The circadian clock protein Rev-erbα provides neuroprotection and attenuates neuroinflammation against Parkinson’s disease via the microglial NLRP3 inflammasome

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Abstract

Background: Circadian disturbance is a common nonmotor complaint in Parkinson’s disease (PD). The molecular basis underlying circadian rhythm in PD is poorly understood. Neuroinflammation has been identified as a key contributor to PD pathology. In this study, we explored the potential link between the core clock molecule Rev-erbα and the microglia-mediated NLR family pyrin domain-containing 3 (NLRP3) inflammasome in PD pathogenesis.

Methods: We first examined the diurnal Rev-erbα rhythms and diurnal changes in microglia-mediated inflammatory cytokines expression in the SN of MPTP-induced PD mice. Further, we used BV2 cell to investigate the impacts of Rev-erbα on NLRP3 inflammasome and microglial polarization induced by 1-methyl-4-phenylpyridinium (MPP+) and αsyn pre-formed fibril. The role of Rev-erbα in regulating microglial activation via NF-κB and NLRP3 inflammasome pathway was then explored. Effects of SR9009 against NLRP3 inflammasome activation, microgliosis and nigrostriatal dopaminergic degeneration in the SN and striatum of MPTP-induced PD mice were studied in detail.

Results: BV2 cell-based experiments revealed the role of Rev-erbα in regulating microglial activation and polarization through the NF-κB and NLRP3 inflammasome pathways. Circadian oscillation of the core clock gene Rev-erbα in the substantia nigra (SN) disappeared in MPTP-induced PD mice, as well as diurnal changes in microglial morphology. The expression of inflammatory cytokines in SN of the MPTP-induced mice were significantly elevated. Furthermore, dopaminergic neurons loss in the nigrostriatal system were partially reversed by SR9009, a selective Rev-erbα agonist. In addition, SR9009 effectively reduced the MPTP-induced glial activation, microglial polarization and NLRP3 inflammasome activation in the nigrostriatal system.

Conclusions: These observations suggest that the circadian clock protein Rev-erbα plays an essential role in attenuating neuroinflammation in PD pathology, and provides a potential therapeutic target for PD treatment.

Keywords: Parkinson’s disease, Circadian rhythm, Rev-erbα, NLRP3, Neuroinflammation

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dopaminergic treatments [2, 3]. Circadian abnormalities in elderly adults are associated with an increased PD risk [4]. In generally, circadian disturbance often occurs decades before the onset of motor symptoms, making it a key factor in poor life quality [4, 5]. Studies have shown that the core clock genes of PD patients are disturbed during the early stage of the disease [6], and interference with the circadian rhythm or core clock genes in animal models of PD leads to exacerbated motor symptoms and increased loss of dopaminergic neurons [7, 8]. However, the causal mechanism underlying circadian disturbance in PD are still poorly understood.

Microglia-mediated neuroinflammation is considered as an important pathological feature of PD [9]. Microglial overactivation by harmful stimuli such as lipopolysaccharide (LPS), toxic misfolded proteins may shift these cells from anti-inflammatory state to a proinflammatory state, with release of proinflammatory cytokines such as Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), leading to further neuronal injury [10]. The NLR family pyrin domain-containing 3 (NLRP3) inflammasome, an important component of the innate immune system, has been increasingly associated with occurrence and progression of PD pathogenesis in PD models and PD patients [11–14]. Exome sequencing analysis of human NLRP3 gene variants revealed that multiple single-nucleotide polymorphisms were associated with a significantly reduced risk of PD development [15]. Moreover, inhibiting the NLRP3 inflammasome can reduce α-synuclein deposition, alleviate dopaminergic neuron damage and improve motor function [11, 16, 17].

There is growing evidence that circadian rhythm and neuroinflammation are closely linked. Studies have reported that astrocyte activation is autonomously regulated by the clock gene Bmal1, and behavioral circadian interference or manipulation of Bmal1 can induce astrocyte hyperplasia and dysfunction, along with oxidative stress, synaptic damage and increased inflammation in the CNS [18, 19]. Another study showed that the loss of Bmal1 or Rev-erba leads to the upregulation of the complement genes C4b and C3 in astrocytes, microglia activation, and increased synaptic phagocytosis [20]. In 2-month-old amyloid precursor protein knock-in (APP-KI) mice, the Clock/Bmal1-driven negative feedback loop of transcription in microglia was impaired, and activation of Rev-erba promoted the expression of inflammatory cytokines and cognitive impairment [21]. In contrast, some researches have shown that Rev-erba activation has an inhibitory effect on neuroinflammation [22, 23]. More interestingly, the link between the circadian clock Rev-erba and neuroinflammation in PD has been reported, as Rev-erba deficiency exacerbates 6-OHDA-induced dopaminergic neurodegeneration, possibly related to microglial proliferation in substantia nigra (SN) [24].

