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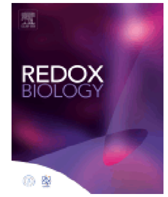

Research Paper

NADPH oxidase-derived H_2O_2 mediates the regulatory effects of microglia on astrogliosis in experimental models of Parkinson's disease

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ABSTRACT

Astrogliosis has long been recognized in Parkinson's disease (PD), the most common neurodegenerative movement disorder. However, the mechanisms of how astroglia become activated remain unclear. Reciprocal interactions between microglia and astroglia play a pivotal role in regulating the activities of astroglia. The purpose of this study is to investigate the mechanism by which microglia regulate astrogliosis by using lipopolysaccharide (LPS) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mouse PD models. We found that the activation of microglia preceded astroglia in the substantia nigra of mice treated with either LPS or MPTP. Furthermore, suppression of microglial activation by pharmacological inhibition or genetic deletion of NADPH oxidase (NOX2) in mice attenuated astrogliosis. The important role of NOX2 in microglial regulation of astrogliosis was further mirrored in a mixed-glia culture system. Mechanistically, H_2O_2, a product of microglial NOX2 activation, serves as a direct signal to regulate astrogliosis. Astrogliosis was induced by H_2O_2 through a process in which extracellularly generated H_2O_2 diffused into the cytoplasm and subsequently stimulated activation of transcription factors, STAT1 and STAT3. STAT1/3 activation regulated the immunological functions of H_2O_2-induced astrogliosis since AG490, an inhibitor of STAT1/3, attenuated the gene expressions of both proinflammatory and neurotrophic factors in H_2O_2-treated astrocyte. Our findings indicate that microglial NOX2-generated H_2O_2 is able to regulate the immunological functions of astroglia via a STAT1/3-dependent manner, providing additional evidence for the immune pathogenesis and therapeutic studies of PD.

1. Introduction

Astrocytes, also known as astroglia, are multifunctional cells and are capable of reacting to any kind of injury in the central nervous system (CNS), leading to activation [1]. The process of astroglial activation remains rather enigmatic and results in so-called astrogliosis. Astrogliosis characterized by hypertrophic morphology and upregulation of intermediate filaments composed of nestin, vimentin, and glial fibrillary protein (GFAP) has long been recognized in multiple neurodegenerative diseases, including Parkinson's disease (PD), the most common neurodegenerative movement disorder [2]. The functions of astrogliosis in PD remain controversial, and both beneficial and detrimental influences on the neural tissues are reported [3]. Despite of this, the cellular and molecular mechanisms leading to astrogliosis remain to be undefined.

In addition to astrogliosis, the activation of microglia, the innate

Abbreviations: BDNF, brain derived neurotrophic factor; GDNF, glia derived neurotrophic factor; GFAP, glial fibrillary protein; Iba-1, ionized calcium binding adaptor molecule 1; IL-1β, interleukin-1β; LPS, lipopolysaccharide; LME, l-leucine-methyl esther; MAPK, mitogen-activated protein kinase (MAPK); MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NF-κB, nuclear factor kappa B; NOX2, NADPH oxidase; PD, Parkinson's disease; PGE_2, prostaglandin E2, SN, substantia nigra; TNFα, tumor necrosis factor α

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immune cells in the CNS, are also detected in the substantia nigra (SN) of PD patients [4] and animal models [5,6]. As the first line of defense in the CNS, microglia are promptly activated and release a variety of cytotoxic factors in response to bacterial infection or CNS injury [7]. Among the factors released from activated microglia, superoxide generated from NADPH oxidase (NOX2) is earliest and plays a special role in neurodegeneration [8]. Multiple evidence revealed that, in contrast to rapid activation of microglia, the astrocyte response is usually delayed [9–11], suggesting that microglia might play a role in astrogliosis. This assumption was supported by the finding that a single pro-inflammatory factor released from activated microglia, such as prostaglandin E2 (PGE_2), interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα), is capable of inducing astroglial activation [12,13]. Moreover, inflammogen lipopolysaccharides (LPS) fails to induce astrogliosis in primary glia cultures once microglia are depleted by l-leucine-methyl esther (LME) [14]. Furthermore, in animal models of chemically induced neurodegeneration, the reduced microglial activation is associated with a reduced astroglial cell number [15–17]. Recently, using an *in vitro* inflammatory PD model, we confirmed that microglia are essential in regulating the immunological functions of astrogliosis [14]. Nevertheless, whether microglia can regulate astrogliosis in animal models of PD and the underlying mechanisms are still not completely understood.

