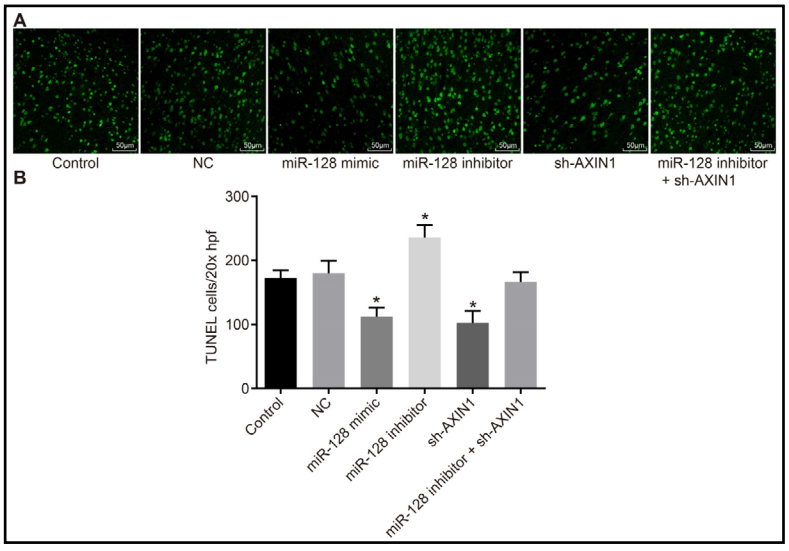

Fig. 6. TUNEL staining demonstrating that miR-128 overexpression or AXIN1 silencing inhibits apoptosis of dopamine neurons in PD mice. A, TUNEL detection of dopamine-neuron apoptosis in each group. B, Histogram showing DA neuron apoptosis; *, P<0.05 vs. the normal group; the data were expressed as the mean ± standard deviation, and were analyzed via one-way analysis of variance; the experiment was repeated three times; miR-128, microRNA-128; AXIN1, axis inhibition protein 1; EAAT4, excitatory amino acid transporter 4; NC, negative control; PD, Parkinson's disease.

increased, while the mRNA and protein levels of AXIN1 were significantly reduced in the miR-128 mimic and sh-AXIN1 groups ($p < 0.05$); the expression of miR-128 was significantly increased in the miR-128 mimic group ($p < 0.05$) and was not significantly different in the sh-AXIN1 group ($p > 0.05$); and the expression of miR-128 and EAAT4 were downregulated, but the expression of AXIN1 was upregulated in the miR-128 inhibitor group ($p < 0.05$). These findings suggested that miR-128 overexpression might increase EAAT4 expression but decrease AXIN1 expression in DA neurons of PD mice.

Overexpressed miR-128 downregulates the positive expression of AXIN1 but reduces that of EAAT4 in DA neurons of PD mice

Immunocytochemistry was employed to evaluate the positive expression of AXIN1 and EAAT4 in DA neurons. As shown in Fig. 4A-B, compared with the control group, the positive expression of AXIN1, caspase-3 and EAAT4 in DA neurons was not significantly different in the NC and miR-128 inhibitor + sh-AXIN1 groups ($p > 0.05$); the positive expression of EAAT4 was significantly increased and positive expression of AXIN1 was decreased in the miR-128 mimic group and sh-AXIN1 group ($p < 0.05$); and the positive expression of EAAT4 was downregulated but that of AXIN1 was upregulated in the miR-128 inhibitor group ($p < 0.05$). These results revealed that overexpressed miR-128 increased the positive expression of EAAT4 and reduced the positive expression rate of AXIN1 in DA neurons of PD mice.

Overexpressed miR-128 and AXIN1 silencing inhibit expression of caspase-3

An immunofluorescence assay was employed to detect the expression of caspase-3 in DA neurons. As shown in Fig. 5A-B, compared with the control group, no significant difference was observed in the expression of caspase-3 in DA neurons in the NC and miR-128 inhibitor + sh-AXIN1 groups ($p > 0.05$); the expression of caspase-3 was significantly downregulated in the miR-128 mimic group and sh-AXIN1 group ($p < 0.05$); and the expression of caspase-3 was upregulated in the miR-128 inhibitor group ($p < 0.05$). These results showed that overexpression of miR-128 and silencing of AXIN1 suppressed the expression of caspase-3.

Overexpressed miR-128 and AXIN1 silencing suppress DA neuron apoptosis

TUNEL staining was adopted to detect DA neuron apoptosis of DA neurons. Compared with the control group, no significant difference was observed in the apoptosis of DA neurons in the NC and miR-128 inhibitor + sh-AXIN1 groups ($p > 0.05$); the apoptosis rate of DA neurons

was significantly decreased in the miR-128 mimic and sh-AXIN1 groups ($p < 0.05$); and the cell apoptosis rate was increased in the miR-128 inhibitor group ($p < 0.05$) (Fig. 6A-B). These findings suggested that overexpressed miR-128 and silenced AXIN1 could repress apoptosis of DA neurons.