Since Rev-erba is closely associated with neuroinflammation in the CNS, and Rev-erba ablation exacerbates pathological changes in PD, we hypothesized that Rev-erba was an important factor that mediated the pathological progression of PD. To test this hypothesis, we first constructed the MPTP-induced PD model, and evaluated the expression patterns of Rev-erba, microglial activation and the expression of inflammatory cytokines. The effects of the Rev-erba-specific small molecule agonist SR9009 on dopaminergic neurons in MPTP mouse were also examined. Furthermore, we examined the effect of SR9009 on glial hyperplasia, microglial polarization and NLRP3 inflammasome activation. Lastly, the potential mechanism by which Rev-erba regulates neuroinflammation induced by 1-methyl-4-phenylpyridinium (MPP+) or asyn pre-formed fibril (asyn PFF) was further investigated in vitro.

Materials and methods

Animals

Two-month-old C57BL/6J male mice were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd., and housed on a 12/12 h light/dark cycle (light–dark, 07:00 light on, 19:00 light off) with food and water ad libitum. All animal experiments were carried out in accordance with the guidelines of the Animal Care and Use Committee of Maximum Efforts of Huazhong University of Science and Technology (HUST).

Experimental design

First, expression patterns of Rev-erba and morphological changes in microglia in the SN in control (n = 36) and MPTP mice (n = 36) were investigated using immunoblotting, immunofluorescence and RT-PCR. Mice in the experimental group received a single intraperitoneal injection of MPTP (25 mg/kg; Sigma) every day for 7 consecutive days. Control mice were given an equal amount of saline at the same time. Mice were harvested the day after the last MPTP injection, and euthanized at 4-h intervals throughout the day (Fig. 1A).

Then, the effects of SR9009 on motor function, dopaminergic neuron loss, glial hyperplasia, microglial polarization, the expression of inflammatory cytokines and NLRP3 inflammasome activation in the MPTP mouse model were examined. Forty mice were randomly assigned to four groups: saline group, SR9009 group, MPTP group, and MPTP + SR9009 group. SR9009 was injected intraperitoneally (100 mg/kg/day) for 7 consecutive days prior to MPTP administration and then continued along with MPTP treatment (Fig. 6A). SR9009 was administered daily at ZT8, followed by MPTP 1 h later.
Behavioral tests began the day after completing 14 days of SR9009 treatment, and mice were killed after behavioral test (day 18).

Behavioral assays

**Balance beam test**

The day after the completion of the 14-day SR9009 treatment, balance beam tests were examined. The balance beam was 0.5 cm wide, 1 m long and 40 cm high. A dark box was placed at the end of the balance beam for the mice to rest. The mice were retested three times in the afternoon with an interval of at least 15 min between tests. During the test, the time each mouse took to cross the balance beam was recorded.

**Pole test**

The day after the completion of the 14-day SR9009 treatment, pole test was examined. The animal was placed face-up near the top of a rough wooden pole (15 mm in diameter and 40 cm in height), and the time it took to
reach the floor was recorded. After 3 days of learning adaptation (3 training sessions per day), each mouse was finally administered 3 formal tests.

Rotarod test
The day after the completion of the 14-day SR9009 treatment, rotarod tests were examined. The accelerated rotarod test was performed over 4 consecutive days with 3 days of training and acclimatization. The rotarod device was used for 3 tests per day at 5–40 rpm for 5 min. There was a break of more than 30 min between each test. The time for each mouse to fall was recorded, and 300 s were recorded for those who did not fall after more than 5 min. Each mouse was tested three times, and the results were then averaged.

BV2 microglial cell culture and treatment
BV2 microglia were cultured in DMEM/high glucose medium containing 10% fetal bovine serum. The cells were kept at 37 °C in a humidified incubator with 5% CO2.

To induce inflammasome activation, BV2 cells were primed with MPP+ (200 uM, Sigma, USA) for 24 h. Alternatively, inflammasome activation was also induced by treatment with sonicated αsyn PFF (5 ug/ml, donated by Zhang’s Laboratory [25]) for 6 h. To examine the effects of Rev-erba, NF-κB and NLRP3 inflammasomes on microglia, cells were pretreated with SR9009 (MedChem Express, USA), SR8278 (MedChem Express, USA), JSH-23 (MedChem Express, USA), or MCC950 (MedChem Express, USA) for 1 h, and then treated with MPP+ or aggregated αsyn PFF for the indicated dose and time.

