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Microglial activation contributes to cognitive impairments in rotenone-induced mouse Parkinson’s disease model

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Abstract

Background: Cognitive decline occurs frequently in Parkinson’s disease (PD), which greatly decreases the quality of life of patients. However, the mechanisms remain to be investigated. Neuroinflammation mediated by overactivated microglia is a common pathological feature in multiple neurological disorders, including PD. This study is designed to explore the role of microglia in cognitive deficits by using a rotenone-induced mouse PD model.

Methods: To evaluate the role of microglia in rotenone-induced cognitive deficits, PLX3397, an inhibitor of colony-stimulating factor 1 receptor, and minocycline, a widely used antibiotic, were used to deplete or inactivate microglia, respectively. Cognitive performance of mice among groups was detected by Morris water maze, objective recognition, and passive avoidance tests. Neurodegeneration, synaptic loss, α-synuclein phosphorylation, glial activation, and apoptosis were determined by immunohistochemistry and Western blot or immunofluorescence staining. The gene expression of inflammatory factors and lipid peroxidation were further explored by using RT-PCR and ELISA kits, respectively.

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Background
Traditionally, Parkinson’s disease (PD) is considered a movement disorder with progressive dopaminergic neurodegeneration and Lewy body formation in the substantia nigra [1]. Current evidence suggests that the pathological processes of PD extend beyond the nigrostriatal system and degeneration of neuronal populations in other brain regions has also been noted [2]. Furthermore, patients with PD display not only motor deficits but also a lot of non-motor symptoms [3]. In the clinic, cognitive impairments are frequently observed in PD patients and are often inadequately treated, which gradually becomes an important determinant of life quality of patients [4, 5]. Currently, the mechanisms of cognitive deficits in PD remain unclear.

Microglia-mediated neuroinflammation has been reported to contribute to the pathogenesis of PD [6]. In addition to nigrostriatal regions, activated microglia and accumulation of inflammatory factors are also noted in the hippocampus and cortex, two critical brain regions that may contribute to cognitive decline in PD [7]. Negative correlations between microglial activation and hippocampal volume or cerebral glucose metabolic rate within hippocampus have been observed in PD patients with dementia [8]. Furthermore, Menza et al. found higher levels of proinflammatory factors, such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) in the plasma of PD patients than that of individuals without PD [9]. A subsequent study demonstrated that increased TNFα and TNF receptor 1 contents in the plasma of PD patients are associated with poor cognitive test scores [9]. Similarly, in Alzheimer’s disease (AD) patients, a positive correlation between microglial activation and the clinical dementia rating score has also been observed [10]. Additionally, activated microglia have been shown to induce neurotoxic reactive astroglia [11]. Neurotoxic astrogial activation is also considered to contribute to neuronal damage and subsequent cognitive impairment in animal models of neurodegeneration [12]. Consistently, blocking microglia-mediated conversion of astroglia to a neurotoxic phenotype by IL-10 or fluorocitrate alleviated LPS-induced depressive-like behavior and cognitive dysfunction in mice [13]. These results suggest that neuroinflammation mediated by microglia may contribute to cognitive decline in neurodegenerative disease.

To provide direct experimental evidence linking neuroinflammation and cognitive impairments in PD, a mouse PD model induced by rotenone was administered PLX3397 or minocycline to deplete brain microglia or inhibit microglial activation, respectively. Cognitive performance as well as neuronal loss, synaptic degeneration, and α-synuclein Ser129-phosphorylation were determined. Then, the underlying mechanisms of how microglial activation contributes to cognitive impairments were further explored. Our results suggested that microglial activation damaged cognitive performance in rotenone-induced mouse PD model by exacerbating neuroinflammation, oxidative stress, and neuronal apoptosis.