The purpose of this study was to investigate whether microglia can regulate astrogliosis in experimental models of PD and underlying mechanisms. In both LPS and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurodegenerative models, we found that microglial activation preceded astrogliosis in the SN of mice. Inhibition of microglial activation by genetic deletion or pharmacological inhibition of NOX2 attenuated astrogliosis induced by LPS and MPTP. Mechanistically, we found that microglial NOX2-derived H_2O_2 played a pivotal role as a paracrine signal to regulate astrogliosis via a STAT1 and 3-dependent pathway. Our data provide the first evidence that NOX2-derived H_2O_2 modulates microglia-to-astrocyte communication in PD.

2. Materials and methods

2.1. Animal treatment

A single systemic LPS (Sigma-Aldrich, St. Louis, MO, USA) injection (15×10^6 EU/kg, i.p.), or repeated MPTP (Sigma-Aldrich, St. Louis, MO, USA) regimen (15 mg/kg, sc. for 6 consecutive days) were administrated to wile type (WT, C57BL/6 J) or gp91$^{phox-/-}$ mice [18]. Mice in control group received an equal volume of 0.9% saline. At different time points after LPS or MPTP treatment, mice were euthanized and brains were collected. Housing, breeding and experimental use of the animals were performed in strict accordance with the Animal Guideline of Dalian Medical University. All experimental protocols were approved by and in agreement with the Ethical Committee of Dalian Medical University.

2.2. Primary cell cultures

Primary mixed glia, astroglia and microglia cultures were prepared according to a previously published protocol [19].

2.3. Reconstituted cell cultures

Microglia were plated into transwells (24-well multi-well insert systems, 1.0 µm pore size, PET membrane) placed above the astroglia layer in culture plates. Although there was no physical contact between microglia and astroglia, secreted soluble factors could move across the transwell membranes. Transwells with LPS-treated microglia would generated environmental neuroinflammation for astroglia layers, and transwells without microglia were used as negative control.

2.4. Immunohistochemistry and immunocytochemistry

Immunohistochemistry and immunocytochemistry were performed as described previously [18]. Briefly, the free-floating coronal sections or primary cultures were immunoblocked with 4–10% normal goat serum and then incubated with primary antibody against ionized calcium binding adaptor molecule 1 (Iba1, 1:5000; Wako Chemicals, Richmond, VA, USA) or GFAP (Dako, Santa Clara, CA, USA) for 24 h, respectively, at 4 °C. Antibody binding was visualized using a Vectastain ABC Kit (Vector Laboratories, Inc. Burlingame, CA, USA) and diaminobenzidine substrate. The densities of Iba-1 and GFAP immunostaining were measured using ImageJ version 1.43 software (National Institutes of Health) [20].

2.5. Real-time PCR analysis

Total RNA was extracted with RNeasy Mini kit (Qiagen, Germantown, MD, USA) and reverse transcribed with an oligo dT primer. Real-time PCR amplification was performed using SYBR Premix Ex Taq™ II (Takara Bio Inc. Kusatsu, Shiga, Japan) and Takara Thermal Cycler Dice™ Real Time System according to manufacturer's protocols. The selected primers are listed in Supplementary Table 1. All the data were normalized with GAPDH.

2.6. Measurement of H_2O_2

The levels of H_2O_2 were examined as described previously [21] by using a H_2O_2 assay kit (Abcam, Cambridge, MA, USA). Briefly, a transwell system in which microglial cells were placed on the upper chamber and astrocytes on the lower chamber was treated with LPS at 37 °C for 1 h. To measure the extracellular levels of H_2O_2 released from activated microglia, the supernatant was collected. To measure the intracellular levels of H_2O_2 in astrocyte, the culture media and microglial cells were removed after 1 h of LPS stimulation. Astrocytes on the lower chamber were washed with cold PBS and were lysed using the assay buffer provided by the kit. After homogenization, the cell samples were centrifuged at 1000×*g* for 15 mins and the supernatant was collected. After deproteinization, the working solution containing OxiRed probe and horse radish peroxidase (HRP) was added to both extracellular and intracellular samples according to the manufacturer's instructions. In the presence of HRP, the OxiRed probe reacts with H_2O_2 to produce product with color (λmax=570 nm). The absorbance at 570 nm was read with a SpectraMax Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The amount of H_2O_2 was calculated and expressed as percentage of vehicle-treated control cultures.

