Mitochondrial glutamine transporter SLC1A5_var, a potential target to suppress astrocyte reactivity in Parkinson’s Disease

Yang Liu1,4, Lei Cao2,4, Yuting Song1, Zhengwei Kang2, Ting Liu3, Jianhua Ding2, Gang Hu1,2✉ and Ming Lu2,3✉
© The Author(s) 2022

SLC1A5 variant (SLC1A5_var) is identified as a mitochondrial glutamine transporter in cancer cells recently. However, the role of SLC1A5_var in Parkinson’s disease (PD) is completely unknown. Here, we found the significant downregulation of SLC1A5_var in astrocytes and midbrain of mice treated with MPTP/MPP+ and LPS. Importantly, overexpression of SLC1A5_var ameliorated but knockdown of SLC1A5_var exacerbated MPTP/MPP+ and LPS-induced mitochondrial dysfunction. Consequently, SLC1A5_var provided beneficial effects on PD pathology including improvement of PD-like motor symptoms and rescue of dopaminergic (DA) neuron degeneration through maintaining mitochondrial energy metabolism. Moreover, SLC1A5_var reduced astrocyte reactivity via inhibition of A1 astrocyte conversion. Further investigation demonstrated that SLC1A5_var restrained the secretion of astrocytic pro-inflammatory cytokines by blunting TLR4-mediated downstream pathways. This is the first study to prove that astrocytic SLC1A5_var inhibits neuroinflammation, and rescues the loss of DA neurons and motor symptoms involved in PD progression, which provides a novel target for PD treatment.

Cell Death and Disease (2022)13:946; https://doi.org/10.1038/s41419-022-05399-z

INTRODUCTION
Parkinson’s disease (PD) is a neurodegenerative disorder characterized by motor and non-motor symptoms. The pathological feature of PD is the progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc), accompanied by the formation of Lewy bodies and the activation of glial cells [1, 2]. Studies of genetics and genomics in PD have demonstrated that oxidative stress and mitochondrial dysfunction play vital roles in the progression of PD [3]. For this reason, theoretically, drugs targeting mitochondria are promising for PD treatment. However, MitoQ as a powerful mitochondrial antioxidant failed to slow the progression of PD in clinical trial [4], indicating a complicated mechanism underlying the mitochondrial dysfunction in PD pathogenesis. Further investigation is warranted to elucidate the sophisticated regulation involved in mitochondrial dysfunction.

Astrocytes are the most abundant cells responsible for maintaining the overall homeostasis in the brain. Our previous studies have proved that dramatic increase in astrocyte reactivity triggers neuroinflammation and accelerates PD progression, while inhibition of astrocyte-derived inflammation is able to improve PD symptoms [5–7], suggesting that retaining astrocyte physiological properties could be a potential strategy for PD therapy. Numerous studies have affirmed mitochondrial dysfunction as a major contributor to astrocyte transformation from resting state to neurotoxic A1 state [8, 9]. Although mitochondrial dysfunction is commonly observed in astrocytes during the development of PD [10], the molecular events taking place within the pathogenic process are poorly documented. Depicting the molecular cascade in astrocyte activated parallel to PD pathogenesis could provide comprehensive understanding of mitochondrial dysfunction and facilitate mitochondria-targetting strategies for PD therapy.

In this study, RNA sequencing was performed to analyze the transcriptome of astrocytes treated with MPP+. Beyond confirming mitochondrial dysfunction and oxidative stress as key pathogenesis of PD, we also found that SLC1A5_variant (SLC1A5_var) located in the mitochondrion is required for mitochondrial energy metabolism. SLC1A5_var is the variant form of SLC1A5 which is a sodium-dependent transporter of neutral amino acids. Unlike SLC1A5 localized in the plasma membrane, SLC1A5_var harbors N-terminal targeting signal for mitochondrial localization that enables it to regulate mitochondrial function [11]. Our study first identified that SLC1A5_var is a crucial regulator of mitochondrial energy metabolism in astrocytes. Meanwhile, overexpression of SLC1A5_var preserved astrocyte physiological properties against neuroinflammation and PD symptoms. Together, these findings broaden our knowledge of the mechanism underlying mitochondrial dysfunction and shed light on mitochondria-targetting therapeutics for PD.

MATERIALS AND METHODS
Experimental animals
C57BL/6J mice (male, 3-month old) used in this experiment were purchased from Nanjing Medical University. The mice used in the experiment were housed with temperatures of 22-25 °C and humidity of 50-60%. Mice were housed at groups of 5 per cage with water and food ad
libitum on a 12:12 h light cycle (lights on at 7 a.m.). All animal procedures were performed in accordance with the guideline of the Institutional Animal Care and Use Committee of Nanjing Medical University.

Stereotoxic surgery

According to previous literature [12], mice were pretreated with sodium pentobarbital anesthesia (40 mg/kg, i.p.), the Lenitivirus (LV) over-expressing SLC1A5_var (NCBI Reference Sequence: NM-001145145.1) and the corresponding empty vector were injected into the mice’s midbrain. The three-dimensional coordinates of the mice’s midbrain are as follows: A/P −3.0 mm, R/L ±1.3 mm, and D/V −4.5 mm. The mice were microinjected with either a SLC1A5_var vector lentivirus (SLC1A5_var vec) or SLC1A5_var overexpression lentivirus (SLC1A5_var OE) (1.5 μL of 2 × 10⁸ viral genome/μL, Hanbio).

Preparation of animal models

For MPTP model, mice were divided into four groups 2 weeks after LV microinjection: SLC1A5_var vec + MPTP, SLC1A5_var OE + MPTP. Mice were weighed and injected subcutaneously with MPTP at 20 mg/kg for 5 consecutive days. 3 days after the completion of modeling, behavioral tests were conducted and then mice were sacrificed for bioassay.