Methods
Reagents
Rotenone was purchased from Sigma-Aldrich, Inc. (R8875, St. Louis, MO, USA). PLX3397 (S7818) and minocycline (S4226) were purchased from Selleck (Shanghai, China). The antibody against Neu-N was purchased from EMD Millipore (MAB377, Temecula, CA, USA). The anti-ionized calcium binding adaptor molecule-1 (Iba-1) and anti-glial fibrillary acidic protein (Continued from previous page)
Morris water maze test
Morris water maze (MWM) test was performed as described previously [19, 20]. The test included the navigation test and the spatial probe test. In the spatial navigation test, a circular platform was placed in the middle of one quadrant (1 cm below the water surface). In one trial, mice were randomly placed in one of the quadrants and were allowed to swim until they found the platform or for a maximum 90 s. Mice were gently guided onto the platform if they could not find the platform within 90 s, and their latencies were recorded as 90 s. All mice were given a break of 5 min on the platform between trials. There were four trials per day for each mouse, and the mice were tested for 4 days. A video camera was used to record the swimming paths of mice. Different parameters for evaluating learning performance, such as latency for escape and traveled distance were analyzed by using the tracking software (NoldusEtho Vision system, version 5, Everett, WA, USA).

On the fifth day, the spatial probe test for spatial memory function was performed. Briefly, the platform was removed and mice were permitted to navigate in the pool freely for 60 s. Then, different parameters, including the time of mice spent in different quadrants, the latency to initially cross the position where the platform was located and the total number of platform crossings were recorded and analyzed by using the tracking software (NoldusEtho Vision system, version 5).

Novel objective recognition
Novel objective recognition (NOR) test was conducted based on a previous protocol [21]. In brief, mice were trained over 3 days (3 times per day) to discriminate a novel object from a familiar one. On the first day, mice were put in an empty chamber and permitted to move freely. On the following day, mice were allowed to explore two the same objects placed in opposite corners of the chamber for 5 min. On the last day, mice were put back in the chamber and were permitted to explore a familiar object (the same object as day 2) and a new object with different colors and shapes from the familiar object. After each test, 70% ethanol was used to thoroughly clean the chamber and objects. The time the mice spent exploring familiar and novel objects was recorded, and the recognition index was calculated by using the following formula.

\[
\text{Recognition index} = \frac{\text{Time spent exploring novel object}}{\text{Time spent exploring both novel and familiar objects}} \times 100\% 
\]

Passive avoidance test
The passive avoidance test was done based on previous protocol [22]. In brief, mice were put in a chamber that was separated into light and dark compartments (the
same size) by a guillotine door. On the first day, the guillotine door between the light and dark compartments was opened and mice were permitted to move freely for 5 min in both compartments of the chamber. The next day, mice were put in the light compartment. After 60 s, the guillotine door was opened, and mice were permitted to move to the dark compartment freely. Once the mice entered, the guillotine door was closed and an electric shock was given (0.3 mA, 5 min). This test was repeated with 10-min intervals until the latency to enter the dark compartment reached 120 s. On the last day, mice were placed in the light compartment. Sixty seconds later, the guillotine door was opened and the latency of mice to enter the dark compartment (step-through latency) and the number of total entrances (step-through number) were recorded.

Immunohistochemistry and immunofluorescence staining
Mice in each group were perfused transcardially with PBS, followed by 4% paraformaldehyde and then the brains were collected. Brain samples were postfixed with 4% paraformaldehyde at 4 °C for 48 h, and then transferred to 30% sucrose in PBS before sectioning. Free-floating coronal sections (30 μm) containing hippocampal and cortical regions were used for immunohistochemistry and immunofluorescence staining as described previously [23, 24]. Briefly, brain sections were blocked in 0.25% Triton/PBS containing 4% goat serum for 2 h and then incubated with anti-PSD-95, anti-Neu-N, anti-Iba-1, anti-GFAP or anti-Ser129-phosphorylated α-synuclein antibodies in PBS containing 0.1% Triton X-100 at 4 °C for an additional 24 h. Then, the sections were washed three times prior to incubation with an appropriate biotinylated or Alexa-594-conjugated secondary antibody. Immunohistochemical staining was visualized by using 3,3'-diaminobenzidine. Digital images were acquired (×10 and ×40 magnification for immunohistochemistry and immunofluorescence staining, respectively) under an Olympus microscope (BX51; Olympus, Tokyo, Japan) using an attached digital microscope camera (DP72; Olympus).

The densities of Iba-1, PSD-95, and GFAP immunostaining from two to three brain sections with 120-μm intervals from each mouse in each group were measured by using ImageJ software [24–26]. Briefly, the image was first converted into the grayscale picture, and the background was adjusted before the quantifying area was selected for the measurement of the total pixels. The relative density of the staining was compared based on the density of the total pixels of a certain brain region (total pixels/area). The quantification of the staining was corrected for background staining by subtracting the pixels without primary antibody.