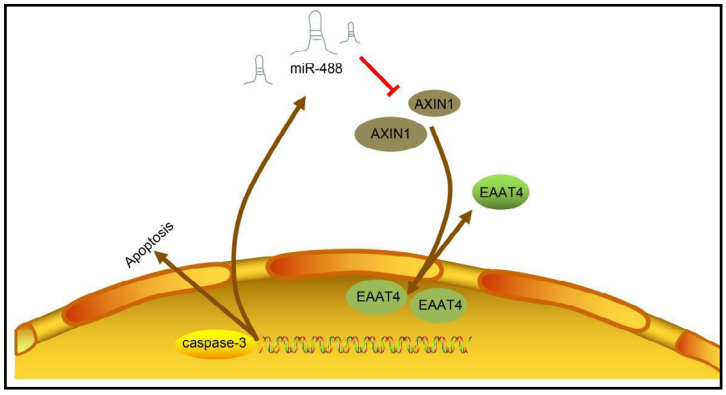

Fig. 7. Mechanism through which overexpressed miR-128 mediates the direct suppression of AXIN1, which may prevent apoptosis of DA neurons and elevate the expression of EAAT4. miR-128, microRNA-128; AXIN1, axis inhibition protein 1; EAAT4, excitatory amino acid transporter 4; DA, dopamine.

Discussion

PD has been defined as the second-most commonly detected neurodegenerative disorder and affects approximately one percent of people older than 60 year of age [26]. PD has been documented to lead to the degeneration of nigrostriatal DA neurons and accompanying neuroinflammation. Although neuroprotective strategies for PD have been explored, there is no effective treatment for preventing or slowing the progression of neurodegeneration [27]. Our study provided evidence that overexpressed miR-128 and downregulated AXIN1 contributed to the upregulation of EAAT4 and rescue of DA neuron from apoptosis, thereby, attenuating PD in the mice.

Initially, we found that there was decreased expression of miR-128 as well as of the mRNA and protein levels of EAAT4 in PD mice, but increased mRNA and protein levels of AXIN1. Moreover, we determined that upregulated miR-128 and downregulated AXIN1 might have the ability to decrease the expression of caspase-3 and, at the same time, protect against apoptosis of DA neurons (Fig. 7). Overexpressed miR-128 decreases neuronal responsiveness, hinders motor activity and alleviates motor abnormalities, which are associated with PD in mice [11]. Furthermore, miR-128 has been shown to be downregulated in glioma, in which overexpressed miR-128 reduces neuroblastoma cell motility and invasiveness by inhibiting Reelin and DCX and inhibits cell proliferation by targeting E2F3a and Bmi-1 (apoptosis-related genes) [28]. Overexpressed miR-128 suppresses the proliferation of neural progenitor cells while facilitating the differentiation of neural progenitor cells to neurons and regulates the neurogenesis of neural precursors by targeting PCM1 in the developing cortex [29]. In addition, Karam et al. reported that miR-128 is induced in the differentiation of neuronal cells and during brain development, which contributes to the inhibition of nonsense-mediated decay, and the regulation of mRNAs that encode proteins are strongly related to neuron differentiation and function [30]. In our study, overexpression of miR-128 inhibited the mRNA and protein levels of AXIN1 at the post-translational level and downregulation of miR-128 expression demonstrated the opposite effect. Therefore, we concluded that AXIN1 is a direct target gene of miR-128 and is negatively mediated by miR-128. AXIN1 is highly expressed in the nucleus to induce neuronal differentiation via beta-catenin activation during development [31]. Previously, miR-128-3p has been reported to modulate the Wnt/beta-catenin signaling pathway in non-small cell lung cancer [32]. Consistent data showed that dynamic changes of AXIN1 subcellular expression and spatial regulation are relevant for neuroepithelial brain tumor development [13].

During early brain development, cell apoptosis is a form of genetically modified cell death that is responsible for the loss of nearly half of DA neurons [33]. Members of the caspase family have been reported to play an essential role in the initiation and progression of cell apoptosis [34]. Among them, caspase-3 is a leading contributor to the execution of neuronal

apoptosis and is notably decreased in the process of brain maturation [33]. EAAT glutamate transporters function not only as secondary active glutamate transporters but also as anion channels [35]. EAATs (EAAT1 - 5) are distributed widely throughout the brain, including the hippocampus of the total protein in the CA1 region, and evidence has been reported the presence of EAAT4 in astrocytes of the hippocampus [36]. The abundance and function of EAAT4 are known to be modulated by the serum and glucocorticoid inducible kinase (SGK) 1 [37]. One study has shown that the neuronal glutamate transporter EAAT4 in Purkinje cells plays a critical role in the extrasynaptic diffusion of climbing fibers (CFs) transmitter, in that EAAT4 not only retrogradely determines the degree of CF-mediated inhibition of GABAergic inputs to the PC via glutamate concentration control for intersynaptic diffusion but also governs synaptic information processing in the cerebellar cortex [38]. The importance of EAATs has been supported by Ogata et al., who demonstrated that EAATs are significant for terminating glutamatergic neurotransmission and rescuing CNS neurons from glutamatergic excitotoxicity [39]. EAAT4 is expressed in astrocytes, and astrocytic localization of neuronal EAAT4 implies a potential role of EAAT4 in the CNS [40]. Consistently, our study provided evidence that the expression of EAAT4 was downregulated in PD mice.

Conclusion

In conclusion, the results of our study offer insights into the mechanism of action of miR-128 in mice with PD; upregulation of miR-128 expression prevented apoptosis of DA neurons and elevated the EAAT4 expression by decreasing the expression of the AXIN1 gene in PD mice. Moreover, these data suggest a therapeutic potential for miR-128 in the treatment of PD, which requires a broadening of the therapeutic perspective through clinical development.

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Disclosure Statement

The authors have declared that no competing interests exist.

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