For LPS model, mice were divided into four groups 2 weeks after LV microinjection: SLC1A5_var vec + LPS, SLC1A5_var overexpression (OE) + LPS, SLC1A5_var var vec − LPS, SLC1A5_var OE − LPS. Injection of LPS into mice’s midbrain was carried out as per the method of the lentivirus injection. LPS was dissolved in saline at a concentration of 2.5 mg/mL, and 1 μL was injected on both sides. 7 days later, behavioral tests were conducted and then mice were sacrificed for bioassay.

Behavioral test

Open field test is mainly used to observe the ability of voluntary movement. The mice were firstly acclimatized in the room for 2 h. Then the overall distance traveled (mm) and speed (cm/s) which represent the spontaneous activities were recorded in 5 min by the software (Top-Scan Version 2.0, American CleverSys Inc).

Pole test is widely used to assess basal ganglia related movement disorders in mice. Time expenditure for climbing from top to bottom of a pole (landing on both front claws) was recorded, and mice with motor coordination defect consume more time. Firstly, the mice were placed on a smooth pole (diameter of 1 cm and height of 50 cm). Then the mice were trained 3 times and the total time consumed from top to bottom was recorded.

Rotarod test is performed to assess motor coordination and balance of mice. Firstly, the mice were trained on an accelerating rotarod in a separate compartment. Then, the mice were tested at 20 rpm for 180 s. The latency to fall off the rod was recorded by the JLBehr-RTTG-5 system.

Cell culture and transfection

Astrocytes and microglia were isolated as previously [7]. Briefly, primary astrocytes were isolated from the brain of neonatal mice (3 days after birth) and cultured in DMEM/F12 medium (#10565018, Gibco) supplied with 10% FBS and 100 unit/mL penicillin/streptomycin. Change the medium every 3 days and culture for 14 days. Primary microglia were isolated from the brain tissues of neonatal mice (3 days after birth). Microglia were seeded on the t75 precoated with poly-lysine and the culture medium is the same as astrocytes. After being cultured for 14 days, microglia were collected by shaking at 200 rpm for 15 min. Then microglia-conditioned medium (MCM) was collected and centrifuged at 12,000 × g for 10 min at 4 °C and the supernatant was collected to stimulate the A1-type astrocytes. Complete (100%) microglia medium was added to the astrocytes, and the volume was 1.0 mL for each well of 6-well plate or 0.5 mL for each well of 12-well plate.

For the cell lines, C6/1A (No. CRL-2541, ATCC) and HEK-293T cells were cultured in DMEM medium (#11965092, Gibco) supplied with 10% FBS and 100 unit/mL penicillin/streptomycin.

For transfection, SLC1A5_var siRNA was purchased from GenePharma (Shanghai, China). Astrocytes were cultured for 14 days in vitro and transfection was performed on Day 14 using Lipofectamine 3000 (Invitrogen, L3000015) according to the manufacturer’s protocol. Then, experiments were carried out 48 hours after transfection. The sequence of SLC1A5_var siRNA was listed in Supplementary Table S1. For over-expression of SLC1A5_var, Slc1a5_var cDNA and its truncations were amplified and subcloned into pcDNA3.1 (PPL, Nanjing, China). Then the plasmid was transfected into the astrocytes and HEK-293T cells.

RNA extraction and library construction

Total RNA was isolated from primary astrocytes (N = 3) with or without MPP+ (500 μM, 24 h) treatment. Then RNA was purified using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s procedure. The RNA amount and purity of each sample was quantified using NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA). The RNA integrity was assessed by Bioanalyzer 2100 (Agilent, CA, USA) with RNA integrity number >7.0, and confirmed by electrophoresis with denaturing agarose gel. Poly (A) RNA is purified from 1 μg total RNA using Dynabeads Oligo (dT)25-61005 (Thermo Fisher, CA, USA) using two rounds of purification. Then the poly(A) RNA was fragmented into small pieces using Magnesium RNA Fragmentation Module (NEB, cat. e6150, USA) under 94 °C 5–7 min. Then the degraded RNA fragments were reverse-transcribed to create the cDNA by SuperScript II Reverse Transcriptase (Invitrogen, cat. 1896649, USA), which were then used to synthesize U-labeled second-stranded DNAs with m. coli DNA polymerase I (NEB, cat.m0209, USA), RNase H (NEB, cat.m0297, USA) and dUTP Solution (Thermo Fisher, cat. R0133, USA). An A-base is then added to the blunt ends of each strand, preparing them for ligation to the indexed adapters. Each adapter contains a T-base overhang for ligating to the A-tailed fragmented DNA. Single- or dual-index adapters are ligated to the fragments, and size selection was performed with AMPureXP beads. After the heat-labile UDG enzyme (NEB, cat.m0280, USA) treatment of the U-labeled second-stranded DNAs, the ligated products are amplified with PCR by the following conditions: initial denaturation at 95 °C for 3 min; 8 cycles of denaturation at 98 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s; and then final extension at 72 °C for 5 min. The average insert size for the final cDNA library was 300 ± 50 bp. At last, we performed the 2 x 150 bp paired-end sequencing (PE150) on an illumina Novaseq™ 6000 (LC-Bio Technology Co., Ltd., Hangzhou, China) following the recommended protocol.