2.7. Western blot analysis

For western blot analysis, equal amounts of protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4% stacking and 10% resolving gel. Following electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk and incubated with primary antibodies (1:1000) against Iba-1, GFAP, phospho-STAT1, STAT1, phospho-STAT3, STAT3 (Cell Signaling Technology, Danvers, MA, USA), GAPDH (Abcam, Cambridge, MA, USA) and HRP-linked anti-rabbit or mouse IgG (1:3000) for 2 h. ECL reagents (Biological Industries, Cromwell, CT, USA) were used as detection system.

2.8. Statistical analysis

All values are expressed as the mean ± SEM. Differences among means were analyzed using one- or two-way ANOVA with treatment as the independent factors. When ANOVA showed significant differences, pair-wise comparisons between means were tested by Tukey's *post hoc*

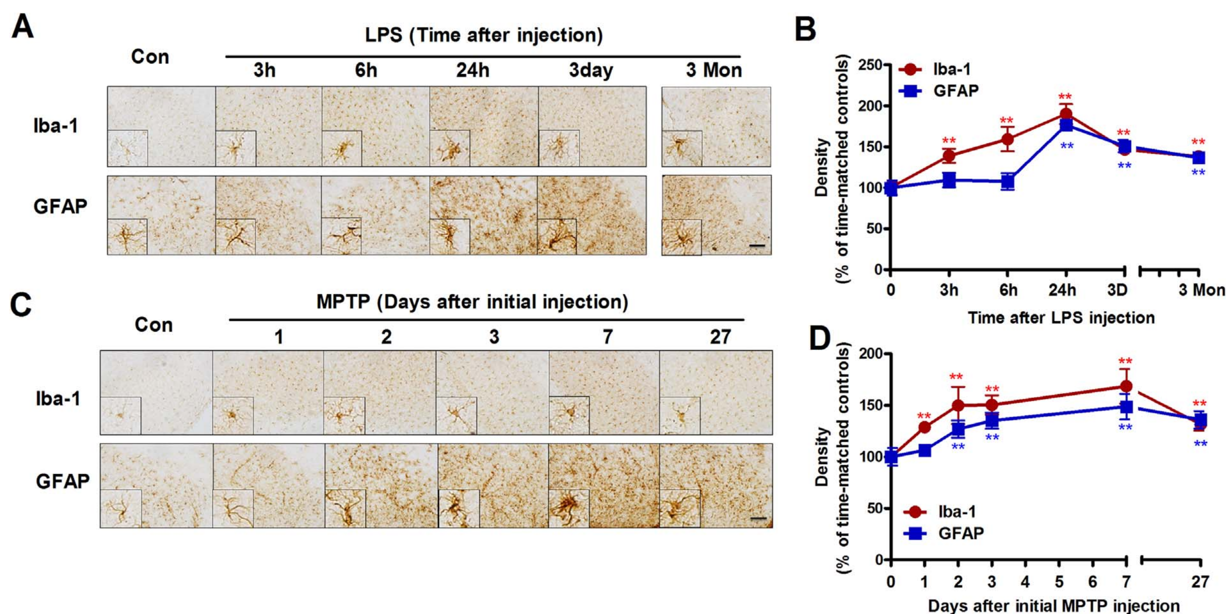

Fig. 1. The activation of microglia in the SN procedes astroglia in both LPS and MPTP-injected mice. (**A**) Microglia and astroglia were stained in the SN by using anti-Iba-1 and anti-GFAP antibody, respectively, at the indicated time points after LPS injection and the representative images were shown. (**B**) The activation of microglia and astroglia was quantified by measuring the pixel density of Iba-1 and GFAP staining, respectively. (**C**) Microglia and astroglia were stained in the SN by using anti-Iba-1 and anti-GFAP antibody, respectively, at the indicated time points after initial MPTP injection and the representative images were shown. (**D**) The density of Iba-1 and GFAP immunostaining was quantified. $^{**}p < 0.01$; n=4–6 in each group; Scale bar=100 µm.

testing. In all analyses, a value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. The activation of microglia in the SN procedes astroglia in both LPS and MPTP-injected mice