Automated counting assessment of neurodegeneration
The number of Neu-N+ neurons in mice among the groups was quantified by the automated counting method in ImageJ software [19, 24].

TUNEL assay
In Situ Cell Death Detection Kit was used to perform TUNEL assays in free-floating coronal sections (30 μm) containing hippocampal and cortical regions [22, 27]. TUNEL+ cells were observed using a fluorescence microscope (×40 magnification). The mean number of TUNEL+ cells in each mouse was obtained by counting three coronal sections with 120-μm intervals by using ImageJ software.

Western blot analysis
For Western blot analysis, mice were perfused transcardially with PBS to remove the blood. The brains were collected and hippocampal, and the cortical brain regions were dissected immediately on ice. Tissue samples including the hippocampus and cortex were homogenized by using ice-cold RIPA buffer with a protease inhibitor mixture and then centrifuged at 10,000×g. After 10 min of centrifugation, protein concentrations were measured in the collected supernatants by using a BCA protein assay kit. Samples containing equal amounts of protein from each group were separated by 4–12% SDS-PAGE, and then, immunoblot analysis was performed using anti-PSD-95, anti-Neu-N, anti-α-synuclein, and anti-α-synuclein (phospho S129) antibodies at 4 °C for 24 h. After washing three times with PBST, the membranes were incubated with appropriate HRP-linked anti-rabbit or mouse IgG. The signal was detected by ECL reagents, and relative density of blots was quantified using ImageJ software.

Real-time PCR analysis
For RT-PCR analysis, mice in each group (n = 6) were perfused transcardially with PBS to remove the blood. The hippocampal and cortical brain regions were dissected immediately on ice and then transferred to liquid nitrogen, followed by a −80 °C freezer. RNA was isolated from the hippocampal and cortical samples by using TRIzol reagent. Quantitative analysis of RNA was performed by using Nanodrop spectrophotometer. A total 1-μg RNA from each sample was used for complementary DNA (cDNA) synthesis using MuLV reverse transcriptase and oligo dT primers according to previous reports [28, 29]. The reaction conditions were set as 25 °C for 5 min, 42 °C for 60 min, and 70 °C for 15 min. SYBR Premix Ex TaqTM II and a Takara Thermal Cycler Dice™ Real Time System were subsequently used for real-time PCR detection based on the manufacturer’s protocols. The primers were designed with Vector NTI.
software and validated for efficacy through melting curve analyses. The sequences of the primers were the follows: GAPDH F (5’-TCTAACGGACAGTCAGGC-3’; 300 nM), GAPDH R (5’-GACCTACGATCTAAGGCC-3’; 300 nM), TNFα F (5’-AACCCCTCAACTACGACGATCC-3’; 300 nM), TNFα R (5’-GCCTTGTGCTTGTTTGCT-3’; 300 nM), IL-1β F (5’-CTGGTGTGTGAGCTCATTAA-3’; 300 nM), IL-1β R (5’-CCTACGGAGGCAGCTT-3’; 300 nM), iNOS F(5’-CTGCCCGCCCTGCTC-3’; 300 nM), and iNOS R (5’-TGGGAGGGGTCGTAAATGC-3’; 300 nM). The following PCR conditions were used: 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s for 40 cycles (for a final reaction volume of 25 μl). All samples were tested in duplicate and normalized to GAPDH using the 2^ΔΔCt method. Fold changes for each treatment were normalized and are shown as percentages of the control.

**GSH and MDA assay**

The tissue samples of mice perfused with PBS only were homogenized in ice-cold lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, and protease inhibitor mixture. After 10 min of centrifugation at 10,000×g (4 °C), the contents of GSH and MDA in the collected supernatant were measured by using commercial kits based on the protocol provided by the manufacturer [30].

**Statistical analysis**

Data were expressed as the mean ± SEM. Except for the MWM test, comparison of means among two or more groups was conducted using one-way (one parameter) or two-way ANOVA (two parameters). Subsequently, Tukey’s post hoc test was used for pairwise comparisons between means once ANOVA showed significant differences. The MWM test data were analyzed using repeated measures ANOVA. Fisher’s PLSD tests were used for comparing group means only when a significant F value was observed in the overall ANOVA. A p value less than 0.05 was considered statistically significant.

