Rapamycin protects dopaminergic neurons by suppressing TLR2 mediated neuroinflammation and enhancing autophagy in rotenone-induced PD mice

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Abstract

Microglia inflammation and autophagy play important roles in the initiation and progression of Parkinson's disease (PD). Toll-like receptor 2 (TLR2) activation is closely related to microglial activation and enhances the cell-to-cell propagation of a-synuclein pathology. Clinical and preclinical samples also observed the impaired autophagy-lysosomal systems. As such, therapeutic strategies that inhibit TLR2 and/or modulate autophagy may be effective for PD treatment. As an autophagy inducer, rapamycin is initially used in the treatment of a variety of tumors by inhibiting mTORC1. Recently, rapamycin was reported to exert the anti-inflammatory effects in a variety of inflammatory diseases. Here, we employed the rotenone-induced Parkinson's disease mouse model and peptidoglycan (PGN) cultured BV-2 cells to investigate whether rapamycin (Rapa) can act on PD by influencing TLR2 activation in vitro and in vivo experiments. The autophagy flux and the expression of inflammation related pathways downstream of TLR2 were examined. Our results showed that rapamycin increased the expression of LC3β to clear the accumulation of α-synuclein S129 phosphorylated(P-α-syn) and improved motor dysfunction in rotenone-induced PD mice, moreover, rapamycin inhibited the expression of TLR2 in microglia, further reduced nuclear translocation of nuclear factor of activated T cells, cytoplasmic 2(NFATc2) and downregulated gene expression of tumor necrosis factor-α (TNF-α) in vitro and in vivo. These results demonstrate that rapamycin exerts therapeutic effects via enhancing autophagy and suppressing the expression of TLR2 in rotenone-induced PD mice.

1. Introduction

Parkinson's disease (PD) is the most common movement disorder affecting more than 6 million people worldwide[1]. Its main pathological characteristics are the loss of dopaminergic neurons in substantia nigra and the formation of Lewy bodies, which main components are α-synuclein aggregates[2, 3]. Increasing evidence shows the critical role of impairment of autophagy and immune responses of microglia in neurodegenerative processes in many central nervous system diseases, include PD[4–6]. However, the reality is that the clinical treatment of PD is only symptomatic, so new therapies of modify the disease to delay the progress of PD are urgently needed.

Autophagy is known to clear misfolded protein[7]. The autophagy-lysosome system was reported to be impaired in postmortem PD patient tissue and PD models, suggesting a candidate role of autophagy in the development and treatment of PD[8, 9]. Studies reported that the mammalian rapamycin target protein (mTOR) pathway has negative regulatory effects on autophagy[10–12]. Toll-like receptor 2 (TLR2)/mTOR pathway has been explored in many disease, TLR2 activation will increase the phosphorylation of mTOR, thereby promoting protein synthesis and inhibit autophagy[13–18]. TLR2 is an important member of the pattern recognition receptor protein family, and recognition includes exogenous pathogens and endogenous pathological proteins. Accumulating evidence from human studies implicated that TLR2 is upregulated in microglia activated in the brain of PD patients and that TLR2 levels are associated with the accumulation of pathological α-synuclein[19]. Selective inhibition of TLR2 reduced glial inflammation,

decreased α-syn transmission, and protected dopaminergic neurons[20–23]. Unfortunately, the application of specific inhibitors of TLR2 in the treatment of PD only stays in the animal experiment staged by now.

Rapamycin (Rapa, an autophagy inducer through inhibiting mTORC1) as a safe and effective immunosuppressant, has been used in patients after renal transplantation since the 1970s. Previous research showed rapamycin protected dopaminergic neurons from rotenone induced cell death in mice primary mesencephalic cell culture, but the molecular mechanisms were not clear yet[24]. Here, we found that rapamycin could strengthen autophagy and inhibit TLR2 expression, further reducing nuclear translocation of NFATc2 and downregulates TNF-α to ameliorate neuroinflammation, prevent dopaminergic neuronal loss and improve motor function in rotenone induced mouse model of PD.

2. Materials and Methods

2.1 Reagents

Rotenone (R8875), peptidoglycan (69554) and DMSO (C6164) were purchased from Sigma-Aldrich. Rapamycin was purchased from MCE (HY-10219, USA). Fetal bovine serum (FBS) was purchased from Invigentech (A6901, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Cytiva (SH30022.01, USA). Penicillin–streptomycin solution (CR2102024) and trypsin (G4004) were purchased from Servicebio (Wuhan, China). DAPI (ab104139) was purchased from Abcam.

2.2 Animals and experimental design

Male C57BL/6J mice aged 10 weeks (22–24g) were purchased from Huaxing Laboratory Animal Research Center, Zhengzhou. The mice were housed four per cage and kept on a standard environmental condition (temperature 22 ± 2°C, humidity 50–60%,12h light/dark cycle) with free access to food and water. All the procedures were performed following the guide to care and use laboratory animals. The animal experiments were approved by the Medical Ethics Committee of the Second Affiliated Hospital of Zhengzhou University (No. 2022142).

The schematic illustration of the animal experimental design is shown as Fig. 3a. A total of 48 mice were randomly and equally divided into four groups: the control group, the rotenone group (i.p. rotenone 0.75mg/kg, every other day for 4 consecutive weeks)[25], the rotenone plus rapamycin group (i.p. rotenone 0.75mg/kg, every other day for 4 consecutive weeks, o.g. rapamycin 2mg/kg, every other day for 4 consecutive weeks begin at week 5)[26–29] and the rapamycin group (o.g. rapamycin 2mg/kg, every other day for 4 consecutive weeks begin at week 5). Rotenone was freshly prepared in a solution of 1% DMSO + 5%Tween-20 (diluted with PBS), rapamycin was diluted in the same way. Control mice received an equivalent volume of vehicle (1% DMSO + 5%Tween-20 diluted with PBS). These mice were weighed weekly for 8 weeks. Behavioral tests were performed at the last four days. All mice were sacrificed after behavioral tests for further analysis.

2.3 Cell culture and treatment

BV-2 murine microglia cells were obtained from Mingjing Biology, shanghai. Cells were cultured in DMEM/high glucose medium contain 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL) and maintained at standard condition (37℃, 5% CO_2). Rapamycin (15nM)[24, 30, 31] and/or peptidoglycan(12.5μM)[32] were added to the cells coculture for 24 hours, then samples were collected for further analysis after washed with PBS for 3 times.

2.4 Behavioral tests

Rotarod and open field tests were employed to evaluate Rotenone-induced motor deficits in mice.

2.4.1 Rotarod test

Mice were placed on the rotarod, and then the rod revolved at a speed of 5–15 rpm /min for 5 minutes to train them. After the training, mice were allowed to rest for 1 hour, then they were placed on the rotating rod revolved at a gradual increasing speed of 5–40 rpm/min for 3 minutes, then 40 rpm/min for 2 minutes. The experiment was ended when the animal slid off the spinner or grabbed the spinner and spun more than 2 turns and the time was defined as latency. Each mouse tested twice a day with an interval of 1 h for 3 consecutive days.