To investigate whether microglia are capable of regulating astrogliosis *in vivo*, we initially determined the time sequence of microglial and astroglial activation in both LPS and MPTP-induced dopaminergic neurodegenerative models. We have previously reported that a single systemic injection of inflammogen LPS generates peripheral inflammation, and some of the circulating proinflammatory factors gain entrance to the brain to initiate proinflammatory response and subsequently lead to the progressive degeneration of nigral dopaminergic neurons [22]. While, in dopaminergic neurotoxin MPTP-induced model, glia also become activated due to response to endogenous factors secreted by damaged neurons, such as µ-calpain and α-synuclein [7]. As shown in Fig. 1**A**, both microglial and astroglial activation were observed throughout the nigral reticulata area in LPS and MPTP-treated mice by showing hypertrophied morphology and intensified Iba-1 and GFAP staining, respectively. Densitometric analysis of immunohistochemical expressions of Iba-1 and GFAP supported the morphological observation (Fig. 1**B**). In LPS model, the elevation of Iba1 expression was firstly observed at 3 h, peaked at 24 h after treatment, and then was maintained about 1.5 fold of control level up to 3 months after LPS injection (Fig. 1**B**). In contrast, the significant increase of GFAP density was not evident by 24 h after LPS injection, indicating that the activation of microglia precedes astroglia (Fig. 1**B**).

A similarly sequential activation of microglia and astroglia was observed in MPTP model. The upregulation of Iba1 and GFAP density in the SN was initially detected 1 and 2 days after MPTP treatment, respectively, and was sustained until the study end-point 27 days (Fig. 1**C and D**).

3.2. Pharmacological inhibition or genetic deletion of NOX2 attenuates LPS and MPTP-induced astrogliosis

Next, we determined whether inhibition of microglial activation interferes with astrogliosis. We recently demonstrated that inhibition of NOX2 markedly reduced microglial activation in both LPS and MPTP-injected mice [18]. To determine whether inhibition of NOX2-mediated microglial activation is associated with suppression of astrogliosis, mice were treated with apocynin, a widely used NOX2 inhibitor, before LPS injection (Fig. 2**A**). Interestingly, apocynin significantly reduced activation of microglia, which was associated with suppression of astrogliosis by showing a decreased GFAP expression compared with LPS alone group (Fig. 2**B and C**).

To determine whether astroglial activation could still be suppressed by targeting microglial NOX2 after onset, an ultra-low dose of DPI that displays potent and specific inhibitory capacity on NOX2 activation [18,23] was used after 3 months of LPS injection, a time point that both microglia and astroglia were activated (Fig. 2**D** and Fig. 1). Consistent with our previous report [18], DPI effectively reduced LPS-induced activation of microglia (**data not shown**). Interestingly, astroglia in DPI/LPS-treated mice also displayed normal morphology and a comparable level of GFAP expression with control group, suggesting that astroglial activation is blocked by DPI (Fig. 2**E and F**). Inhibition of astroglial activation by DPI was not transient but continually observed up to 10 months after LPS injection (Fig. 2**E and F**).

Consistently, attenuated astrogliosis by DPI was also observed in the SN of MPTP model by showing reduced expression of GFAP in combined DPI and MPTP-treated mice compared with MPTP alone group (Fig. 2**G–I**).

To further verify the role of microglial NOX2 in regulating astrogliosis, NOX2-deficient mice ($gp91^{phox-/-}$) were used and were treated with MPTP. We found that genetic deletion of NOX2 markedly decreased MPTP-induced elevation of GFAP expression (Fig. 2**G–I**), further confirming the essential role of NOX2 in astrogliosis. In agreement, no significant effect of DPI on astroglial activation in MPTP-treated $gp91^{phox-/-}$ mice was observed (Fig. 2**H–J**).