**Results**

**Rotenone dose-dependently impairs cognitive capacity of mice**

To determine whether rotenone-treated mice displayed cognitive dysfunction, the MWM, novel objective recognition, and passive avoidance tests were performed after 3 weeks of rotenone exposure (Fig. 1a). In MWZ test, mice in the control group exhibited normal spatial learning ability as indicated by the time-dependent decreases in escape latency and traveled distance (Fig. 1b). We did not find a significant difference in escape latency and traveled distance between 0.75 mg/kg rotenone-treated mice and vehicle controls, although an increasing trend was observed (Fig. 1b). However, 1.5 mg/kg rotenone significantly elevated the escape latency as the rotenone-treated mice showed a longer time to locate the platform than the control mice (Fig. 1b). Consistently, 1.5 mg/kg rotenone also markedly increased the traveled distance of mice (Fig. 1c), indicating impaired spatial learning function. No significant difference was observed in swimming speed for the mice (Fig. 1d), which excluded the possibility that the differences in MWM performance among the groups were due to locomotor deficits. The spatial probe test was subsequently performed to evaluate the spatial memory capacity of mice. Mice that received 1.5 mg/kg rotenone displayed increased latency for the first platform crossing and a reduced total number of platform crossings and percentage of time spent in the target quadrant compared with control group (Fig. 1e–g), indicating impaired memory ability. However, mice in the 0.75 mg/kg rotenone and control group failed to show significant differences in the spatial probe test (Fig. 1e–g).

To further confirm the impaired cognitive capacity of mice, the novel objective recognition and passive avoidance tests were subsequently performed. Consistently, rotenone at 1.5 mg/kg significantly reduced the recognition index and step-through latency and increased the error times in step-through compared to vehicle controls (Fig. 1h–j). No significant difference in recognition index, step-through latency, and number was observed between 0.75 mg/kg rotenone-treated mice and control group. These results suggest that 1.5 mg/kg rotenone-induced PD model mice displayed impaired cognitive performance.

**Rotenone dose-dependently induces neuronal damage**

Cognitive dysfunction in PD is associated with abnormal neuronal damage, especially in the hippocampal and cortical regions [31, 32]. To investigate whether rotenone-induced cognitive deficits were associated with hippocampal and cortical neuron damage, neuronal cells were immunostained with neuron-specific anti-Neu-N antibody. As illustrated in Fig. 2a–e, 1.5 mg/kg rotenone treatment significantly reduced the number of Neu-N+ neurons in the hippocampal and cortical regions of mice compared with the vehicle control treatment. Additionally, synapses are critical for neuron-neuron communication and cognitive function. Reduced expression of key synaptic proteins, such as PSD-95, is significantly correlated with cognitive decline in neurodegenerative disorders [19, 20]. In agreement with neuronal loss, immunohistochemical analysis revealed a reduced density of hippocampal and cortical PSD-95 immunostaining in 1.5 mg/kg rotenone-treated mice (Fig. 2a–e and Supplementary Fig. 1).
To further confirm the damage to neurons in rotenone-treated mice, the levels of Neu-N and PSD95 were determined. As shown in Fig. 2f–h, rotenone at 1.5 mg/kg reduced the protein levels of Neu-N and PSD-95 in the hippocampal and cortical regions of mice. The comparison of Neu-N+ cell number, PSD95 immunostaining density and expression of Neu-N and PSD95 between the 0.75 mg/kg rotenone-treated mice and vehicle-treated controls showed no significant differences (Fig. 2a–h).

In addition to neuronal damage, α-synuclein accumulation and phosphorylation, especially at the Ser129 site, also contribute to cognitive deficits in PD [33]. Immunofluorescence staining showed elevated phosphorylation of α-synuclein at Ser129 in the hippocampus and cortex of 1.5 mg/kg rotenone-treated mice compared with control group (Fig. 2i). Fluorescence density analysis of Ser129-phosphorylated α-synuclein immunostaining confirmed this observation (Fig. 2j).