Quantitative real-time PCR (qRT-PCR)
Total RNA was extracted by using TRIzol reagent (Takara, Japan) according to the manufacturer’s instructions. After the RNA was reversely transcribed into cDNA, qRT-PCR was carried out. qRT-PCR was performed on a StepOne Plus system (Applied Biosystems) and analyzed using StepOne 2.3 software. The experimental operating conditions were 95 °C for 5 min, 95 °C for 5 s and 60 °C for 30 s for 40 cycles. ACTB served as an internal standard. The primers used for PCR are shown in Table 1.

Protein isolation and western blot
RIPA lysis buffer containing phosphorylase inhibitors, cocktails, and PMSF was used to digest tissue for protein extraction. Western blotting was performed as described previously [26]. The following primary antibodies were used: rabbit anti-NR1D1 (ab174309, Abcam), rabbit anti-NR1D1 (14506-1-AP, Proteintech), rabbit anti-tyrosine hydroxylase (TH) (25859-1-AP, Proteintech), rabbit anti-IBA1 (10904-1-AP, Proteintech), mouse anti-GFAP (60190-1-Ig, Proteintech), rabbit anti-NF-kB p65 (#8242, Cell Signaling Technology), rabbit anti-phospho-NF-kB p65 (#3033, Cell Signaling Technology), rabbit anti-NLRP3 (BA3677, BOSTER), mouse anti-ASC (sc-514414, Santa Cruz Biotechnology), rabbit anti-caspase-1 (22915-1-AP, Proteintech), rabbit anti-IL-1β (A16288, Abclone), rabbit anti-IL-6 (A0286, Abclone), mouse anti-actin (66009-1-Ig, Proteintech), mouse anti-TNF Alpha (60291-1-Ig, Proteintech), rabbit anti-iNOS (22226-1-AP, Proteintech), rabbit anti-IL-18 (10663-1-AP, Proteintech), mouse anti-Agarinase-1 (66129-1-Ig, Proteintech), rabbit anti-CD163 (16646-1-AP, Proteintech), rat anti-CD68 (MCA1957, BIO-RAD), Anti-Alpha-synuclein (ab138501, Abcam).

Immunofluorescence
Immunofluorescent stain was performed on paraffin-embedded sections or frozen sections of brain tissue. Paraffin sections were dewaxed and hydrated and underwent antigen retrieval, but frozen sections did not require this process. The sections were treated with 5% bovine serum albumin and sealed at room temperature for 30 min. Then, diluted primary antibody

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Table 1: Mouse primer sequences for quantitative real-time PCR (qPCR)

| Gene   | Forward (5’-3’ sequence) | Reverse (3’-5’ sequence)       |
|--------|--------------------------|---------------------------------|
| Rev-erba | TTTTCCGCCGAGAGCATCCA | ATCTCGGCAAGCATCCGTTG          |
| IL-1β  | AATGCCACCTTTGACAGTGATG | AGCTTCCTCCAGCAGCCACAAT        |
| IL-18  | TCAAGGTGGCAGAAGCCCCC | GGTCAAGAGGCTGTCCCTTAC         |
| IL-6   | ATCCAGTGCCCTCCCTCTGGAAG | TGAAGGCTCCGACTTTGAGAAGG        |
| TNF-a  | AGC AAA CCA CCA AGT GGA GGA | GCT GCC ACC ACT AGT TGG TTG T |
| Nlrp3  | ATTACCCGGCGAGAAAGG | TGCCGAGAAAGATCCACACACAG       |
| Arg-1  | TGCTCAGACTGACATCAAACAT | TCTAGCCTAGCGAAAGCCAAATGAC     |
| iNOS   | GCC AAA CCC AAG GTC TAG GTT | TCG CTC AAG TTC AGC TGG GT     |
| Actin  | GCCCTCACTGTCACCCTTCCA | AGCCATGCGCAATGTGCTCTT         |
was added to each section and incubated overnight in a wet box at 4 °C. After the excess primary antibody was washed away, the diluted fluorescent secondary antibody was added to the brain slice and incubated at 20–37 °C for 1 h in the dark. 4ʹ,6-diamino-2-phenylindole (DAPI) staining was performed for 5–10 min to label the nuclei. The sections were observed with an Olympus Automatic Scanning System SV120. The following primary antibodies were used: rabbit anti-IBA1 (NO. 019-19741, Wako), rabbit anti-iNOS (18985-1-AP, Proteintech), mouse anti-Arg-1 (66129-1-Ig, Proteintech), mouse anti-IBA1 (GB12105, Servicebio), and mouse anti-NLRP3 (AG-20B-0014-C100, Adipogen).