Bioinformatics analysis of RNA-seq

Fastp software (https://github.com/OpenGene/Fastp) was used to remove the reads that contained adapter contamination, low-quality bases, and undetermined bases with default parameter. Then sequence quality was also verified using fastqs. We used HISAT2 (https://ccb.jhu.edu/software/hisat2) to map reads to the reference genome of Mus musculus (GRCm38). The mapped reads of each sample were assembled using StringTie (https://ccb.jhu.edu/software/stringtie) with default parameters. Then, all transcriptomes from all samples were merged to reconstruct a comprehensive transcriptome using gffcompare (https://github.com/gpertea/gffcompare). After the final transcriptome was generated, StringTie and was used to estimate the expression levels of all transcripts. StringTie was used to use perform expression level for mRNAs by calculating FPKM (FPKM = [total exon/fragments/mapped_reads(millions) × exon_length(kb)]) The differentially expressed mRNAs were selected with fold change > 2 or fold change < 0.5 and with parametric F-test comparing nested linear models (p-value < 0.05, q value was used for the p-value correction) by R package edgeR (https://bioconductor.org/packages/release/bioc/html/edgeR.html). The differentially expressed genes associated with mitochondrial function and oxidative stress, two major causes of PD, were selected further and enriched using Gene Ontology (http://geneontology.org). Genes were further validated by qRT-PCR experiments.

Measurement of mitochondrial oxygen consumption rate (OCR)

The oxygen consumption rate of primary astrocytes was measured using the Seahorse XF96 (Agilent, CA, USA). Astrocytes were seeded on the plates (20,000 cells/per well) and grouped into four: SLC1A5_var vec, SLC1A5_var OE, SLC1A5_var vec + LPS, and SLC1A5_var OE + LPS. Oxygen consumption rates were measured after injection of oligomycin (1 μM), FCCP (1 μM), and antimycin A (Mitochondrial Complex III inhibitor, 0.5 μM) plus rotenone (Mitochondrial Complex I inhibitor, 0.5 μM). The non-mitochondrial respiration was not subtracted for the quantification.

Immunofluorescence and immunohistochemistry

Immunofluorescence and immunohistochemistry were performed as described in our previous study [13]. Cells or brain slices (frozen sections, 25 μm) were fixed in 4% paraformaldehyde and blocked with 5% BSA in PBS (0.3% Triton X-100), and then they were incubated with the primary antibody at 4 °C overnight. After that, the corresponding secondary antibodies were incubated for 1 h at room temperature. The fluorescence intensity was quantified by Image J software (v.1.8.0). Briefly, same color threshold was
Mitochondria isolation
Astrocytes and HEK-293T cells were transfected with SLC1A5_var and mitochondria were isolated using Cell Mitochondrial Extraction Kit (C3601, Beyotime) as per the manufacturer’s protocol. Briefly, cells were harvested and the mitochondrial isolation reagent provided by the kit was added and incubated for 15 min on the ice. Then, the cells were homogenized and centrifuged for 10 min at 4 °C, 1000 × g. The supernatant was collected and centrifuged for 10 min at 4 °C, 3500 × g. After that, the pellet was collected as mitochondria and lysed using the mitochondrial lysis buffer provided by the kit. BCA assay was performed to determine the concentration of mitochondrial protein.

Mitotracker
Cells were transfected with overexpression of SLC1A5_var and then incubated with Mitotracker Red solution (Concentration: 1 μM, Ex/Em = 581 nm/664 nm) at 37 °C for 30 min. Then colocalization of Mitotracker Red (Thermo, M22425) and SLC1A5_var was captured by CarlZeiss LSM710 confocal microscope.

Detection of ATP
The ATP content in astrocytes was detected using the Enhanced ATP Assay Kit (Beyotime Biotechnology, S0027) following manufacturer protocol.

Mitochondrial membrane potential (MMP)
MMP was measured by JC-1 probe (Invitrogen) to determine the mitochondrial membrane potential. Astrocytes were stained with JC-1 dye (Concentration: 5 μM; Red: Ex/Em = 535/590 nm; Green: Ex/Em = 485/530 nm) for 30 min at 37 °C. Then they were analyzed by flow cytometer (FACS Calibur, BD).

Mitochondrial reactive oxygen species (ROS)
For mitochondrial ROS measurements, cells were incubated with Mitosox Red (Concentration: 5 μM, Ex/Em = 510/580 nm, Invitrogen) or H2DCFDA (Concentration: 5 μM, Ex/Em = 488/525 nm, Beyotime) for 30 min. Cells were analyzed by confocal microscopy and the level of Mitochondrial ROS was evaluated by a flow cytometer (FACS Calibur, BD).

Transmission electron microscopy (TEM) analysis
The procedure was described in our previous study [13]. Primary astrocytes are perfused with 2.5% glutaraldehyde. Specimens were fixed in 1% osmium tetroxide, stained in aqueous uranyl acetate, and then dehydrated and embedded in epoxy resin. Ultrathin sections were stained using lead citrate and examined with the transmission electron microscope. Healthy mitochondria have a clear structure of the membrane. The inner membrane was folded to form normal mitochondrial cristae including lamellar, tubular, and vesicular cristae. Damaged mitochondria harboring morphologies including the disappeared double membrane structure and mitochondrial cristae, and the blurred edges. Each group randomly selected 4-5 visual fields to be photographed, then the percentage of damaged mitochondria was calculated.

Annexin V/PI analysis
Cells were performed by Annexin V/PI apoptosis detection kit (Vazyme, A211-02) according to the manufacturer’s manual. The apoptosis rate was measured by flow cytometer (FACS Calibur, BD).

Statistical analysis
Statistical analyses were performed using SPSS version 22.0 or GraphPad Prism 8.0 software, and all data were presented as mean ± S.E.M. Statistical significance was assessed with two-way ANOVA followed by Tukey’s post hoc test. p < 0.05 was predetermined as the threshold for statistical significance.