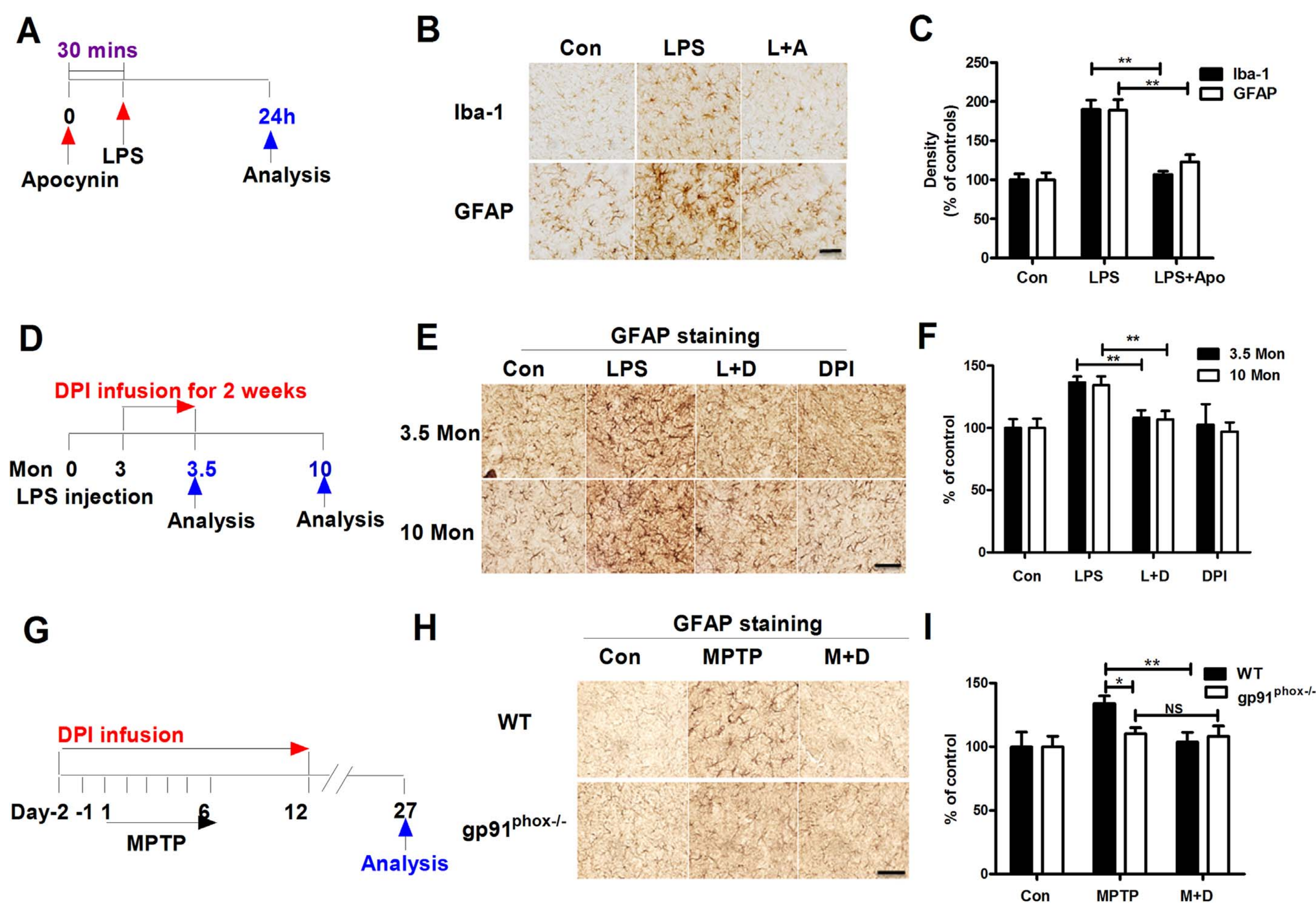

Fig. 2. Pharmacological inhibition or genetic deletion of NOX2 attenuates astroglial activation induced by LPS and MPTP. (A) Experimental design. C57BL/6J mice were treated with apocynin 30 mins prior to LPS. Twenty-four hours later, brain samples were dissected and analysized. (B) Microglia and astroglia were stained in the SN by using anti-Iba-1 and anti-GFAP antibody, respectively, and the representative images were shown. (C) The pixel density of Iba-1 and GFAP immunostaining was analyzied. (D) Experimental design. After 3 months of LPS injection, mice were infused with either vehicle or DPI for 2 weeks and brain samples were collected immediately or 7 months after DPI infusion. (E) Astroglia in the SN were immunostained by using anti-GFAP antibody and the representative images were shown. (F) The pixel density of GFAP immunostaining was analyzied. (G) Experimental design. DPI was administrated to WT (C57BL/6J) or NOX2-deficient ($gp91^{phox-/-}$) mice 2 day before MPTP and brain samples were collected after 27 days of initial MPTP injection. (H) Astroglia in the SN were immunostained by using anti-GFAP antibody and the representative images were shown. (I) The pixel density of GFAP immunostaining was analyzied. *$p < 0.05$, **$p < 0.01$; n=4–6 in each group; Scale bar=50 µm.