**Immunohistochemistry**

Immunohistochemical staining was performed on paraffin-embedded sections. After the sections were dewaxed and rehydrated, 3% hydrogen peroxide solution was used to inactivate endogenous peroxidase. The next steps were the same as those for immunofluorescence staining until HRP-labeled secondary antibodies were administered, followed by the diaminobenzidine reaction. Nuclei were stained with hematoxylin as needed. For immunohistochemical staining, the primary antibodies used were as follows: rabbit anti-TH (25859-1-AP, Proteintech), rabbit anti-IBA1 (NO. 019-19741, Wako), and mouse anti-GFAP (60190-1-Ig, Proteintech).

**Statistical analysis**

All data are presented as the means ± SEM and were analyzed using Prism 9 (GraphPad Software). Two-way ANOVA was used to analyze the differences in clock genes between the control group and MPTP group at different time points. Other data were analyzed using Student’s t test or one-way ANOVA with Dunnett’s post hoc test. A value of p < 0.05 was considered statistically significant throughout the study.

**Results**

**A aberrant diurnal Rev-erba rhythms in the SN of MPTP-induced PD mice**

The mRNA levels of Rev-erba in the SN were examined at different times within 24 h from the control and MPTP groups (Fig. 1A). As shown in control mice, Rev-erba showed diurnal changes throughout the day, with the highest expression at ZT6 and the lowest expression at ZT18 (Fig. 1B). Contrarily, the diurnal oscillation of Rev-erba in MPTP group disappeared (Fig. 1B; p < 0.05). Furthermore, the mean expression level of Rev-erba in the MPTP group was significantly decreased (Fig. 1C; p < 0.001). To further verify these findings, we examined changes in protein levels corresponding to Rev-erba mRNA expression. Similar results were found and mRNA expression of Rev-erba lost diurnal fluctuations (Fig. 1D, E; p < 0.01). In addition, we detected the mRNA level changes of core circadian clock molecule Bmal1 and Per2 in the SN within 24 h, and the amplitude of Bmal1 and Per2 were also slightly attenuated (Additional file 1: Fig. S1A and C; both p < 0.05).

**Diurnal changes in microglia-mediated inflammatory cytokines expression in the SN of MPTP-induced PD mice**

The microglia-mediated neuroinflammation plays a vital role in PD pathogenesis [27]. Therefore, we examined morphological changes in microglia at different times to determine the relationship between Rev-erba and microglial activation in the MPTP model. We first performed Iba1 immunofluorescent stain in SN at ZT6 and ZT18 time, which indicated the highest and lowest expression of Rev-erba, respectively. Compared to control group, the diurnal change in the SN was abrogated in MPTP mice, as microglial size was significantly increased at ZT6 and ZT18, with no difference in the two time points (Fig. 2A, B). In addition, the number of microglia in the SN in the MPTP group was also significantly increased (Fig. 2C, D). Next, we used qRT-PCR to examine microglia-associated inflammatory cytokines, including IL-1β, NLRP3, IL-6 and TNF-α (Fig. 2E–H). The results showed that the average mRNA expression levels of these genes in the SN of MPTP mice were significantly higher than those in control group, indicating an activation of microglia-mediated neuroinflammation in MPTP mice.

Thus, we hypothesized that the loss of daily Rev-erba fluctuations was the main factor contributing to the development of a proinflammatory microglial phenotype in MPTP mice, thereby mediating PD pathologic progression. Therefore, we further explored the effect and mechanism of Rev-erba on microglia activation in BV2 cell, a mouse microglia cell line.

**Rev-erba inhibited microglial activation induced by MPP+ and asyn PFF**

BV2 cells were pretreated with different concentrations of Rev-erba agonist SR9009 (2 uM, 5 uM, 10 uM) for 1 h, and then treated with MPP+. Compared with control group, western blot showed that treatment with MPP+ significantly activated NF-kB and NLRP3 inflammasome in BV2 cells and promoted the expression of proinflammatory factors such as iNOS, IL-1β, IL-6 and TNF-α (Fig. 3A–D). However, these inflammatory responses were dose-dependently suppressed by SR9009, with increased expression of the anti-inflammatory cytokines Arg-1 and CD163 (Fig. 3A–D), indicating that SR9009 promoted MPP+-induced microglial transition from M1 to M2 type. Furthermore, we assessed the effects of SR9009 on the inflammatory response induced by asyn PFF (Fig. 3E).
Consistently, SR9009 effectively inhibited αsyn PFF-induced activation of NF-κB and NLRP3 inflammasome pathways (Fig. 3F–H). Furthermore, SR9009 reduced the deposition of αsyn PFF in BV2 cells (Fig. 3H). We further examined the phagocytosis of microglia, which was increased upon addition of αsyn PFF but decreased upon administration of SR9009 (Fig. 3H), suggesting that Rev-erba may promote αsyn clearance by regulating microglial phagocytosis.