RESULTS
Glutamine transporter SLC1A5_var is located in mitochondria of astrocytes and significantly downregulated in MPTP and LPS mouse models
RNA sequencing was performed in astrocytes stimulated with or without MPP+ to gain PD-related genes. From the volcano plot,
1858 upregulated and 2561 downregulated genes were obtained in the context of MPP+ stimulation (Fig. 1A). In the volcano plot, we found the gene slc1a5 was significantly upregulated and it has been demonstrated closely correlated with the progression of PD in our previous study. Moreover, the differentially expressed genes associated with mitochondrial function and oxidative stress, two major causes of PD, were selected further and enriched using Gene Ontology (http://geneontology.org/). GO analysis enriched pathways was shown in Fig. 1B and validated by qRT-qPCR. Interestingly, we found slc1a5 was significantly increased consistently with RNA sequencing while the mitochondrial variant SLC1A5_var was significantly decreased (Fig. 1C). Therefore, the
gene SLC1A5 var has aroused our interest and it was selected for further study. Recent evidence has shown that SLC1A5 var is a mitochondrial transporter of glutamine in cancer cells [11], while the function of SLC1A5 var in astrocytes and PD is completely unknown. To this end, we explored the expression pattern of SLC1A5 in the context of PD. Firstly, RNA scope showed the existence of SLC1A5 var in the midbrain of mice (Supplementary Fig. 1A). Then, the SLC1A5 var was found in the mitochondrial fractions of astrocytes (Fig. 1D) which is consistent with the finding in cancer cells [11]. Moreover, after treatment with peptide-N-glycosidase F (PNGase F), endogenous SLC1A5 var were detectable in the whole cell lysates of astrocytes using an anti-SLC1A5 antibody (Fig. 1E). To give a visualization of cellular location of SLC1A5 var, HEK-293T cells (much higher transfection efficiency than astrocytes) were used to transfected with Slc1a5 var (Supplementary Fig. S1B). Overexpression of SLC1A5 var was easily detected in the cell lysates (Fig. 1F) and clearly observed in the mitochondria of HEK-293T cells (Fig. 1G). Therefore, we confirmed that SLC1A5 var is located in the mitochondrion. More importantly, we found that SLC1A5 var was decreased significantly in both midbrain-and astrocytes-derived mitochondria upon LPS or MPTP/MPP+ exposure (Fig. 1H, I and Supplementary Fig. S1C–F), suggesting a potential role of SLC1A5 var in PD. Intriguingly, although overexpression of SLC1A5 var was observed in both astrocytes (marked by GFAP in Fig. 1J) and DA neurons (marked by TH in Supplementary Fig. S1G), dramatic decline of SLC1A5 var was mostly found in astrocytes (Fig. 1K, L) but not in DA neurons (Supplementary Fig. S1H–I), indicating astrocytic SLC1A5 var may play crucial roles in PD progression. Thus, we investigated the function of astrocytic SLC1A5 var in the following study.

Astrocytes transfected with SLC1A5 var plasmid exhibited elevated mRNA level of SLC1A5 var (Supplementary Fig. S2A) and increased protein level of mitochondrial SLC1A5 var (Supplementary Fig. S2B). Importantly, overexpression of SLC1A5 var rescued LPS-induced ATP depletion (Fig. 1M), and reversed AMPK activation reflected by decreased phosphorylation of AMPKα (Fig. 1N and Supplementary Fig. S2C) which acts as a sensory signal of cellular energy [15]. Similarly, the effect of SLC1A5 var overexpression on energy metabolism was replicable in MPP+–stimulated astrocytes (Fig. 1O, P and Supplementary Fig. S2D). These results suggest that the mitochondrial glutamine transporter SLC1A5 var is a key regulator of energy metabolism in astrocytes, and loss of SLC1A5 var may contribute to PD progression.

SLC1A5 var ameliorates disrupted mitochondrial energy metabolism in LPS-treated astrocytes

To investigate the role of SLC1A5 var in mitochondrial energy metabolism, oxygen consumption rate (OCR) of astrocytes was traced by Seahorse XF analyzer. Overexpression of SLC1A5 var reversed LPS-induced reduction in basal oxygen consumption and maximal respiration (Fig. 2A–C). Meanwhile, reduced ATP production and proton leak were improved by SLC1A5 var overexpression in LPS-stimulated astrocytes (Supplementary Fig. S3A–C). To validate the effect of SLC1A5 var on mitochondrial energy metabolism, astrocytes were transfected with siSlc1a5 var. Transfection of siSlc1a5 var decreased the mRNA level of SLC1A5 var but not Slc1a5 in astrocytes (Supplementary Fig. S3D, E), and the protein level of mitochondrial SLC1A5 var was reduced as well in siSlc1a5 var–transfected astrocytes (Supplementary Fig. S3F). Consistent with the findings in SLC1A5 var overexpressed astrocytes, knockdown of SLC1A5 var aggravated LPS-induced reduction in basal oxygen consumption and maximal respiration (Fig. 2D–F). Although no change in ATP production and non–mitochondrial oxygen consumption were observed, knockdown of SLC1A5 var further reduced the proton leak in astrocytes with LPS treatment (Supplementary Fig. S3G–I). These results highlight the importance of SLC1A5 var in mitochondrial energy metabolism.

Besides energy metabolism, we also found the beneficial effect of SLC1A5 var on mitochondrial integrity. The mitochondrial morphology was observed in astrocytes by transmission electron microscope (TEM). As shown in Fig. 2G and Supplementary Fig. S4A, LPS treatment led to the accumulation of damaged mitochondria with disappeared double membrane structure and mitochondrial cristae, and the blurred edges. Intriguingly, overexpression of SLC1A5 var reduced the amount of abnormal mitochondria in LPS-treated astrocytes. In contrast, knockdown of SLC1A5 var aggravated LPS-induced accumulation of injured mitochondria (Supplementary Fig. S4B, C). The above results reveal a potential role of SLC1A5 var in maintaining mitochondrial integrity which is critical for mitochondrial function.