2.4.2 Open field test

Locomotor activities were evaluated in the open-field apparatus consisting of a light-yellow plastic board (45 × 45 cm) surrounded by light yellow walls (40 cm in height). each mouse was gently placed in the center of the open field area to explore it for the next 5 minutes. The movements were recorded by a camera tracking system (Smart5. System, the Netherlands). Total distances moved were calculated.

2.5 Western blotting.

The midbrain and caudate putamen tissues of mice were dissected in cold PBS. All the samples were stored at -80℃ refrigerator. The tissues and cells were respectively homogenized in cytoplasmic lysate containing 1mM sucrose, 100nM DTT, 5mM EGTA, 10mM Tris-HCl (pH 7.4), 5mM $MgCl_2$, protease inhibitors and phosphatase inhibitor cocktails. The homogenate was centrifuged at 2,500 × rpm for 10 min at 4°C. The supernatant obtained after centrifugation used to detect the expression of cytoplasmic and membrane proteins. The precipitate was dissolved in nucleus lysate containing cytoplasmic lysate,0.1% TritonX-100, 1.5% SDS. The mixture was further ultrasonic crushed on ice for detect the expression of nucleus proteins. Equal amounts of proteins were electrophoresed in 10% or 12% SDS-PAGE and transferred onto a Poly-vinylidene fluoride (PVDF) membrane. We blocked the membranes with 5% BSA at room temperature for 2 hours and then probed them with the following primary antibodies: anti-P-α-synuclein(Ser129 phosphorylated, 1:2000, Abcam, #ab51253), Anti-LC3B (1:1000, Abcam, #ab48394), Anti-Tyrosine hydroxylase (TH, 1:2000, Proteintech, #25859-1-AP), anti-TNF-α (1:2000, Proteintech, #60291-1-Ig), anti-TLR2 (1:1500, Invitrogen, #14-9922-82), anti-NFATc2 (1:1000, CST, #5861), anti-P-NF-κB p65(Ser536 phosphorylated, 1:500, #WL02169) and H3/β-actin (1:5000, ab1791, Abcam;1:2000, GB11001, Serviebio,) at 4°C overnight. The membranes were then washed 6 minutes for

four times with TBST and probed with horseradish peroxidase-linked anti-rabbit IgG (1:5000, Abbkine) antibody or horseradish peroxidase-linked anti-mouse IgG (1:5000, Abbkine) antibody at room temperature for 2 hours. The blot signals were detected by ECL reagents (biosharp, BL520A). Band intensities were quantified using ImageJ software (USA), All blots were compared to H3 or β-actin.

2.6 Real-time PCR.

Total RNA was extracted from midbrain/cells using the Universal RNA kit (Cofitt, LBD9515) following the manufacturer's protocol. Then, the RNA was reverse transcribed with the High speed-strand cDNA Synthesis Plus Kit (Cofitt, LBQ7513). Real-time PCR was carried out in QuantStudio sequence detection system with the SYBR qPCR Master Mix obtained from Vazyme (Q511-00) based on the manufacturer's protocols. Use the glyceraldehyde-3-phosphate dehy-drogenase (GAPDH) gene as the internal control. All datas were normalization of GAPDH using the $2^{-\Delta\Delta Ct}$ method.

The following primer sequences were used:

α-synuclein: forward: 5'-TGGCTTTGTCAAGAAGGACCAGATG-3'

reverse: 5'-CCACAGGCATGTCTTCCAGGATTC-3'

TLR2: forward: 5'-CTCCCAGATGCTTCGTTGTTCCC-3'

reverse: 5'-GTTGTCGCCTGCTTCCAGAGTC-3'

TNF-α: forward: 5'-CACCACGCTCTTCTGTCTACTGAAC-3'

reverse: 5'-TGACGGCAGAGAGGAGGTTGAC-3'

GAPDH: forward: 5'-GGTTGTCTCCTGCGACTTCA-3'

reverse: 5'-TGGTCCAGGGTTTCTTACTCC-3'.

2.7 Immunofluorescence.

After anesthesia, mice were perfused transcardially with normal saline(NS) and then with cold 4% paraformaldehyde (PFA). The brains were then kept in 4% PFA at 4°C for 4 hours. The brains were dehydrated with 20% sucrose for 1 day then dehydrated with 30% sucrose for an additional 2 days. 25μm coronal sections were cut from the the brain in a cryotome and blocked with 0.3% Triton-100/PBS containing 10% goat serum for 1 hour. In the case of cell samples (BV-2), these were washed 3 times with cold PBS and then fixed with 4% paraformaldehyde for 20 minutes. Samples were permeated in PBS containing 1% Triton X-100 for 10 minutes then blocked with 5% goat serum in PBS for 1 hour at room temperature. After that, probed them with the following primary antibodies: anti-P-α-syn (1:500), Anti-TH (1:500), anti-TNF-α (1:500), anti-TLR2 (1:250), anti-NFATc2 (1:50), anti-glial fibrillary acidic protein (GFAP, 1:500, Proteintech, #16825-1-AP) and anti-ionized calcium binding adaptor molecule-1 (Iba-1,1;500, Affinity, #DF6442) at 25°C overnight. The next day, the samples were washed 5 minutes for four times

with PBS and further probed with Cy3- or 488-labeled secondary antibodies (all 1:300, Servicebio) for 2 hours at room temperature. All immunofluorescence-stained images were examined using a Leica DMI4000 fluorescence microscope and captured with a DFC365FX camera. The amounts of TH immunoreactive neurons and Iba-1 immunoreactive microglial in the region of SN were calculated under a microscope (100×). ImageJ software to measure the fluorescence intensity of each target protein in the SN/striatum region or each group of cells.

3. Statistics.

Statistics were performed using GraphPad Prism v8.0.2. Values are expressed as mean ± SD. At least three independent experiments were calculated for each analysis. The normally distributed and equal variance values were analyzed by Shapiro–Wilk and Bartlett's tests, respectively. Differences between groups were analyzed using one-way ANOVA. A *P*-value no more than 0.05 was considered statistically significant.

4. Results

4.1 Rapamycin improved rotenone induced-motor deficits and rescued the loss of TH + neuron in SN and their fibers to striatum in rotenone treated mice.

Epidemiological and biochemical studies suggested that chronic systemic inhibition of complex I by the lipophile pesticide rotenone leads to highly selective nigrostriatal dopaminergic degeneration, simulating the clinical and pathological features of PD[33, 34]. In our study, we performed rotenone-induced PD in mice and investigated the effect of rapamycin on PD treatment. As shown in Fig. 3a, rotenone was intraperitoneally administered at the dose of 0.75 mg/ kg for 4 weeks to induce PD-like symptom, followed by the administration of rapamycin (2 mg/ kg) for another 4 weeks in treatment groups. Consistent with early reports, mice exposed to rotenone showed significant motor deficits, as demonstrated by the reduced time spent on the rod and decreased distances travelled in the field as compared to those in the control groups (Fig. 1a). Whereas rapamycin treatment could strikingly improve rotenone-induced motor deficits by prolonging the duration on the rod and increasing the total distance(Fig. 1a). Taken together, rotenone administration led to PD-like behavior, which can be effectively alleviated by rapamycin treatment.