**Rev-erba regulates microglial activation via NF-κB and NLRP3 inflammasome pathway**

We further elucidated the potential mechanism by which Rev-erba regulates microglial activation through SR8278 (Rev-erba inhibitor) and JSH-23 (NF-κB inhibitor). As shown in Fig. 4A, p-NF-κB p65, NLRP3, ASC, IL-18, cleaved caspase-1 and IL-1β, as well as other inflammatory cytokines iNOS, IL-6, and TNF-α, were increased in the MPP⁺ group compared with the control group (Fig. 4B–D, all $p < 0.05$). In addition, the activation of microglia was more pronounced after SR8278 pretreatment while the inflammatory cytokines decreased and anti-inflammatory factors increased after JSH-23 pretreatment, suggesting that Rev-erba may alleviate MPP⁺-induced microglial activation and promote the transformation of microglia from M1 type to M2 type through the NF-κB pathway.

(See figure on next page.)

**Fig. 3** Activation of Rev-erba inhibited microglial activation induced by MPP⁺ and αsyn PFF. The representative western blot bands (A) and the statistical graph (B–D) of p-NF-κB p65, NLRP3, ASC, cleaved caspase-1, IL-1β, IL-18, IL-6, TNF-α, iNOS, Arg-1 and CD163 protein expressions. BV2 cells were pretreated with SR9009 (2 μM, 5 μM, 10 μM) for 1 h, then incubated with MPP⁺ for 24 h. The representative western blot bands (E) and the statistical graph (F–H) of p-NF-κB p65, NLRP3, ASC, cleaved caspase-1, IL-1β, CD68 and αsyn protein expressions. BV2 cells were pretreated with SR9009 (2 μM, 5 μM, 10 μM) for 1 h, then incubated with αsyn pre-formed-fibril for 6 h. The p-NF-κB p65 level was normalized to the total of NF-κB p65, and the rest protein levels were normalized to β-actin. Data were presented as mean ± SEM (n = 3). ($p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** or $p < 0.0001$ by One-way ANOVA test)
Fig. 3 (See legend on previous page.)
To further investigate whether Rev-erba modulates the inflammatory phenotype of microglia via the NLRP3 inflammasome, we pretreated BV2 cells with MCC950, a classic NLRP3 inflammasome inhibitor. Western blot results (Fig. 5A) showed that compared with the MPP$^+$ group, MCC950 administration significantly inhibited the activation of NLRP3 inflammasome, decreased the expression of proinflammatory factors (iNOS, IL-6 and TNF-α), and increased the expression of anti-inflammatory factors such as Arg-1 and CD163 (Fig. 5B, p > 0.05). In comparison with the SR9009 + MPP$^+$ group, the MCC950 + SR9009 + MPP$^+$ group also showed a similar trend, further suggesting that the NLRP3 inflammasome pathway plays a key role in the regulation of Rev-erba on neuroinflammation.

SR9009 improves motor function in MPTP-induced PD mice

Based on the above studies, our next step was to explore the regulatory effects of SR9009 on motor function, dopaminergic neuron loss and microglia-mediated neuroinflammation in the MPTP model (Fig. 6A). Behavioral tests were performed including pole test, rotarod test and balance beam test. In comparison with that of the control group, the pole test indicated a significantly prolonged time in MPTP group (p < 0.0001, Fig. 6B). Similar results were found in the balance beam test (p < 0.0001, Fig. 6C). It should be noted that Rev-erba agonist SR9009 partially reversed these effects (both p < 0.05, Fig. 6B, C). Similarly, SR9009 partially reversed MPTP-induced latency shortening on the rotational axis (p < 0.01, Fig. 6D). In addition, SR9009 was tested to be no influence on body weights of mice (Fig. 6E). These data indicate that SR9009 improves motor function of MPTP-induced mice.
SR9009 protects against nigrostriatal dopaminergic degeneration in the SN of MPTP-induced mice

To assess whether SR9009 affects MPTP-dependent neural loss, we evaluated nigrostriatal dopaminergic degeneration. Immunohistochemical analysis and western blotting showed that dopaminergic neurons in the striatum (all \(p < 0.05\), Fig. 7A–D) and SN (all \(p < 0.05\), Fig. 7E–H) in the MPTP group were severely damaged in comparison with those in the control group, but this effect was largely reversed by SR9009. The neuroprotective effect of SR9009 was further confirmed by Nissl staining (Fig. 7I), which demonstrated partial preservation of dopaminergic neurons in the SN in the MPTP + SR9009 group. These results suggest that SR9009 partially prevents MPTP-induced dopaminergic loss.