SLC1A5 var reduces overproduction of mitochondrial ROS and maintains mitochondrial membrane potential in the LPS model

During the last decade, accumulating evidence suggested that mitochondrial dysfunction, ROS levels, and aberrations in mitochondrial morphology are interconnected [16]. To determine whether SLC1A5 var attenuates LPS-induced ROS production, the mitoSOX assay was performed. As shown in Fig. 3A, B, mitochondrial ROS (mito-ROS) was significantly increased upon LPS exposure in astrocytes, while overexpression of SLC1A5 var markedly suppressed LPS-induced mito-ROS production. From the flow cytometry data shown in Fig. 3C, D, LPS caused a significant increase (92.25 ± 1.78% relative to the SCL1A5 var vec group) in mitoSOX-positive astrocytes, and overexpression of SLC1A5 var reduced the amount of mitoSOX-positive astrocytes (22.65 ± 2.25% relative to the SCL1A5 var vec-i–LPS group). Meanwhile, overexpression of SLC1A5 var promoted the scavenging of intracellular ROS indicated by DCFH-DA probe in the LPS-treated astrocytes (Supplementary Fig. S5A–C). On the contrary, the knockdown of SLC1A5 var aggravated ROS production (Supplementary Fig. S5D, E). As LPS treatment and overexpression of SLC1A5 var showed no effects on apoptosis of astrocytes and C8-D1A cells (Supplementary Fig. S6A, B), the effect of SLC1A5 var on ROS scavenging should not be attributed to cell death–caused ROS reduction. Besides, the markedly reversed mitochondrial membrane potential (MMP) induced by LPS (80.23 ± 1.54% relative to the SCL1A5 var vec group) was visualized by the JC-1 green fluorescence, and it was significantly decreased (40.18 ± 1.10% relative to the SCL1A5 var vec-i–LPS group) after overexpression of SLC1A5 var (Fig. 3E–H). Together, these data demonstrate that SLC1A5 var attenuates LPS-induced mitochondrial ROS accumulation and low MMP.

SLC1A5 var inhibits activation of NF-κB signaling in LPS-stimulated astrocytes

Next, we explored the downstream effects of SLC1A5 var–mediated mitochondrial function in LPS-treated astrocytes. Accumulation of mitochondrial ROS triggers activation of NF-κB signaling which is a critical regulatory mechanism involved in neuroinflammation [17, 18]. We then examined the phosphorylation and nuclear translocation of p65 in astrocytes with LPS treatment. Our data showed that overexpression of SLC1A5 var reduced (Fig. 4A, B) but knockdown of SLC1A5 var–LPS group) after overexpression of SLC1A5 var (Fig. 4C, D). The remarkable reversed NF-κB signaling in SLC1A5 var–transfected astrocytes, DAPI staining showed that SLC1A5 var significantly decreased p-p65 and p-IκB in astrocytes (Fig. 4F–H). Consistently, knockdown of SLC1A5 var enhanced NF-κB signaling in astrocytes (Fig. 4E). This finding is crucial for understanding the roles of SLC1A5 var in LPS-induced astrocytes. Moreover, the NF-κB signaling was further stimulated by co-treatment with IL-1β and LPS (Fig. 4I, J). Results showed that the knockdown of SLC1A5 var enhanced NF-κB signaling in astrocytes (Fig. 4K, L). These findings further support the role of SLC1A5 var in regulating NF-κB signaling in LPS-treated astrocytes.
activation of NF-κB signaling (Fig. 4H–J). Therefore, SLC1A5_var acts as a negative regulator of NF-κB activation.

**SLC1A5_var reduces A1 astrocyte reactivity in the LPS model**

As is known, increased intracellular ROS production activates microglia to secrete pro-inflammatory cytokines and exacerbate inflammation [19]. A1 reactive astrocytes can be induced by neuroinflammatory microglia [20]. Since we had demonstrated that SLC1A5_var attenuates the level of ROS stimulated by LPS, Next, we determined whether SLC1A5_var had an effect on the astrocyte reactivity by assessing mRNA levels of A1- and A2-specific markers. The expression of A1-specific markers (H2-D1, Ligp1, Serping1, C3, Fbnl5, Fkbp5, Pmb8, Amigo2, and Ugt1a) were increased by LPS stimulation, which was reversed by overexpression of SLC1A5_var (Fig. 5A). However, LPS stimulation failed to affect the levels of A2-specific markers (Clcf1, Ptx3, ...
SLC1A5_var inhibits the release of astrocytic pro-inflammatory cytokines through blunting TLR4-mediated signaling in the LPS model

As A1 is the pro-inflammatory astrocyte phenotype, we next examined levels of pro-inflammatory cytokines in LPS-stimulated astrocytes. Pro-inflammatory cytokines including TNF-α, IL-6, IL-1β, and IL-18 were significantly increased in mRNA levels (Fig. 6A–D) and proteins levels (Fig. 6E–H) upon LPS exposure, while overexpression of SLC1A5_var reduced upregulation of these pro-inflammatory cytokines. Then, we sought to explore the mechanism underlying SLC1A5_var-mediated inhibition of inflammation. Toll-like receptor 4 (TLR4) recognizes LPS and activates NF-κB signaling.
to enhance inflammatory cytokine production [21]. Our data showed that overexpression of SLC1A5_var inhibited LPS-induced upregulation of TLR4, p-p38, p-JNK, and p-AKT (Fig. 6I–M). Besides, knockdown of SLC1A5_var increased the expression of TLR4, p-p38, p-JNK, and p-AKT (Fig. 6N–R). Therefore, these data suggest that the anti-inflammatory activity of SLC1A5_var is attributed to the inhibition of TLR4-mediated signal transduction.