3.3. NOX2 activation contributes to microglia-mediated astroglial activation in vitro

To investigate the mechanisms of how microglial NOX2 regulates astrogliosis, a mixed-glia culture system that contains ~20% microglia and ~80% astrocyte [24], was used and was treated with LPS. As seen in Fig. 3**A**, LPS at all the concentrations activated microglia by showing elevated expression of Iba-1. While, increased GFAP expression was only observed in cultures treated with LPS at 10 ng/ml or higher, indicating a threshold exists for astroglial activation. LPS-induced astroglial activation was further verified by immunofluorescence staining using anti-GFAP antibody (Fig. 3**B**).

To determine the sequence of microglial and astroglial activation, the expressions of Iba-1 and GFAP were determined at different time points after LPS treatment. Consistent with that of *in vivo*, the activation of microglia preceded astroglia (Fig. 3**C**). Western blot analysis showed that the increase of Iba-1 expression was evident by 1 day after LPS treatment (Fig. 3**C**). In contrast, the increase ($p < 0.01$) of GFAP expression was not observed until 3 days after LPS treatment (Fig. 3**C**). Deletion of microglia by LME abolished LPS-induced increase of GFAP expression (Fig. 3**D**), indicating the presence of microglia is required for astrogliosis induced by LPS.

The role of microglial NOX2 in astroglial activation was subsequently examined by using two inhibitors, DPI and apocynin. As shown in Fig. 3**E**, DPI or apocynin treatment significantly attenuated LPS-induced astroglial activation by showing a decreased level of GFAP compared with LPS alone group. The results of immunofluorescence staining were consistent with Western blot analysis (Fig. 3**B**).

3.4. H_2O_2, a product of microglial NOX2 activation, serves as a direct signal to regulate astrogliosis

NOX2 activation increases extracellular superoxide, which can spontaneously convert to H_2O_2. Previous reports indicate that redox reactions are capable of stimulating astroglial activation [25]. We therefore hypothesized that H_2O_2 generated from NOX2 might be a key signal for the regulation of astrogliosis by microglia. The changes in concentrations of extracellular (supernatant) H_2O_2 were initially measured. We found that LPS dose-dependently increased the levels of H_2O_2 in the supernatant of LPS-treated mixed glia culture (Fig. 4**A**). To determine whether LPS could increase the levels of H_2O_2 in astrocyte, a trans-well system with enriched microglia on the upper chamber and enriched astroglia on the lower chamber was used. Consistently, LPS stimulation also increased the **intracellular** levels of H_2O_2 in astroglia (Fig. 4**B**).

To determine whether activation of NOX2 contributes to the elevated levels of H_2O_2 in both supernatant and cytosolic fractions of astrocyte, DPI and apocynin were applied to the trans-well culture