SR9009 ameliorates microgliosis and astrocytosis in the SN of MPTP-induced mice

Glial activation is an important pathological marker of PD neuroinflammation [28, 29]. Therefore, the effect of SR9009 on the activation of microglia and astrocytes in the SN was examined. Immunohistochemical staining showed that compared with that in the control group, the number of microglia and astrocytes in the SN in MPTP group was significantly increased, indicating an activated state (Fig. 8A–D, the control group vs. the MPTP group, both \(p < 0.05\)). Moreover, we found that glial activation was partly abolished in the SN in the MPTP + SR9009 group (Fig. 8A–D, the MPTP + SR9009 group vs. the MPTP group, both \(p < 0.05\)). In comparison with the control, MPTP strongly increased IBA1 and GFAP expression.
Fig. 6  SR9009 ameliorates behavioral impairments in MPTP-induced Mice. A Schematic representation of SR9009 intervention therapy. SR9009 was injected intraperitoneally (100 mg/kg/day) for 7 consecutive days prior to MPTP administration and was continued along with MPTP treatment. Behavioral tests began the day after completing 14 days of SR9009 treatment, and mice were killed after behavioral test (day 18). B Time spent in climbing of the pole test. C The time taken to cross the balance beam. D Latency to fall of the rotarod test. E Body-weight changes over time. n = 10 for each group. Data were presented as mean±SEM. (*p < 0.05, **p < 0.01, or ****p < 0.0001 by One-way ANOVA test)

Fig. 7  SR9009 protects against dopaminergic neurons degeneration in MPTP-induced mice. The representative immunohistochemical staining of TH (A) and the OD of TH staining (B) in the striatum. Scale bar, 1 mm. Representative western blot bands (C) and the statistical graph (D) of TH in the striatum. The representative immunohistochemical staining of TH (E) and the number of TH positive neurons (F) in the SN. Scale bar, 500 μm. Representative western blot bands (G) and the statistical graph (H) of TH in the SN. I The representative Nissl staining for neurons in the SN. Scale bars, 200 μm for the top row and 50 μm for the bottom row. The TH protein level was normalized to β-actin. n = 3–4 for each group. Data were presented as mean±SEM. (*p < 0.05, **p < 0.01, or ***p < 0.001 by one-way ANOVA test)
Compared with those in the MPTP group, the protein levels of IBA1 and GFAP in the SN in the MPTP + SR9009 group were decreased (Fig. 8E–G, both \( p < 0.05 \)). These results indicate that SR9009 partially ameliorates MPTP-induced glial cell overactivation in the SN of MPTP-induced mice.

**SR9009 ameliorates microgliosis and astrocytosis in the striatum of MPTP-induced mice**

In addition to SN area, the striatum is also an important part of the dopaminergic system. Immunohistochemical staining showed that compared with that in the control group, microglia and astrocytes in the striatum in
the MPTP group was significantly activated (Fig. 9A–D, the control group vs. the MPTP group, both p < 0.05). However, the glial activation was partly abolished in the striatum in the MPTP + SR9009 group (Fig. 9A–D, the MPTP + SR9009 group vs. the MPTP group, both p < 0.05). These results further indicate that SR9009 can partially ameliorate MPTP-induced glial cell overactivation in the nigro striatum of MPTP-induced mice.

SR9009 reverses the phenotypic polarization of microglia in the SN of MPTP-induced mice

Next, we sought to determine whether SR9009 could modulate the phenotypic polarization of microglia. To examine phenotypic polarization, immunofluorescence stain was performed against iNOS (M1 phenotypic marker) and Arg-1 (M2 phenotypic marker). INOS+ IBA1+ cells and Arg+ IBA1+ cells were observed, as shown in Fig. 10A and D. The amount of iNOS co-localized with IBA1 in the MPTP + SR9009 group was less than that in the MPTP group (Fig. 10A), while the amount of Arg-1 co-localized with IBA1 in the MPTP + SR9009 group was higher than that in MPTP group (Fig. 10D).

Similar to these results, the mRNA levels of iNOS and Arg-1 showed the same trend (Fig. 10B, C, both p < 0.05). In addition, the PCR results showed that SR9009 significantly reduced the microglia-associated proinflammatory factors TNF-α and IL-6 in MPTP group (Fig. 10E, F, both p < 0.05). These results demonstrated that SR9009 promoted transformation from the M1 phenotype to the M2 phenotype in MPTP-induced mice.