SLC1A5_var alleviates neuroinflammation and attenuates dopaminergic neurons loss in the MPTP and LPS mouse models

Both loss of dopaminergic neurons in the SNpc and enhanced neuroinflammation are involved in the progression of PD [22, 23]. To evaluate the neuroprotective role of SLC1A5_var in PD, we conducted the MPTP mouse model. Briefly, C57BL/6 mice were microinjected with SLC1A5_var LV in the SNpc and received MPTP treatment (20 mg/kg) for 5 days afterward the LV injection for 2 weeks (Fig. 7A). Successful overexpression of SLC1A5_var was validated by Western blot using striatum samples (Fig. 7B). Overexpression of SLC1A5_var mainly distributed in mitochondria of astrocytes, and inhibited astrocyte activation and proliferation in the midbrain of MPTP-treated mice (Fig. 7C–E). Importantly, overproduction of pro-inflammatory cytokines including TNF-α, IL-6, IL-1β, and IL-18 were significantly reduced by SLC1A5_var overexpression (Fig. 7F, G), which was attributed to the inhibition of astrocyte phenotype polarization to A1 reactive astrocytes (Fig. 7H). Together, these results strengthen the anti-inflammatory effect of SLC1A5_var.

Fig. 4 SLC1A5_var significantly blocks the nuclear translocation of p-p65 in LPS-stimulated astrocytes. Astrocytes were transfected with SLC1A5_var OE (A, B) or SLC1A5_var siRNA (C, D) for 48 h and then stimulated with LPS for 6 h. Phosphorylation of nuclear translocation of NF-κB (p65) was assessed by fluorescence microscopy (n = 5–8). Scale bar represents 20 μm. Phosphorylation of IKKβ and p65 was determined by western blot analysis (E, H) and quantified by densitometry (F, G and I, J). Data are shown as the mean ± S.E.M. ***P < 0.001 vs control, #P < 0.05, ##P < 0.01 and ###P < 0.001 vs LPS group, two-way ANOVA followed by Tukey’s post hoc test.
To evaluate the therapeutic effect of SLC1A5_var on PD, we performed behavioral analyses of PD-like motor symptoms. In the open field test, MPTP-treated mice exhibited shorter travel distance in central area and slower speed relative to saline-treated mice, while overexpression of SLC1A5_var improved the locomotor activity (Fig. 8A–C). Motor coordination was evaluated by rotarod test and pole test. Overexpression of SLC1A5_var had no effect on rotarod test but improved the performance in pole test (Fig. 8D, E). Overall, SLC1A5_var facilitates the improvement of motor symptoms in PD mice. More importantly, loss of dopaminergic neurons in the SNpc and striatum were rescued by SLC1A5_var overexpression (Fig. 8F–J). As SLC1A5_var is a mitochondrial glutamine transporter, we measured the glutamine content by HPLC. Glutamine was increased significantly in the striatum of mice with SLC1A5_var overexpression (Fig. 8K). The expression of p65/p-p65 and TH was examined by Western blot while SLC1A5_var was overexpression (D) or knockdown (F) and quantified by densitometry (E, G) (n = 3). Data are shown as the mean ± S.E.M. ***P < 0.001 vs control, #P < 0.05, ##P < 0.01 vs LPS group, two-way ANOVA followed by Tukey’s post hoc test.

**Fig. 5 SLC1A5_var decreases A1 astrocyte reactivity in the LPS-stimulated model.** Astrocytes were transfected with SLC1A5_var OE or siRNA for 48 h and then replaced with the conditioned medium of microglia stimulated with LPS for 6 h. Then cell lysate was analyzed by QT-PCR. Heatmap of A1-specific and A2-specific markers in astrocytes while SLC1A5_var was overexpression (A) (n = 3) or knockdown (B) (n = 3). C Colocalization of C3 (green) and Serping1(red) while SLC1A5_var was overexpressed. Scale bar, 50 μm. Expression of C3 was determined by Western blot while SLC1A5_var was overexpression (D) or knockdown (F) and quantified by densitometry (E, G) (n = 3). Data are shown as the mean ± S.E.M. **P < 0.01 vs control, #P < 0.05, ##P < 0.01 vs LPS group, two-way ANOVA followed by Tukey’s post hoc test.

To evaluate the therapeutic effect of SLC1A5_var on PD, we performed behavioral analyses of PD-like motor symptoms. In the open field test, MPTP-treated mice exhibited shorter travel distance in central area and slower speed relative to saline-treated mice, while overexpression of SLC1A5_var improved the locomotor activity (Fig. 8A–C). Motor coordination was evaluated by rotarod test and pole test. Overexpression of SLC1A5_var had no effect on rotarod test but improved the performance in pole test (Fig. 8D, E). Overall, SLC1A5_var facilitates the improvement of motor symptoms in PD mice. More importantly, loss of dopaminergic neurons in the SNpc and striatum were rescued by SLC1A5_var overexpression (Fig. 8F–J). As SLC1A5_var is a mitochondrial glutamine transporter, we measured the glutamine content by HPLC. Glutamine was increased significantly in the striatum of mice with SLC1A5_var overexpression (Fig. 8K). The expression of p65/p-p65 and TH was examined by Western blot while SLC1A5_var was overexpression (D) or knockdown (F) and quantified by densitometry (E, G) (n = 3). Data are shown as the mean ± S.E.M. ***P < 0.001 vs control, #P < 0.05, ##P < 0.01 vs LPS group, two-way ANOVA followed by Tukey’s post hoc test.

Next, we confirmed the therapeutic effect of SLC1A5_var on PD using LPS-induced neuroinflammation model which is also widely used to replicate PD phenotype [24]. In the LPS mouse model, overexpression of SLC1A5_var failed to improve the locomotor activity evaluated by open filed test (Supplementary Fig. S8A, B), and rotarod test was not sensitive enough to examine motor...
coordination in this context (Supplementary Fig. S8C). However, the defect of motor coordination of LPS-treated mice was observed in pole test, and attenuated by SLC1A5_var overexpression (Supplementary Fig. S8D). Meanwhile, from the HPLC analysis, the reduced dopamine level was reversed by SLC1A5_var overexpression (Supplementary Fig. S8E). Consistent with the findings in the MPTP model, overexpression of SLC1A5_var also rescued loss of dopaminergic neurons in the LPS model (Supplementary Fig. S8F, G). Overall, these data suggest that SLC1A5_var elicits the neuroprotective effect via alleviating the neuroinflammation in the LPS-induced PD model.