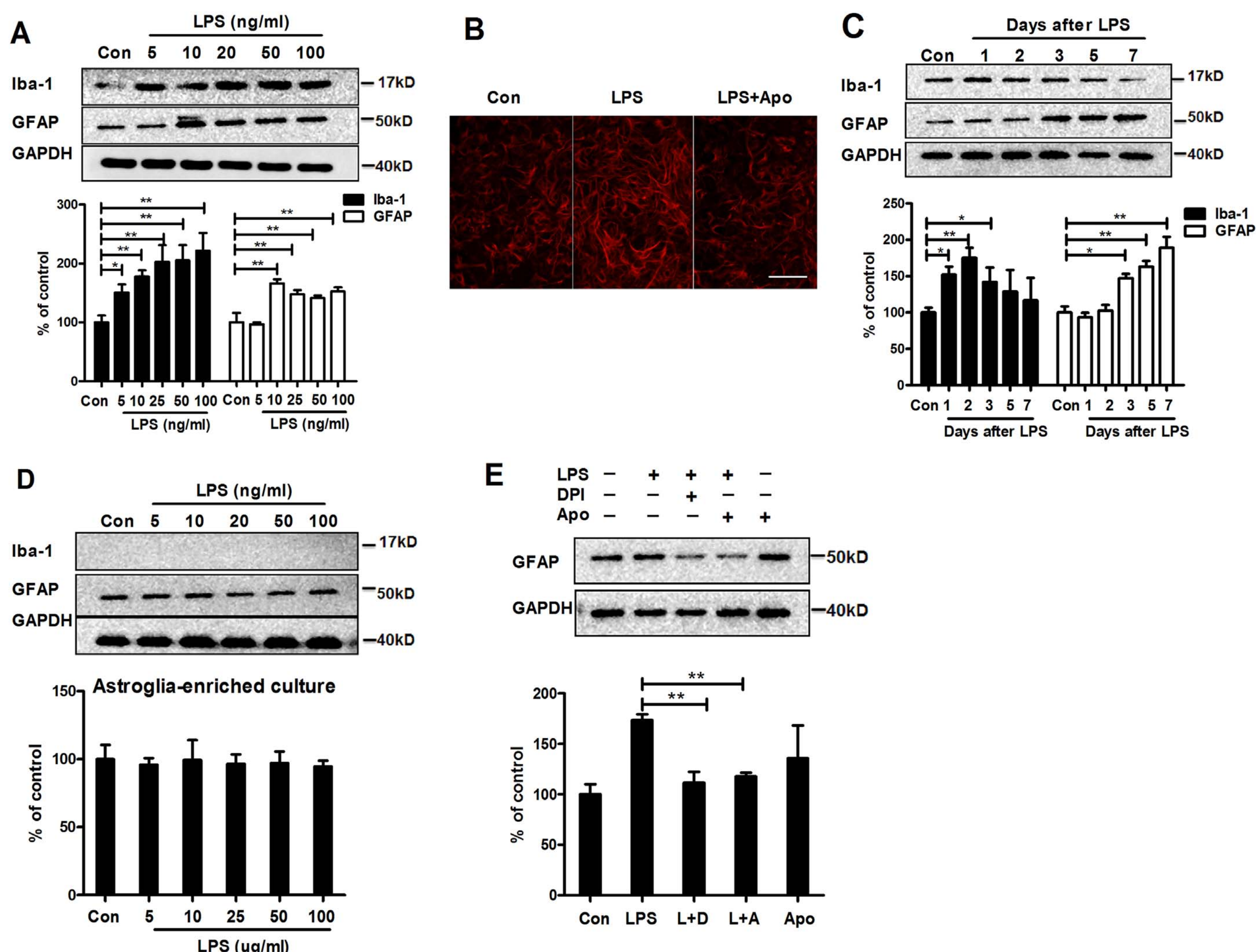

Fig. 3. Pharmacological inhibition of NOX2 attenuates LPS-induced activation of astroglia *in vitro*. (A) The expressions of Iba-1 and GFAP were determined in mixed glial cultures treated with LPS for 3 days by Western blot and the density of the blot was quantified. GAPDH was used as an internal control. (B) Mixed glial cultures treated with LPS with or without apocynin pre-treatment were immunostained with anti-GFAP antibody and the representative images were shown. (C) The expressions of Iba-1 and GFAP were determined in mixed glial cultures at the indicated time points after LPS treatment by Western blot and the blot density was quantified. (D) LPS-induced expressions of Iba-1 and GFAP were determined in microglia-deleted cultures by Western blot and the density of the blot was quantified. (E) The expressions of GFAP were determined in LPS-treated mixed glia cultures in the presence of DPI or apocynin by Western blot and the density of the blot was quantified. The results are expressed as the percentage of controls from three experiments performed in duplicate. *$p < 0.05$. **$p < 0.01$; Scale bar=50 μm.

system before LPS challenge. As seen in Fig. 4**B,** DPI and apocynin markedly suppressed the elevation of H_2O_2 in both fractions, suggesting that NOX2 is the main source of H_2O_2.

Exogenous H_2O_2 was further used to test its capacity to stimulate activation of astroglia. As shown in Fig. 4**C**, H_2O_2 at 10, 20, 50 and 100 μM dose-dependently stimulated astroglial activation as shown by 47.6% (±16.5%), 60.7% (±14.1%), 94.8% (±12.4%) and 92.6% (±13.6%) increase of GFAP expressions, respectively, in astrocyte culture, compared with vehicle controls. No significant toxicity of H_2O_2 on astrocyte was observed in the current conditions (Fig. 4**D**)· H_2O_2-induced up-regulation of GFAP expression was further confirmed by immunostaining with anti-GFAP antibody (Fig. 4**E**). Time course study revealed that a time-dependent increase of GFAP expression was seen till 24 h in H_2O_2–treated astrocyte culture (Fig. 4**F**).