SR9009 partially inhibits NLRP3 inflammasome activation in the SN of MPTP-induced mice

Since there is increasing evidence that the NLRP3 inflammasome is involved in PD progression [12, 30], we next investigated the effect of SR9009 on NLRP3 inflammasome activation in the MPTP model. The effects of SR9009 on NF-κB, which is an essential priming effector for inflammasome activation and the core components of the NLRP3 inflammasome, were examined by western blotting (Fig. 11A–F). In comparison with that in control mice, the expression of p-NF-κB p65, NLRP3, cleaved caspase-1 and ASC in the SN of MPTP-induced mice was significantly increased (all p < 0.05), suggesting the activation of the NLRP3 inflammasome. However, SR9009 effectively reversed the increase in p-NF-κB p65, NLRP3, ASC and cleaved caspase-1 in the SN (MPTP + SR9009 group vs. MPTP group, all p < 0.05). In addition, the PCR data also showed that SR9009 effectively reversed the production of the inflammatory cytokines IL-1β and IL-18 in the MPTP-induced mice (Fig. 11G, H, the MPTP + SR9009 group vs. the MPTP group, all p < 0.05). Immunofluorescence colocalization analysis also showed that NLRP3 was mainly expressed in microglia in the MPTP group, and this effect was partially reversed in the MPTP + SR9009 group (Fig. 11I, J). These data suggest that MPTP-induced NLRP3 inflammasome activation in the SN can be partially blocked by SR9009.

Discussion

In this study, we showed for the first time that the normal daily fluctuations of Rev-erbα were absent in subacute MPTP-induced PD model. In addition, the diurnal variations in microglial immunoactivity in the SN disappeared, suggesting that decreased expression of Rev-erbα may be responsible for microglial activation and elevated neuroinflammation. Then we demonstrated that Rev-erbα can regulate microglial activation and polarization through the NF-κB and NLRP3 inflammasome pathways in vitro. Moreover, our results further revealed that activation of Rev-erbα by the small molecule agonist SR9009 could improve motor function, ameliorate dopaminergic neurons loss, inhibit gliosis and microglial polarization by regulating NLRP3 inflammasome activation in vivo. In summary, our results indicate that Rev-erbα is involved in the regulation of neuroinflammation in the pathological process of PD and is a potential new target for PD treatment (Fig. 12).

A great deal of researches indicates that circadian disturbance may not only be a concomitant symptom, but also a cause of neurodegenerative disease [31, 32]. Our previous study also confirmed that most PD patients were associated with disturbed sleep–wake cycles, indicating circadian rhythm disruption [33]. Rev-erbα, a known circadian modulator playing a pivotal role in the cyclic Bmal1 and CLOCK transcription [34, 35], has been implicated in a variety of physiological and pathophysiological processes, such as metabolism [36], cancers [37, 38], and inflammatory responses [39]. Moreover, Rev-erbα seems to be a double-edged sword in neurodegenerative disease, exacerbating amyloid-β (Aβ) deposition in Alzheimer’s disease while suppressing neuroinflammation in a model of epilepsy [40, 41], but its role in PD pathology is poorly understood. In the present study, we are the first to demonstrate that Rev-erbα is reduced and loses diurnal fluctuations in the SN in the MPTP model.

It is well known that microglial activation is an important part of the pathogenesis of PD [42]. Our previous studies have shown that microglia play a key role in the delivery of α-synuclein via exosomes [26], and activated microglia facilitate the transport of exosomal α-synuclein through Toll-like receptor 2 [43]. Microglia-mediated neuroinflammation in PD has been the focus of our attention [44]. In this study, we observed changes in microglial volume over time in the control group, which was significantly higher at ZT18 than at ZT6.
Fig. 9 SR9009 inhibits microgliosis and astrocytosis in striatum of MPTP-induced mice. **A** The representative immunohistochemistry staining of IBA1 in the striatum. Scale bars, 1 mm for the top row and 100 μm for the bottom row. **B** The representative immunohistochemistry staining of GFAP in the striatum. Scale bars, 1 mm for the top row and 100 μm for the bottom row. **C, D** The statistical graph of IBA1 and GFAP in the striatum. (*p < 0.05, **p < 0.01, or ***p < 0.001 by One-way ANOVA test)
Fig. 10 SR9009 reversed the phenotypic polarization of microglia in the SN of MPTP-induced mice. 

A. Representative double-immunofluorescent staining of IBA1 (green) and iNOS (red) in the SN. Scale bar, 50 μm. 

B. Representative double-immunofluorescent staining of IBA1 (green) and iNOS (Arg-1) in the SN. Scale bar, 50 μm. The real-time PCR results of iNOS (B), Arg-1 (C), TNF-α (E) and IL-6 (F) in the SN. n = 3–4 for each group. Data were presented as mean ± SEM. (*p < 0.05, **p < 0.01, or ***p < 0.001 by One-way ANOVA test).
(corresponding to the times of the lowest and highest Rev-erbα expression, respectively). However, this diurnal change was abrogated in the MPTP group, and microglial volume was significantly increased at ZT6 and ZT18. This finding is similar to that of a study showing that the deletion of Rev-erbα caused microglia to lose time-of-day changes and switch to a proinflammatory state [22], suggesting that Rev-erbα may mediate neuroinflammation in the MPTP model.