**DISCUSSION**

Mitochondrial dysfunction and oxidative stress have been recognized as major contributors to the death of DA neurons in PD. Although it has been increasingly clear that mitochondrial dysfunction in astrocytes triggers neuroinflammation underlying the development of PD [25, 26], sophisticated regulation of mitochondrial function within astrocytes remains largely unknown. In this study, by replicating mitochondrial dysfunction and oxidative stress in astrocytes, we identified that SLC1A5_var is required for mitochondrial energy metabolism. More importantly, genetic manipulation of SLC1A5_var further validated its beneficial effects on PD pathology.
SLC1A5 has been widely studied as a neutral amino acid transporter located in the plasma membrane of most mammalian cells [27], while intriguingly a novel variant of SLC1A5 (SLC1A5_var) was discovered recently [11]. The SLC1A5_var contains an N-terminal targeting signal for mitochondrial localization by which SLC1A5_var mediates mitochondrial glutamine metabolism in cancer [11]. Consistently, SLC1A5_var also acts as a mitochondrial glutamine transporter in astrocytes demonstrated by our findings.

To investigate the role of SLC1A5_var in PD progression, both MPTP/MPP⁺ and LPS models were performed by in vivo and in vitro experiments. The expression of SLC1A5_var was decreased significantly in mitochondria of astrocytes reflected by both of the two PD models. Thereafter, we studied the effect of SLC1A5_var on mitochondrial energy metabolism by overexpression and knockdown of SLC1A5_var in astrocytes. Our data prove that SLC1A5_var preserves mitochondrial oxidative phosphorylation and reduces ROS production thus maintaining mitochondrial morphology and function in astrocytes treated with LPS. Simultaneously, we observed that genetic manipulation of SLC1A5_var expression showed no impact on the baseline...
parameters. The phenomenon that overexpression or knockdown of certain gene does not affect baseline parameters exists not only in our current study but also in other literatures [28, 29], which may indicate a disease-associated function of this gene.

Mitochondrial dysfunction-driven neuroinflammation has been tested as the major accelerator of the development of PD [25, 30]. To explore the regulatory role of SLC1A5_var in astrocyte-derived neuroinflammation, LPS model was performed to examine astrocyte reactivity and neuroinflammation-associated signaling pathway in the context of overexpression and knockdown of SLC1A5_var. Intriguingly, we found that SLC1A5_var inhibits astrocyte polarization from resting state into pro-inflammatory A1 phenotype but not anti-inflammatory A2 phenotype. Mechanistically, SLC1A5_var inactivates TLR4-mediated p38/JNK/AKT signaling pathway thus reducing the production and release of pro-inflammatory cytokines in response to LPS treatment.

Owing to the powerful enhancement of mitochondrial function and inhibition of neuroinflammation, we explored the effect of SLC1A5_var on PD pathology replicated by both MPTP and LPS mouse models. As expected, overexpression of SLC1A5_var...
significantly improves PD motor symptoms and rescues the loss of DA neurons in the midbrain and striatum regions, suggesting potential therapeutic effect of SLC1A5 var on PD. Further experiments are warranted to gain a comprehensive view of SLC1A5 var-driven mitochondrial transport of other neutral amino acids excluding glutamine. Additionally, cell type-specific effect of SLC1A5 var should be explored in future experiments. Nevertheless, this is the first study of the function of SLC1A5 var in PD which makes substantial noteworthy contribution to mitochondria-targeting strategy for PD therapy.

DATA AVAILABILITY
The raw data supporting the conclusions of this article will be made available by the authors.