Microglial activation precedes neuronal damage in rotenone-treated mice
To investigate whether microglial activation contributes to rotenone-induced cognitive deficits in mice, microglia were immunostained with a microglial marker, Iba-1, after 3 weeks of rotenone exposure. As seen in Fig. 3a, microglial cells in the hippocampus and cortex of 1.5 mg/kg rotenone-injected mice exhibited hypertrophic morphology and increased Iba-1 expression, indicating microglial activation. Quantitative analysis revealed 45.4% and 39.7% increases Iba-1 density in the hippocampus and cortex, respectively, of 1.5 mg/kg rotenone-treated mice compared with controls (Fig. 3b, c). No
significant microglial activation was observed in 0.75 mg/kg rotenone-injected mice (Fig. 3a–c).

To determine the time sequence of microglial activation and neuronal damage in rotenone-treated mice, a time course study was performed at the indicated time points of rotenone intoxication (Fig. 3d). The results showed that microglial activation in the hippocampal and cortical regions of mice occurred as early as 1 week after rotenone exposure and was sustained up to 3 weeks (Fig. 3e). Quantitative analysis of Iba-1 immunostaining density revealed 21.1%, 32.6%, and 54.9% increases in the hippocampus and 22.8%, 42.0%, and 65.3% increases in the cortex after 1, 2, and 3 weeks of rotenone injection, respectively (Fig. 3f, g). In contrast, neuronal damage as evidenced by decreased Neu-N⁺ cell number and PSD95 immunostaining density was observed after 2 weeks of rotenone injection (Fig. 3e–g). These results suggested that rotenone-induced microglial activation precedes neurodegeneration.

**PLX3397 and minocycline attenuate rotenone-induced cognitive deficits in mice**

To determine whether microglial activation contributes to cognitive impairment, rotenone-injected mice were administered PLX3397 or minocycline. PLX3397 is an inhibitor of colony-stimulating factor 1 receptor (CSF1R) and can efficiently reduce the number of microglia in the brains of mice [17]. Minocycline is a widely used antibiotic that could suppress neuroinflammation in a variety of rodent models of neurodegenerative diseases.
Consistent with a previous report, PLX3397 treatment significantly reduced the number of microglia in the hippocampus and cortex when compared to vehicle-treated mice (Supplementary Fig 2A-C). Minocycline also efficiently blocked rotenone-induced microglial activation in the hippocampus and cortex of mice (Supplementary Fig 2D-F). Subsequently, cognitive performance was measured in mice treated with rotenone with or without PLX3397 and minocycline. In agreement with the results of Fig. 1, impaired learning and memory performance of mice was detected in MWM test after rotenone (1.5 mg/kg) treatment. Interestingly, PLX3397 and minocycline significantly ameliorated rotenone-induced learning and memory impairments by showing reduced escape latency, traveled distance, and latency for first platform crossing as well as the recovered platform crossing number and percentage of time spent in target quadrant in combined PLX3397 or minocycline and rotenone-treated mice compared with rotenone alone group (Fig. 4a–c and e–g). There were no significant differences in swimming speed among the groups, which excluded the possibility that the protective effects of PLX3397 and minocycline-afforded were caused by recovered motor activity (Fig. 4d).

To further confirm the protective effects of PLX3397 and minocycline against rotenone-induced cognitive deficits, novel objective recognition and passive avoidance tests were performed. In agreement with that of MWZ test, PLX3397, and minocycline significantly elevated the recognition index of rotenone-treated mice in the novel objective recognition test (Fig. 4h). Minocycline also elevated the step-through latency and decreased the error times of step-through in rotenone-treated mice (Fig. 4i). Although reduced error times in step-through and increased step-through latency were observed in PLX3397 and rotenone cotreated mice compared with rotenone...
alone group, the difference did not reach statistical significance (Fig. 4i). These results suggested that microglial activation contributed to rotenone-induced cognitive deficits in mice.

**PLX3397 and minocycline attenuate rotenone-induced neuronal damage and Ser129 phosphorylation of α-synuclein**

The neuroprotective effects of PLX3397 and minocycline were subsequently determined. Consistent with their attenuation of cognitive decline, PLX3397 and minocycline also mitigated rotenone-induced neuronal damage in mice. Restored Neu-N$^+$ cell numbers and increased PSD95 staining densities were observed in both the hippocampal and cortical regions of PLX3397 or minocycline and rotenone cotreated mice compared with rotenone alone group (Fig. 5a–e and Supplementary Fig. 3), indicating neuroprotection. This conclusion was further supported by analysis of the expression of Neu-N and PSD95 (Fig. 5f–h).