Dopaminergic neurons are crucial in the development of PD. The effects of rapamycin on dopaminergic neurons were also examined. The expression of TH, an enzyme involved in dopamine production, was observed by western blot and immunofluorescence staining. We found that the number of TH-positive neurons in the midbrain and TH-positive fibers in the striatum were dramatically decreased in rotenone treated mice, which was reversed by administration of 2 mg/kg rapamycin (Fig. 1b-g). These findings

suggest that rapamycin may abolishes rotenone-induced motor deficits by preventing the loss of TH-positive neurons and fibers, at least partially.

4.2 Rapamycin decreased the aggregation of P-α-syn and elevated the depressed autophagy induced by rotenone.

To confirm whether rapamycin has an action on the aggregation of P-α-syn, a protein implicated in the pathogenesis of PD by enhancing autophagy in rotenone treated mice, we further detected the expression of P-α-syn and LC3-β/LC3-α ratio in midbrain of mice. As expected, western blotting analysis showed that compared with the control group, rotenone increased the level of brain P-α-syn deposition in mice, both the control group and the rotenone group showed low expression of LC3β and low LC3-β/LC3-α ratio. Under normal physiological conditions, autophagy was at a low steady state, but the level of autophagy did not increase accordingly to clear the misfolded protein when the level of P-α-syn increased in the midbrain of mice after rotenone treatment (Fig. 2c, 2d), while rapamycin reversed this change (Fig. 2a-d). Interestingly, the mRNA level of α-syn in midbrain samples with rotenone injection showed no difference as compared to the either group (Fig. 2g), which was consistent with the previous report. These results indicate that the Rapamycin treatment strengthening autophagy and decrease the aggregation of P-α-syn induced by rotenone.

4.3 Rapamycin prevented TLR2/ NFATc2/TNF-α pathway in microglia of rotenone-induced PD mice.

TLR2 has been found in activated microglia of Parkinson's disease brains, and was linked to the accumulation of pathological α-synuclein.[19] Studies show that activation of TLR2 will increase the phosphorylation of mTOR, thereby promoting protein synthesis and inhibit autophagy[13–18]. To investigate whether rapamycin can affect the activation of TLR2 to play wider roles in mice model of PD, we evaluated the expression of TLR2 and its downstream pathways. As expected, RT-PCR, western blot and immunofluorescence analysis showed that the expression of TLR2 in SN was significantly increased in rotenone group compared to the control group, and rapamycin treatment inhibited the change (Fig. 3b-3f). In addition, double labeling immunofluorescence analysis showed that TLR2 colocalized with IBA1-labeled microglia cells, but not with TH-positive neurons in SN (Fig. 3d,3e). Notably, rotenone treated mice showed a significant increase IBA-1 compared to the control group in SN(Fig. 3e,3g,3h). Compared to control group, the number and soma areas of IBA-1^+ microglial cells were increased in the rotenone group, showing a reactive microglia morphology, while rapamycin treatment exhibited the inhibition effect on IBA-1^+ microglial cells (Fig. 3e). These results indicate that rapamycin protects against rotenone-induced activation of microglia in SN.

TLR2 has been known to modulate NF-κB signaling pathways[23]. The NFATc2 pathway is another downstream signaling pathway of TLR2, and has also been proved play a crucial role in neuroinflammation[35]. Consistently, our results showed that there was no difference in the expression of

p-NF-κB p65 in nuclear tissue between the three groups (Fig. 4a,b (right)), but with the increased levels of nuclear translocation of NFATc2 in the midbrain in rotenone treated mice as compared with the control group, which was decreased by rapamycin treatment (Fig. 4a,b(left)). Immunofluorescent staining further revealed the elevated levels of nuclear translocation of NFATc2 in microglia of SN, which was in line with western blot observation (Fig. 4c,4d). Similarly, we found a similar pattern of TNF-α gene expression, with increased expression of the TNF-α gene in rotenone group and decreased expression in rapamycin group (Fig. 4e-4h). Overall, the data demonstrated that rapamycin inhibits7 the activation of TLR2 /NFATc2/TNF-α inflammatory signaling caused by rotenone.

4.4 Rapamycin prevented peptidoglycan-induced TLR2 activation, decreased nuclear translocation of NFATc2, and further lowered gene expression of TNF-α in BV-2.

Based on the above in vivo findings, we also conducted in vitro experiments to verify our results. Previous studies demonstrated that PGN binds to innate immune TLR2, and initiates signaling leading to inflammation[36]. Rapa (15nM)[24, 30, 31] and/or PGN(12.5μM)[32] were added to the cells coculture for 24 hours, then samples were collected for further analysis after washed with PBS for 3 times. The expression of TLR2 and nuclear translocation of NFATc2 in BV-2 cells was measured by western blot (Fig. 5a-5c). Bands density analysis showed that the levels of TLR2 and nuclear translocation of NFATc2 were significantly increased in PGN-exposed cells compared to the control group (Fig. 5b,5c). Besides, the gene expression of TNF-α were raised in PGN-cultured group quantified by real-time PCR. (Fig. 5d). In contrast, co-cultured with Rapa and PGN markedly inhibited the PGN-induced upregulation of TLR2 and nuclear translocation of NFATc2, further downregulated gene expression of TNF-α in BV-2 cells (Fig. 5a-5d), which was also observed by immunofluorescence analysis (Fig. 5e-5h), consistent with we previous results (Fig. 4). Taken together, the data suggest that rapamycin inhibits PGN-induced activation of TLR2/NFATc2/TNF-α inflammatory signaling.

5. Discussion

The finding in our study confirmed that intraperitoneal injection of rotenone for 4 weeks induced Parkinson's disease model in mice and microglia in substantia nigra of the model group were significantly activated. Our study demonstrated that rapamycin exerts protective effects by increasing the clearance of pathological α-synuclein may through enhancing autophagy, and inhibiting TLR2/NFATc2/TNF-α inflammatory pathway of microglia. Interestingly, in immunofluorescence experiment, the results of the rapamycin alone treatment group are different from those in western blot experiment, which may be caused by the different range of materials. It also does not rule out that rapamycin may have potential mild neurotoxicity, although previous studies have shown that it has no neurotoxicity[24, 26, 27, 37, 38].