REFERENCES
1. Bloem BR, Okun MS, Klein C. Parkinson’s disease. Lancet. 2021;397:2284–303.
2. Le H, Zeng W, Zhang H, Li J, Wu X, Xie M, et al. Mean apparent propagator MRI is better than conventional diffusion tensor imaging for the evaluation of Parkinson’s disease: a prospective pilot study. Front Aging Neurosci. 2020;12:563595.
3. Juncara A. Insights into the pathogenesis of neurodegenerative diseases: focus on mitochondrial dysfunction and oxidative stress. Int J Mol Sci. 2021;22:11847.
4. Snow BJ, Rolfe FL, Lockhart MM, Frampton CM, O’Sullivan JD, Fung Y, et al. A double-blind, placebo-controlled study to assess the mitochondria-targeted antioxidant MitoQ as a disease-modifying therapy in Parkinson’s disease. Mov Disord. 2010;25:1670–4.
5. Wei Y, Lu M, Mei M, Wang H, Han Z, Chen M, et al. Pyridoxine induces glutathione synthesis via PKM2-mediated Nrf2 transactivation and confers neuroprotection. Nat Commun. 2020;11:941.
6. Zhu J, Hu Z, Han X, Wang D, Jiang Q, Ding J, et al. Dopamine D2 receptor restricts astrocytic NLRP3 inflammasome activation via enhancing the interaction of beta-arrestin2 and NLRP3. Cell Death Differ. 2018;25:2037–49.
7. Zhu J, Sun T, Zhang J, Liu Y, Wang D, Zhu H, et al. Drd2 biased agonist prevents neurodegeneration against NLRP3 inflammasome in Parkinson’s disease model via a beta-arrestin2-biased mechanism. Brain Behav Immun. 2020;90:79–81.
8. Ding ZB, Song LJ, Wang Q, Kumar G, Yan YQ, Ma CG. Astrocytes: a double-edged sword in neurodegenerative diseases. Neural Regen Res. 2021;16:1702–10.
9. Giovannoni F, Quintana FJ. The role of astrocytes in CNS inflammation. Trends Immunol. 2020;41:805–19.
10. Dauer W, Przedborski S. Parkinson’s disease: mechanisms and models. Neuron. 2003;39:889–905.
11. Yoo HC, Park SJ, Nam M, Kang J, Kim K, Yeo JH, et al. A variant of SLC1A5 is a mitochondrial glutamine transporter for metabolic reprogramming in cancer cells. Cell Metab. 2020;31:267–83, e212.
12. Han X, Sun S, Sun Y, Song Q, Zhu J, Song N, et al. Small molecule-driven NLRP3 inflammation inhibition via interplay between ubiquitination and autophagy: implications for Parkinson disease. Autophagy. 2019;15:1860–81.
13. Song N, Fang Y, Zhu H, Liu J, Jiang S, Sun S, et al. Kim6.2 is essential to maintain neurite features by modulating PM20D1-reduced mitochondrial ATP generation. Redox Biol. 2021;47:102168.
14. Maley F, Trimble RB, Tantoreno AL, Plummer TH Jr. Characterization of glycoproteins and their associated oligosaccharides through the use of endoglycosidases. Anal Biochem. 1989;180:195–204.
15. Lee H, Zandiarifini F, Zhang Y, Meena JK, Kim J, Zhuang L, et al. Energy-stress-mediated AMPK activation inhibits ferroptosis. Nat Cell Biol. 2020;22:225–34.
16. Willems PH, Rossignon R, Dieteren CE, Murphy MP, Koopman WJ. Redox homeostasis and mitochondrial dynamics. Cell Metab. 2015;22:207–18.
17. Pang Y, Wu D, Ma Y, Cao Y, Liu Q, Tang M, et al. Reactive oxygen species trigger NF-kappaB-mediated NLRP3 inflammasome activation in low-dose CdTe QDs exposure-induced hepatotoxicity. Redox Biol. 2017;4:102157.
18. Bang E, Kim DH, Chung HY. Protease-activated receptor 2 induces ROS-mediated inflammation through Akt-mediated NF-kappaB and FoxO6 modulation during skin photoaging. Redox Biol. 2021;44:102022.
19. Liu H, Han Y, Wang T, Zhang H, Xu Q, Yuan J, et al. Targeting microglia for therapy of Parkinson’s Disease by using biomimetic ultrasound nanoparticles. J Am Chem Soc. 2020;142:21730–42.
20. Lidderlow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schimerl L, et al. Neurotoxic reactive astrocytes are induced by activated microglia. Nature 2017;541:481–7.

Y. Liu et al.

21. Ciesielska A, Matyjek M, Kwiatkowska K. TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. Cell Mol Life Sci. 2021;78:1233–61.
22. Zhang C, Zhao M, Wang B, Su Z, Guo B, Qin L, et al. The Nrf2-NLRP3-caspase-1 axis mediates the neuroprotective effects of Celastrol in Parkinson’s disease. Redox Biol. 2021;47:102134.
23. Ramirez Al, de Hoz R, Salobrar-Garcia E, Salazar JJ, Rojas B, Ayop D, et al. The role of microglia in retinal neurodegeneration: Alzheimer’s Disease, Parkinson, and glaucoma. Front Aging Neurosci. 2017;9:214.
24. Han X, Xu T, Fang Q, Zhang H, Yue L, Hu G, et al. Quercetin hinders microglial activation to alleviate neurotoxicity via the interplay between NLRP3 inflammation and mitophagy. Redox Biol. 2021;44:102100.
25. Di Martino R, Stialli MJ, Sirabella R, Della Notte S, Borzacchiello D, Feliacci A, et al. Noc3-induced mitochondrial dysfunction in midbrain leads to neuroinflammation in strain of A53t-alpha-synuclein transgenic old mice. Int J Mol Sci. 2021;22:8177.
26. Sarkar S, Malovic E, Harischandran DS, Ngwa HA, Ghosh A, Hogan C, et al. Manganes exposure induces neuroinflammation by impairing mitochondrial dynamics in astrocytes. Neurotoxicology. 2018;64:204–18.
27. Scalise M, Pochini L, Console L, Lusio MA, Indiveri C. The human SLC1A5 (ASCT2) amino acid transporter: from function to structure and role in cell biology. Front Cell Dev Biol. 2018;6:96.
28. Zhao M, Wang B, Zhang C, Su Z, Guo B, Zhao Y, et al. The DJ1-Nrf2-STATING axis mediates the neuroprotective effects of Withaferin A in Parkinson’s disease. Cell Death Differ. 2021;28:2157–31.
29. Han X, Liu Y, Dai Y, Xu T, Hu Q, Yi X, et al. Neuronal SH2B1 attenuates apoptosis in an MPTP mouse model of Parkinson’s disease by promoting PLIN4 degradation. Redox Biol. 2022;52:103208.
30. Mani S, Sevanan M, Krishnamoorthy A, Sekar S. A systematic review of molecular approaches that link mitochondrial dysfunction and neuroinflammation in Parkinson’s disease. Neurol Sci. 2021;42:4459–69.

AUTHOR CONTRIBUTIONS
YL and LC designed and performed the experiments, analyzed the data, and wrote the manuscript. YS, ZK and TL performed experiments and analyzed the data. JD provides technical support. GH and ML conceived the study concept and designed the experiments. LC revised the manuscript. All authors discussed and contributed to the final manuscript.

FUNDING
This research was supported by grants from the National Key R&D Program of China (No.2021YFE020030), the National Natural Science Foundation of China (No. 81922066, No. 82003725, No. 82173797, No. 81991523), and the Natural Science Foundation of Nanjing University of Chinese Medicine (No. NZY2003725).

COMPETING INTERESTS
The authors declare no competing interests.

ETHICS APPROVAL
The animal study was reviewed and approved by Nanjing Medical University’s Institutional Animal Care and Use Committee (IACUC).

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41419-022-05399-z.

Correspondence and requests for materials should be addressed to Gang Hu or Ming Lu.

Reprints and permission information are available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Cell Death and Disease (2022) 13:946