In addition to neuroprotection, PLX3397 and minocycline also mitigated the Ser129-phosphorylation of α-synuclein by showing reduced expression of Ser129-phosphorylated α-synuclein in PLX3397 or minocycline and rotenone cotreated mice compared with rotenone alone group (Fig. 5i, j).

**PLX3397 and minocycline attenuate rotenone-induced astroglial activation and proinflammatory factor production**

A recent study revealed that microglial activation can induce astrocytes into a neurotoxic activation status in
multiple neurological disorders [11]. To determine whether microglial depletion by PLX3397 or inactivation by minocycline could suppress astroglial activation, immunohistochemistry using anti-GFAP antibody, was performed in mice. As seen in Fig. 6a, astroglial cells in the hippocampus and cortex of rotenone-treated mice displayed hypertrophied morphology and intensified GFAP immunostaining, which indicated astroglial activation. Densitometric analysis of GFAP staining further supported this conclusion (Fig. 6b, c). Interestingly, astrocytes in PLX3397 or minocycline and rotenone-coated treated mice showed normal morphology and reduced expression of GFAP compared with that of rotenone alone group, suggesting that the activation of astrocytes is blocked by PLX3397 or minocycline (Fig. 6a–c).

The production of proinflammatory factors is a common mechanism by which activated glial cells damage neurons in the central nervous system. To determine whether microglial depletion by PLX3397 or inactivation by minocycline could dampen the production of proinflammatory factors, the gene expression levels of iNOS, TNFα, and IL-1β were measured. As seen in Fig. 6d, e, rotenone injection increased the gene transcript levels of iNOS, TNFα, and IL-1β in both the hippocampus and cortex of mice, and these increases were significantly reduced by PLX3397 and minocycline.

**PLX3397 and minocycline attenuate rotenone-induced oxidative stress**

Oxidative stress usually coexists with neuroinflammation. To determine whether microglial depletion by PLX3397 or inactivation by minocycline could dampen oxidative stress, the contents of GSH and MDA were measured. In agreement with reduced production of
proinflammatory factors, rotenone-induced lipid peroxidation was also mitigated by PLX3397 and minocycline by showing decreased MDA levels (Fig. 7a, c) and recovered GSH contents (Fig. 7b, d) in PLX3397 or minocycline and rotenone-cotreated mice compared with rotenone alone group.

PLX3397 and minocycline attenuate rotenone-induced neuronal apoptosis

The effects of PLX3397 and minocycline on neuronal apoptosis were further determined in rotenone-treated mice. TUNEL staining revealed a high number of TUNEL+ cells in the hippocampal and cortical regions of rotenone-treated mice compared with those of control group (Fig. 8a, b), indicating that apoptosis occurs after rotenone exposure. Immunofluorescence staining with antibodies against MAP2, Iba-1, and GFAP antibodies combined with TUNEL labeling was further performed to observe the distribution of apoptosis among neurons, microglia, and astrocytes, respectively. Results showed that rotenone-induced increase in TUNEL staining in the hippocampus and cortex was mainly concentrated in MAP2+ cells, indicating neuronal apoptosis (data not shown). Interestingly, PLX3397 and minocycline treatment markedly decreased rotenone-induced neuronal apoptosis by showing reduced number and percentage of TUNEL+ cells in PLX3397 or minocycline and rotenone co-treated mice compared with rotenone alone group (Fig. 8a, b).

It is well documented that apoptosis is controlled by multiple proteins, such as the Bcl-2 family and caspase [35]. Bcl-xL and Bax, two key members of the Bcl-2 family, display anti-apoptotic and proapoptotic functions, respectively [36]. Caspase-3, a member of the caspase family, is the main executor of apoptosis [35]. The effects of PLX3397 and minocycline on the expression of these apoptotic regulators were determined by using Western blotting. Consistent with the increase in TUNEL staining, rotenone treatment caused an increase of active caspase-3 and Bax expression and a decrease in
Bcl-xL expression in mice (Fig. 8c–e). In contrast, mice treated with combined PLX3397 or minocycline and rotenone displayed reduced the expression of caspase-3 and Bax as well as elevated expression of Bcl-xL compared with those of mice treated with rotenone alone (Fig. 8c–e), indicating attenuated apoptosis.