PD is the second most common neurodegenerative disease. Impaired autophagy are thought to contribute to PD pathogenesis, restoring autophagy therefore be a potential treatment strategy[4–6]. Rapamycin as an autophagy inducer through inhibit mTORC1 used for the treatment of various tumors, in recent years, its application in the research of neurodegenerative diseases has also attracted attention[39]. Studies show that rapamycin treatment ameliorated neurodegenerative diseases through enhanced autophagy in MPTP treated mice[37, 38, 40]. Moreover, rapamycin induce autophagy clearance by inhibiting mTOR, reducing the expression of α-synuclein aggregates in α-synuclein transgenic mice[41, 42]. Our experimental results demonstrated that the LC3-β/LC3-α ratio was upregulated and the deposition levels of P-α-syn were decreased by rapamycin treatment, which confirmed that autophagy was promoted. This observation suggested that rapamycin may protect dopaminergic neurons through restoring the autophagic flux impaired and decreasing accumulation of the cytotoxic protein.

Excessive microglia activation and the subsequent release of proinflammatory cytokines drive progressive neuropathology in neurodegenerative diseases[43, 44]. Recognition of damage- or pathogen-associated molecular patterns (PAMPs, DAMPs) by Toll-like receptors (TLRs) is crucial to the activation of microglia[45, 46]. Inhibition of TLR2/MyD88/NF-κB pathway was reported to reduce alpha-synaptic nucleoprotein transmission in bv2 cells cultured with prefabricated alpha-SYN fibers (PFF) and in PFF-A53T mice[23]. Most importantly, TLR2/NFATc2/TNF-α was also found to implicate in inflammatory pathway independently [47–50]. And rapamycin treatment ameliorated neurodegenerative diseases through enhanced autophagy and anti-inflammation via IL2 or IL-6 signaling pathway in MPTP treated mice[37, 38, 40]. In addition, rapamycin suppresses TLR2-induced inflammatory responses by down-regulation of Erk and NF-κB signaling in monocytic THP-1 cells reported by Sun R et[31]. Here, we found that rapamycin can inhibit the expression of TLR2, reduce the nuclear transfer of NFATc2, and thus decrease the gene expression of TNF-α in PGN treated BV-2 cells and in rotenone-induced PD mice. Interestingly, results showed the levels of P-NF-κB p65 in nuclear have no difference among three groups (Fig. 4a,4b(right)), this may be caused by different modeling methods.

Despite we found a new molecular mechanism of rapamycin to alleviate PD, we still have some limitations in our research. For example The pathological changes in the brain of Parkinson's disease are extensive, including the movement related substantia nigra striatum area, cerebral cortex, marginal area and other non motor brain areas. In addition to movement disorders, there are many non motor symptoms, which also seriously affect the quality of life of patients. Our study mainly focuses on the effects of rapamycin on the movement symptoms of PD mice and the movement related areas in the brain, to further explore the effect of rapamycin on non motor symptoms and its brain regions of diffirent PD models are our future research interest.

In conclusion, our study demonstrated that rapamycin could ameliorate motor impairments and dopaminergic neuronal loss in rotenone-induced PD mice, promoting autophagy to remove abnormal proteins is one of the mechanisms of its neuroprotective effect, and inhibiting TLR/NFATc2/TNF-α inflammatory pathway of microglia to reduceneuroinflammation could constitute an underlying

mechanism for its neuroprotective effect in PD mice. This work provides a therapeutic strategy to alleviate the progress of neurodegenerative diseases.

Declarations

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Disclosure statement

The authors declare that they have no competing interests.