The NLRP3 inflammasome, an important component of inflammation, is a complex of multiple proteins, including NLRP3, ASC and caspase-1, which are expressed abundantly in microglia [17, 45]. When stimulated, the NLRP3 complex leads to caspase-1 activation, promotes the maturation and secretion of the proinflammatory cytokines IL-1β and IL-18, and induces the shift of microglia into an anti-inflammatory state [46], while inhibition the NLRP3 inflammasome suppresses microglial polarization to a proinflammatory state [47]. The link between Rev-erbα and microglial activation was investigated in BV2 cell. We found that activation of Rev-erbα could attenuate microglial activation and promote the transformation of microglia from proinflammatory M1 to anti-inflammatory M2 state through NF-κB and microglial polarization to a proinflammatory state [47]. The link between Rev-erbα and microglial activation was investigated in BV2 cell. We found that activation of Rev-erbα could attenuate microglial activation and promote the transformation of microglia from proinflammatory M1 to anti-inflammatory M2 state through NF-κB and microglial polarization to a proinflammatory state [47]. The link between Rev-erbα and microglial activation was investigated in BV2 cell. We found that activation of Rev-erbα could attenuate microglial activation and promote the transformation of microglia from proinflammatory M1 to anti-inflammatory M2 state through NF-κB and microglial polarization to a proinflammatory state [47]. The link between Rev-erbα and microglial activation was investigated in BV2 cell. We found that activation of Rev-erbα could attenuate microglial activation and promote the transformation of microglia from proinflammatory M1 to anti-inflammatory M2 state through NF-κB and microglial polarization to a proinflammatory state [47]. The link between Rev-erbα and microglial activation was investigated in BV2 cell. We found that activation of Rev-erbα could attenuate microglial activation and promote the transformation of microglia from proinflammatory M1 to anti-inflammatory M2 state through NF-κB and microglial polarization to a proinflammatory state [47].

Fig. 11 SR9009 suppresses NLRP3 inflammasome activation in the SN of MPTP-induced mice. The representative western blot bands (A) and the statistical graph (B) of p-NF-κB p65 and NF-κB p65 in the SN. The representative western blot bands (E) and the statistical graph (C–D, F) of NLRP3, ASC and cleaved-caspase-1 in the SN. The real-time PCR results of IL-1β (G) and IL-18 (H) in the SN. I Representative double-immunofluorescent staining of IBA1 (red) and NLRP3 (green) in the SN and the statistical graph (J) of IBA1+ NLRP3+/IBA1+ cells. Scale bar, 50 μm. n = 3–4 for each group. The p-NF-κB p65 level was normalized to the total of NF-κB p65, and the rest protein levels were normalized to β-actin. Data were presented as mean ± SEM. (*p < 0.05, **p < 0.01, or ***p < 0.001 by One-way ANOVA test).
Fig. 11  (See legend on previous page.)
Conclusions
In summary, abnormal expression of Rev-erbα is a key factor in regulating neuroinflammation in the SN in the MPTP model, and restoring stable circadian rhythm may be an effective strategy for delaying or even stopping the progression of neurodegenerative disease.

Abbreviations
PD: Parkinson’s disease; MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridyl; SN: Substantia nigra; SNpc: Substantia nigra pars compacta; CNS: Central nervous system; NLRP3: NLR Family Pyrin Domain Containing 3; APP-KI: Amyloid precursor protein knock-in; RT-PCR: Quantitative real-time PCR; ASC: Apoptosis-associated speck-like protein; TH: Tyrosine hydroxylase; HUST: Huazhong University of Science and Technology; IL-1β: Interleukin-1β; TNF-α: Tumor necrosis factor-α.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12974-022-02494-y.

Acknowledgements
The authors would like to thank the Nature Research Editing Service for the English language editing and the BioRender for the drawing of graphic abstracts.

Author contributions
LK was involved in the entire study, including the experimental design, experimental implementation, data analysis, and article writing. YX critically revised the draft before submission. XC and YS contributed to data analysis. CH, FW, JH, SY, JW, YL, QZ, WZ, JH and NX contributed to the experimental implementation. The entire research process was conducted under the direction of TW and YX. All authors read and approved the final manuscript.

Funding
This work is supported by grants 81671260 and 81974201 from the National Natural Science Foundation of China (to TW), grants 2017YFC1310200 and 2016YFC1306000 from the National Key Plan for Scientific Research and Development of China (to TW) and grants 2019HSA02 from Chinese Sleep Research Society Hansoh Project (to JH).

Availability of data and materials
The data used during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
Not applicable.
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