**Discussion**

In this study, we provided strong evidence to support that microglial activation contributed to cognitive decline in a mouse PD model generated by rotenone. Four salient features were observed: (1) rotenone-induced microglial activation preceded neurodegeneration in mice; (2) microglial depletion by PLX3397 and inactivation by minocycline significantly ameliorated rotenone-induced cognitive deficits and neuronal damage in mice; (3) PLX3397 and minocycline suppressed astroglial activation, production of proinflammatory factors, and oxidative stress in rotenone-treated mice; and (4) PLX3397 and minocycline abrogated rotenone-induced neuronal apoptosis in mice.

PD is traditionally recognized as a movement disorder with progressive loss of nigral dopaminergic neurons [37]. However, accumulating evidence suggests that PD is a heterogeneous multisystem disorder and patients with PD display both motor deficits and multiple nonmotor symptoms [19, 38]. Cognitive impairments are one of the most common nonmotor symptoms in patients with PD. Clinical studies have revealed that 25~46.8% of newly diagnosed patients exhibit mild cognitive deficits, and up to 80% exhibit dementia in the late stage of the disease [39, 40]. A postmortem study in PD patients indicates that limbic and cortical neuron damage and Lewy body pathology correlate with cognitive decline [41]. However, studies in widely used parkinsonian animal model induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) generated mixed results, which greatly hampered the progress of mechanical studies on cognitive deficits. Although cognitive decline was reported in MPTP-induced mouse model in previous reports [42, 43], Fifel et al. found no significant decrease in cognitive alterations in either acute or chronic MPTP-treated mice [44]. Our preliminary data also showed that MPTP failed to damage learning and memory capacity in mice (data not shown), although significant dopaminergic neurodegeneration was reported in this model [45, 46]. The rotenone-induced mouse PD model is also widely used. However, mixed results regarding the effects of rotenone on cognition in mice have been generated. Alabi et al. reported that daily doses of rotenone (2.5 mg/kg, i.p) for 4-weeks impaired memory in mice as shown by a significantly impaired...
performance in the Y-maze test compared with that of vehicle controls [47]. In contrast, Jia and colleagues found that intragastric delivery of rotenone (5 mg/kg) for 3 months improved spatial learning and memory abilities of mice in the MWM test, although spatial memory ability was impaired at 1 month after treatment [48]. In this study, we further investigated the effects of rotenone on cognition in mice by using a subchronic dosing regimen. Results showed that rotenone dose-dependently decreased novel object recognition, passive avoidance, and MWM performance in mice after 3 weeks of treatment. Furthermore, elevated neuronal damage, loss of synapses, and Ser129-phosphorylation of α-synuclein were also detected in rotenone-injected mice, which was consistent with observations in PD patients.