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

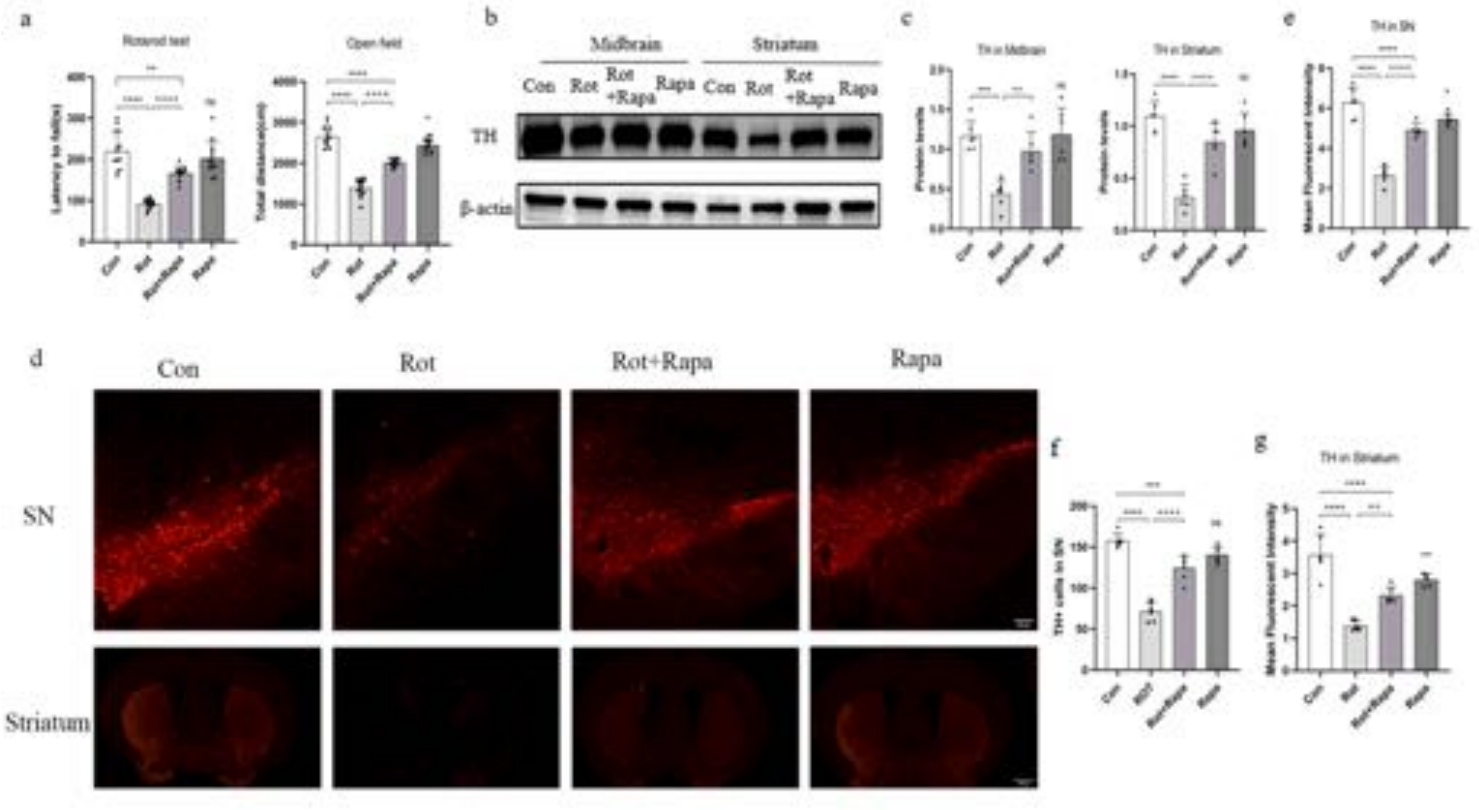

Figure 1

Rapamycin treatment improved motor deficits via saving rotenone induced loss of TH+ neuron in SN and their fibers to striatum. Latency to fall in rotarod test (a(left), F (3,44) = 36, P <0.0001 for control vs rotenone, P= 0.0012 for control vs rotenone + rapamycin and P < 0.0001 for rotenone vs rotenone + rapamycin. The total distance traveled in open field test. (a(right), F (3,48) = 113.6, P < 0.0001 for control vs rotenone, P < 0.0001 for control vs rotenone + rapamycin, P = 0.0308 for control vs rapamycin and p < 0.0001 for rotenone vs rotenone + rapamycin).The expression of TH in midbrain and striatum evaluated by western blot (b, c, F (3,20) = 12.63, P = 0.002 for control vs rotenone, P = 0.048 for rotenone vs rotenone + rapamycin (c, left) and F (3,20) = 28.66, P < 0.0001 for control vs rotenone, P < 0.0001 for rotenone vs rotenone + rapamycin(c, right)). Immunofluorescence show the expression of TH in SN and striatum (d). The analysis of TH fluorescence intensity measured by ImageJ (e, F (3,20) = 60, P < 0.0001 for control vs rotenone, P = 0.005 for control vs rotenone + rapamycin, P = 0.0393 for control vs rapamycin and P< 0.0001 for rotenone vs rotenone +rapamycin; f, F (3,20) = 63,79, P< 0.0001 for control vs rotenone, P = 0.0003 for control vs rotenone + rapamycin and P < 0.0001 for rotenone vs rotenone + rapamycin; g, F (3,20) = 39.4, P < 0.0001 for control vs rotenone, P < 0.0001 for control vs rotenone + rapamycin, P = 0.0065 for control vs rapamycin and P = 0.001 for rotenone vs rotenone + rapamycin). Data were analyzed using one-way ANOVA followed by Tukey’s multiple comparison tests. * P < 0.05, ** P <0.01, *** P <0.001, **** P <0.0001, compared to control; ++ P < 0.01, ++++ P < 0.0001, compared to rotenone. Values are given as mean ± SD. (n = 6-12independent experiment). Con: Control, Rot: Rotenone, Rot +Rapa: Rotenone + rapamycin, Rapa: rapamycin.

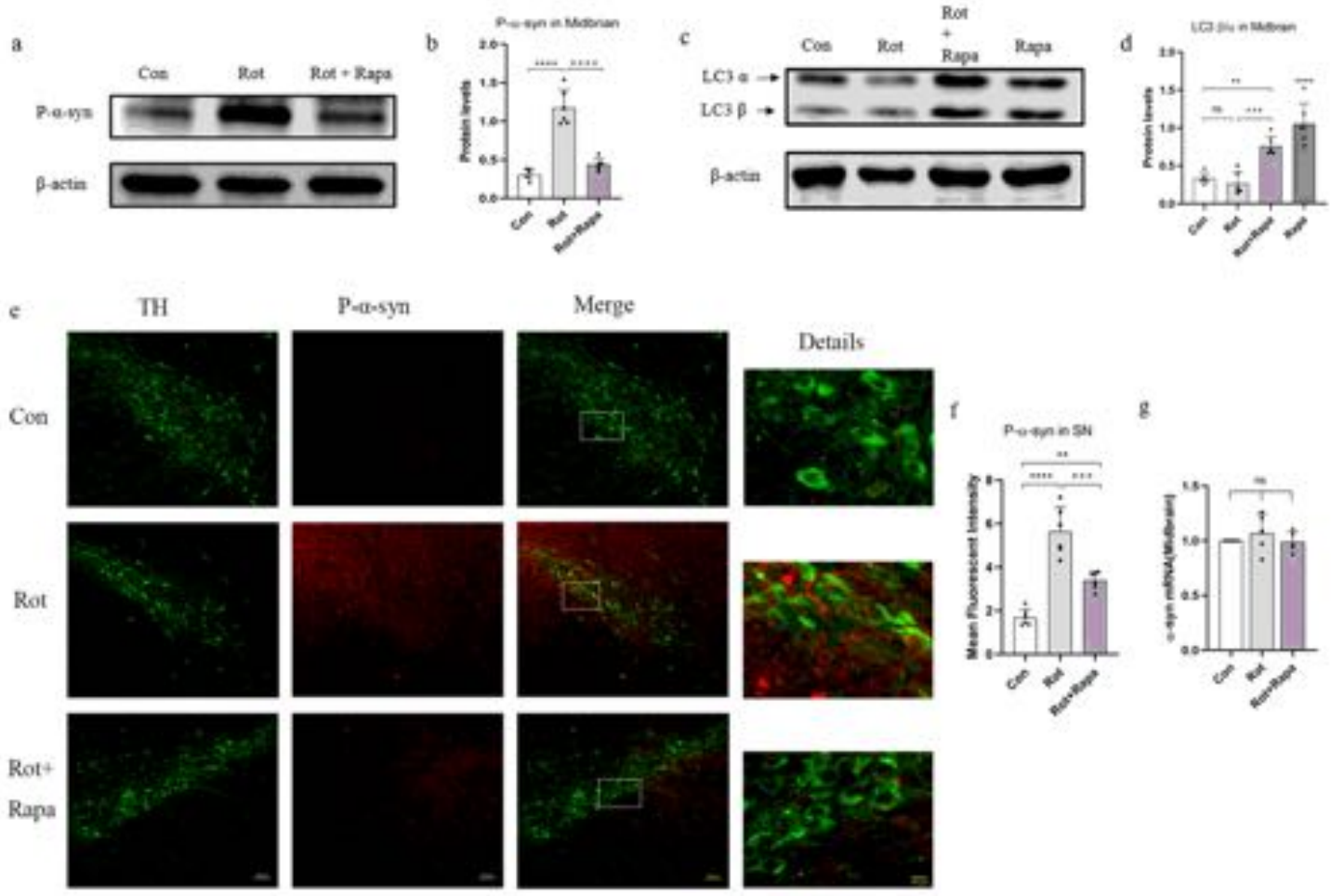

Figure 2

Rapamycin treatment restored autophagy and decreased the deposition of P-α-syn. The expression of P-α-syn in midbrain evaluated by western blot (a, b, F (2,15) = 60.21, $P < 0.0001$ for control vs rotenone and $P < 0.0001$ for rotenone vs rotenone + rapamycin). The LC3-β/LC3-α ratio in midbrain evaluated by western blot (c, d, F (3,20 = 28.63, $P = 0.9137$ for control vs rotenone, $P = 0.0016$ for control vs rotenone + rapamycin, $P < 0.0001$ for control vs rapamycin and $P = 0.0004$ for rotenone vs rotenone + rapamycin). Immunofluorescence show the expression of P-α-syn in SN (e, f, F (2,15) = 45.48, $P < 0.0001$ for control vs rotenone, $P = 0.0025$ for control vs rotenone + rapamycin and $P = 0.0002$ for rotenone vs rotenone + rapamycin). The gene expression of α-syn were quantified by real-time PCR (g, F (2,12) = 0.7181, $P = 0.6095$ for control vs rotenone, $P = 0.9912$ for control vs rotenone + rapamycin and $P = 0.5347$ for rotenone vs rotenone + rapamycin). Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison tests. ** $P < 0.01$, **** $P < 0.0001$, compared to control; +++ $P < 0.001$, ++++ $P < 0.0001$, compared to rotenone. Values are given as mean ± SD. (n = 5-6 independent experiment).

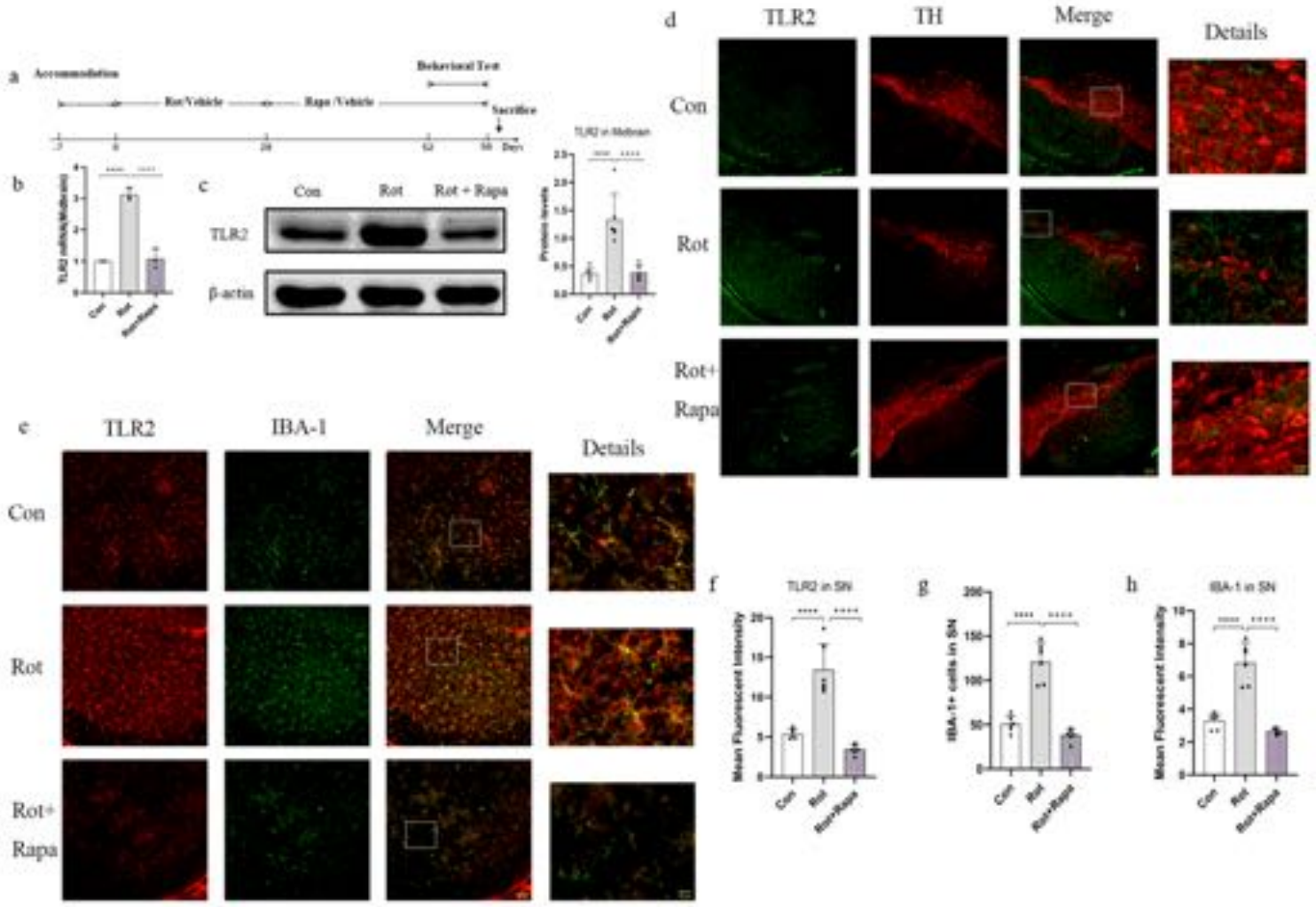

Figure 3

Rapamycin prevented TLR2 activation in microglia and decreased microglia infiltration in SN of rotenone-induced PD mice. Treatment schedule(a). The gene expression of TLR2 in midbrain evaluated by RT-PCR (b, F (2,6) = 94.7, $P < 0.0001$ for control vs rotenone and $P < 0.0001$ for rotenone vs rotenone + rapamycin). The expression of TLR2 in midbrain evaluated by western blot (b, c, F (2,15) = 44.42, $P < 0.0001$ for control vs rotenone and $P < 0.0001$ for rotenone vs rotenone + rapamycin). Immunofluorescence double labeling showed no co localization relationship between TLR2 and TH neurons in SN(d). Immunofluorescence double labeling show the expression of TLR2 and IBA-1 and colocalization of TLR2 and IBA-1 in SN(e). The analysis of TLR2 and IBA-1 measured by ImageJ (f, F (2,15) = 41.58, $P < 0.0001$ for control vs rotenone and $P < 0.0001$ for rotenone vs rotenone +rapamycin; g, F (2,15) = 56.87, $P < 0.0001$ for control vs rotenone and $P < 0.0001$ for rotenone vs rotenone + rapamycin, h, F (2,15) = 49.96, $P < 0.0001$ for control vs rotenone and $P < 0.0001$ for rotenone vs rotenone + rapamycin). Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison tests. **** $P < 0.0001$, compared to control; ++++ $P < 0.0001$, compared to rotenone. Values are given as mean ± SD. (n =3-6 independent experiment).