The mechanisms underlying rotenone-induced cognitive dysfunction remain unclear. Inflammation has long been found to be inversely correlated with cognitive decline [20, 49]. Increasing evidence suggested that activated microglia is a key causative factor in inflammation-mediated neurodegeneration and behavioral deficits [50]. Dysregulated microglial activation is reported to be able to increase pathological protein aggregation and impair synaptic pruning and neuron plasticity in key brain regions subserving cognition [51, 52]. In the present study, activated microglia were detected in the hippocampus and cortex of rotenone-induced mouse PD model. A time experiment revealed that microglial activation induced by rotenone preceded neuronal damage and α-synuclein pathology in mice. Furthermore, microglial depletion by PLX3397 or inactivation by minocycline significantly reduced neuronal damage in the hippocampus and cortex of mice treated with rotenone. Consistently, neuroprotective effects of both PLX3397 and minocycline against rotenone-induced dopaminergic neurodegeneration were also observed in mice (data not shown). More importantly, PLX3397 and minocycline-affected neuroprotection were associated with improved cognitive performance in rotenone-treated mice, indicating an important role of activated microglia in mediating cognitive dysfunction. In agreement with our findings, Cope and colleagues recently found that microglial activation plays an active role in obesity-associated cognitive decline since blocking microglial activation by minocycline prevented loss of dendritic spines and cognitive decline in obese mice [53]. Depletion of microglia by CSF1R inhibitors, such as PLX3397 and PLX5622, was also associated with improved cognitive performance in
experimental mouse models of AD [54], intracerebral hemorrhage [55], and irradiation-induced memory deficits [56]. Microrglia can become overactivated in response to certain injuries and release a variety of cytotoxic factors that cause neurotoxicity [11]. In addition, activated microglia have recently been shown to be able to induce astrocytes into a neurotoxic A1 status by releasing TNFα, IL-1α, and C1q to amplify neuronal damage [11]. Proinflammatory factors and reactive oxygen species (ROS) are considered to be critical to mediate neuroinflammatory damage since neutralization of proinflammatory factors or inhibition of ROS during neuroinflammation showed potent neuroprotection in a variety of neurodegeneration models [57]. Consistent with these findings, in this study, elevated astrogial activation, production of proinflammatory cytokines, and oxidative stress were observed in rotenone-treated mice. Furthermore, we demonstrated that microglial depletion by PLX3397 and inactivation by minocycline suppressed these toxic events and was accompanied by decreased apoptosis of neurons and expression of cleaved caspase-3 and Bax as well as elevated expression of Bcl-xL. Our findings suggested that microglial activation contributed to cognitive deficits and neuronal apoptosis through neurotoxic astroglial activation, neuroinflammation, and oxidative damage. Consistent with our findings, endotoxin LPS, an inflammatory stimulator, has been reported to induce cognitive deficits in mice through neuroinflammation and neuronal apoptosis [21]. Inhibition of inflammatory cytokines, oxidative stress, and neuronal apoptosis in the hippocampus also ameliorated cognitive deficits in mouse model of AD [58], vascular dementia, and sepsis [59]. Notably, a pharmacological manipulation method (PLX3397 and minocycline) was used to block microglial activation in the current study, and we could not exclude the possibility that PLX3397 and minocycline might be have direct effects on astrogial activation, oxidative stress, or neuronal apoptosis. Further study focusing on this issue should be performed in the future.

It is interesting to compare the similar protective effects of minocycline and PLX3397 in this study. Minocycline is a semisynthetic tetracycline derivative that displays potent anti-inflammatory effects. Multiple studies have revealed that minocycline can dampen proinflammatory microglial activation (M1) and might simultaneously promote microglial M2 polarization in neuropathological conditions [60–62]. In contrast, PLX3397 is reported to directly deplete microglia in an apoptosis-dependent manner in the brains of mice [17]. Although microglial elimination by PLX3397 is not accompanied by an inflammatory response in the brain, PLX3397-treated mice exhibited robust reductions in the expression of many inflammatory genes, including TNF-α and other cytokines, in response to LPS [17]. Consistently, Liang et al. reported that PLX3397 treatment significantly reduced the number of M1 phenotype-like microglia and production of proinflammatory factors in a mouse model of high-fat-diet (HFD)-induced obesity [63]. Our preliminary data showed reduced expression of iNOS, a marker of M1 microglia, and elevated expression of the M2 microglial marker Arg-1 in the brains of mice treated with combined PLX3397 and rotenone compared with those of mice treated with rotenone alone (Supplementary Fig. 5), suggesting that PLX3397 might prefer to target/eliminate M1-trended microglia in neuroinflammatory or pathological conditions. Thus, we believe that the equal potency of PLX3397 and minocycline in dampening M1 microglial activation by microglial depletion and inhibition, respectively, might be one of the potential reasons for similar neuroprotective effects of these two compounds.

Conclusions

Our results reveal an important role of microglial activation in rotenone-induced cognitive deficits in mice through astrogial activation, neuroinflammation, oxidative stress, and apoptosis. Our study adds to strong experimental evidence for the connection between microglial activation and cognitive decline in PD and provides a new therapeutic target for the treatment of the nonmotor symptoms in patients in the future.

Supplementary Information

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Authors’ contributions

D.Z. performed the experiments. S.L and L.H. guided the experiments and gave critical comments for the research. L.H. wrote the original draft and analyzed data. L.J., Z.R., B.P., and X.Z. helped to perform the experiments. J.Z. gave critical comments for the research. R.Z. gave comments and provided reagents. Q.W. designed the research and edited the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

All animal procedures and their care were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Dalian Medical University.

Consent for publication

Not applicable.

Competing interests

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