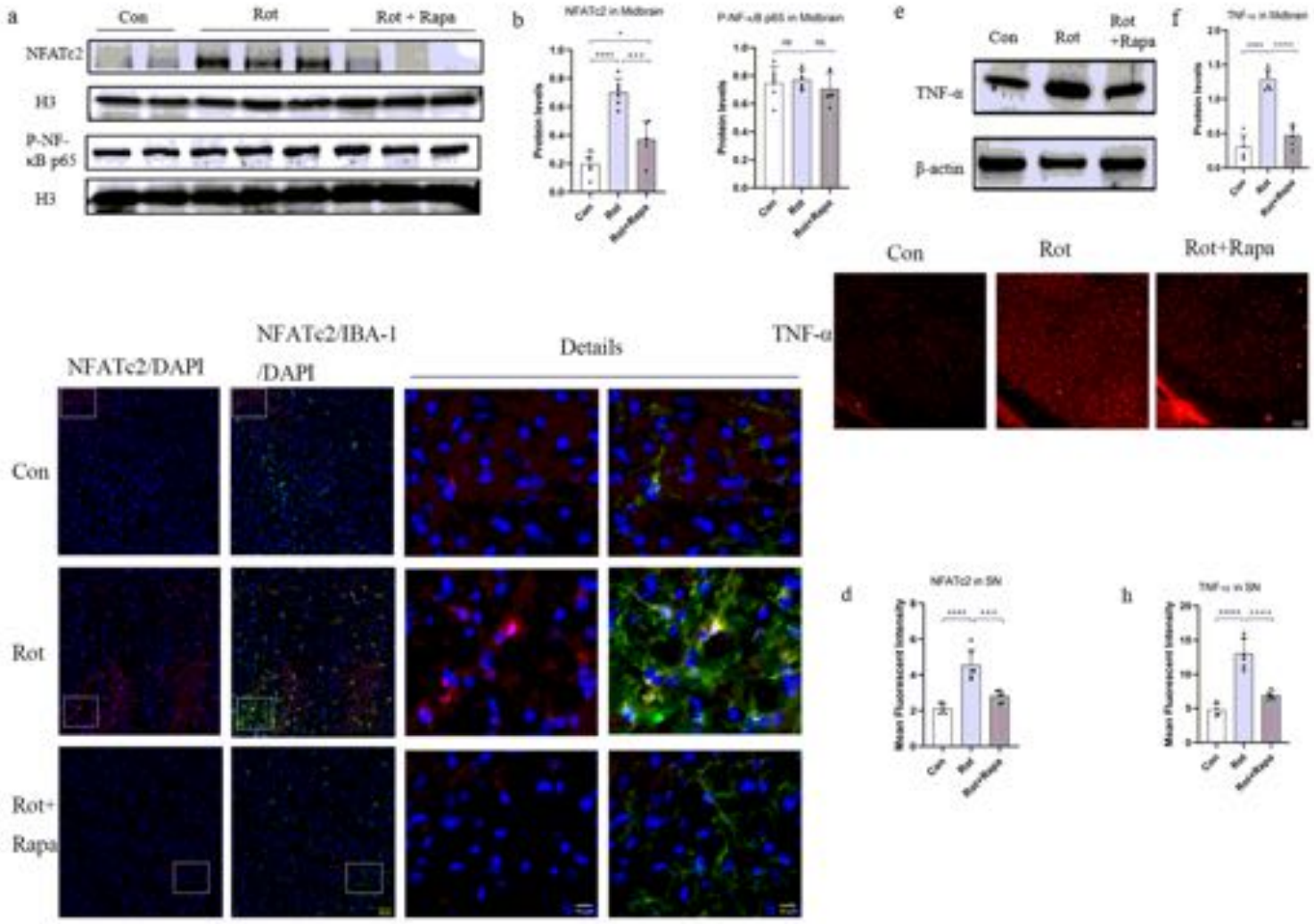

Figure 4

Rapamycin reduced the nuclear translocation of NFATc2, and further lowerd gene expression of TNF-α in SN of rotenone-induced PD mice. The nuclear translocation of NFATc2 and P-NF-κB p65 in midbrain evaluated by western blot (a, b(left) F(2,15) = 36.32, $P < 0.0001$ for control vs rotenone, $P = 0.0297$ for control vs rotenone + rapamycin and $P < 0.001$ for rotenone vs rotenone + rapamycin; b F(2,15) = 0.5874, $P = 0.5681$). Immunofluorescence show the expression of NFATc2 and colocalization of NFATc2, IBA-1 and DAPI in SN(c). The expression of NFATc2 analyzed by ImageJ (d, F (2,15) = 30.37, $P < 0.0001$ for control vs rotenone and $P < 0.001$ for rotenone vs rotenone + rapamycin). The expression of TNF-α in midbrain evaluated by western blot (e, f, F (2,15) = 65.25, $P < 0.0001$ for control vs rotenone and $P < 0.0001$ for rotenone vs rotenone + rapamycin). Immunofluorescence show the expression of TNF-α in SN (g, h, F (2,15) = 53.51, $P < 0.0001$ for control vs rotenone and $P < 0.0001$ for rotenone vs rotenone + rapamycin). Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison tests. * $P < 0.05$, **** $P < 0.0001$, compared to control; +++ $P < 0.001$, ++++$P < 0.0001$compared to rotenone. Values are given as mean ± SD. (n = 6 independent experiment).

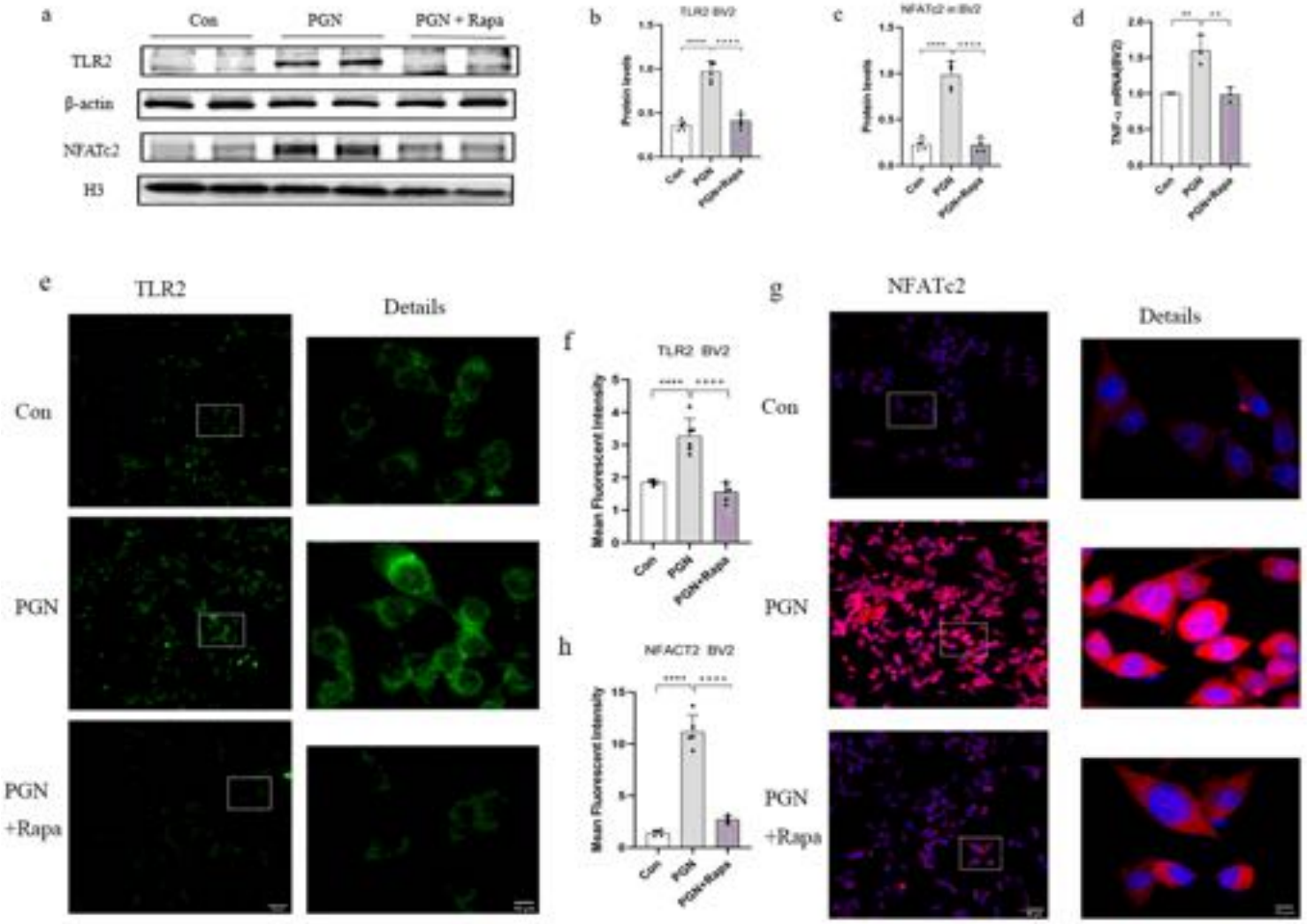

Figure 5

Rapamycin inhibited PGN-induced activation of TLR2/NFATc2/TNF-α inflammatory signaling in BV-2 cells. The expression of TLR2 and nuclear translocation of NFATc2 evaluated by western blot (a, b, F (2,15) = 104, $P < 0.0001$ for control vs PGN and $P < 0.0001$ for PGN vs PGN + rapamycin; c, F (2,12) = 99.48, $P < 0.0001$ for control vs PGN and $P < 0.0001$ for PGN vs PGN + rapamycin). The gene expression of TNF-α were quantified by real-time PCR. (d, F (2,6) = 20.4, $P = 0.0038$ for control vs PGN and $P = 0.0033$ for PGN vs PGN + rapamycin). The expression of TLR2 and nuclear translocation of NFATc2

measured by immunofluorescence (e, f, F (2,15) = 41.58, $P < 0.0001$ for control vs PGN and $P < 0.0001$ for PGN vs PGN + rapamycin; g, h F (2,12) =151.3, $P < 0.0001$ for control vs PGN and $P < 0.0001$ for PGN vs PGN + rapamycin). Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison tests. ** $P < 0.01$, **** $P < 0.0001$, compared to control; ++$P < 0.01$, ++++ $P < 0.0001$, compared to PGN. Values are given as mean ± SD. (n = 3-6 independent experiment). Con: Control, PGN: peptidoglycan, PGN + Rapa: peptidoglycan + rapamycin, Rapa: rapamycin.

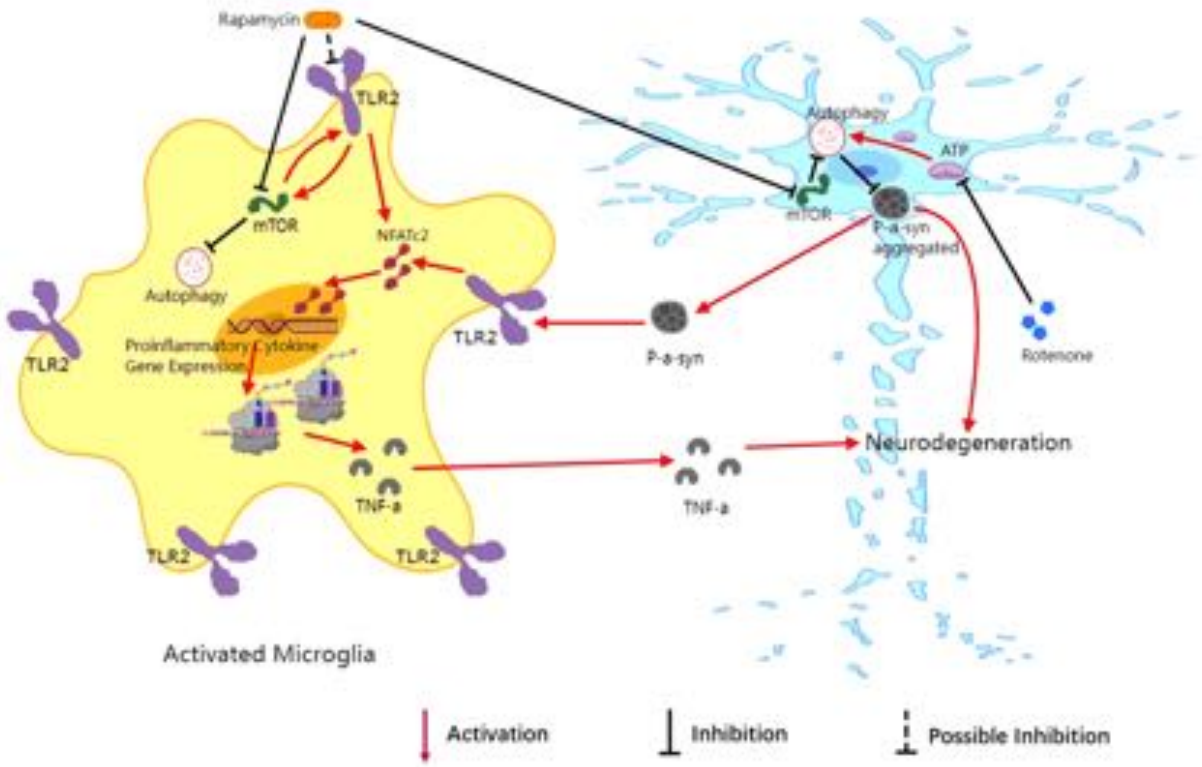

Figure 6

Mechanism's diagram. Rapamycin inhibits rotenone-induced activation of TLR2/NFATc2/TNF-α inflammatory signaling and impairement of autophagy. Rotenone inhibits the oxidative respiratory chain, and then suppress what should be enhanced autophagy, which leads to the degradation of abnormal protein(P-α-syn) and leads to the degeneration of neurons. P-α-syn activates mTOR and then inhibits autophagy by activating TLR2 of microglia, activatiion of TLR2 also causes the activation of proinflammatory signal pathway downstream of TLR2, which leads to the increase of inflammatory factor synthesis and further aggravates the degeneration of neurons. However, rapamycin treatment reversed the above changes, at least partially.