3.5. NOX2-generated H_2O_2 interfere with the immunological functions of astrogliosis

To determine whether inhibition of microglial NOX2 alters the functions of astrogliosis, we detected the changes of the mRNA levels of TNFα, iNOS, GDNF and BDNF in astroglia treated with conditioned medium (CM) freshly prepared from LPS-treated microglia with or without NOX2 inhibitor, apocynin. As seen in Fig. 5**A**, LPS-derived CM significantly elevated the mRNA levels of TNFα, iNOS, GDNF and BDNF in astrocyte, which was significantly reduced in astrocyte treated with LPS/Apocynin-derived CM.

We further assessed the mRNA levels of TNFα, iNOS, GDNF and BDNF in H_2O_2-treated astrocyte culture to directly reflect the effects of H_2O_2 on the functional changes of astrogliosis. As shown in Fig. 5**B**, H_2O_2 significant increased the mRNA levels of both proinflammatory factors TNFα, iNOS and neurotropic factors GDNF and BDNF in astrocyte, suggesting that H_2O_2 alone is enough to regulate the immunological functions of astrogliosis.

3.6. H_2O_2 regulates the immunological functions of astrogliosis via a STAT1/3-dependent manner

We subsequently determined the mechanism by which NOX2-generated H_2O_2 regulates astrogliosis. JAK/STAT signaling pathway appeared to be a likely candidate because it is linked to enhanced expression of GFAP, *in vitro*, in a model of gliogenesis [26]. We therefore analyzed the expression and phosphorylation of STAT1 and

Fig. 4. H_2O_2, a product of microglial NOX2 activation, mediates LPS-induced astroglial activation *in vitro*. (A) Mixed glial cultures were treated with different concentrations of LPS and the levels of H_2O_2 in the supernatant were determined by using a commercial kit. (B) The levels of H_2O_2 in the supernatant and in astroglia were determined in a trans-well co-culture system treated with LPS with or without DPI or apocynin. (C) The expressions of GFAP were determined in H_2O_2-treated astroglia cultures by Western blot and the density of the blot was quantified. (D) The toxicity of H_2O_2 on astroglia was determined by MTT assay. (E) Astroglia were stained with anti-GFAP antibody after 24 h of H_2O_2 treatment and the representative images were shown. (F) The expressions of GFAP were determined in 50 μM H_2O_2-treated astroglia cultures at the indicated time points by Western blot and the density of the blot was quantified. The results are expressed as the percentage of controls from three experiments performed in duplicate. *$p < 0.05$. **$p < 0.01$; Scale bar=50 μm.

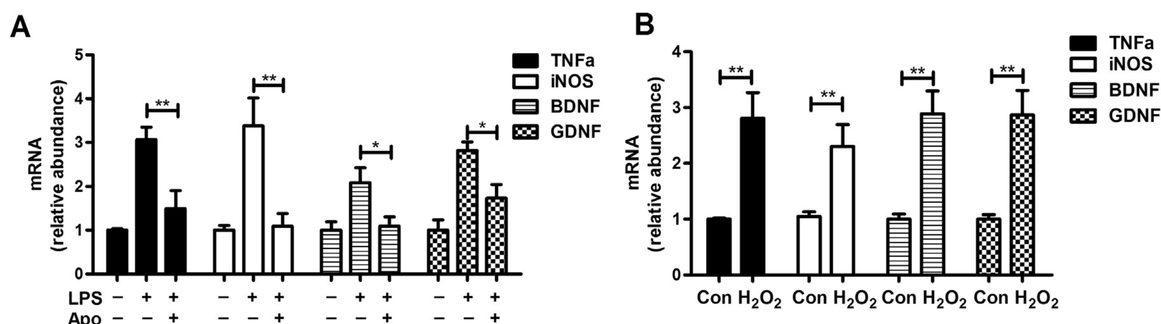

Fig. 5. NOX2-generated H_2O_2 stimulates astroglia to produce proinflammatory and neurotrophic factors. (A) The mRNA levels of TNFα, iNOS, GDNF and BDNF were determined in astrocyte treated with conditioned medium freshly prepared from LPS-treated microglia with or without apocynin pre-treatment. (B) Astrocyte cultures were treated with 50 μM H_2O_2 and the mRNA levels of TNFα, iNOS, GDNF and BDNF were determined by RT-PCR. The results are expressed as the percentage of controls from three to six experiments. *$p < 0.05$. **$p < 0.01$.

STAT3 in astroglia culture treated with H_2O_2. Representative immunoblot data for phosphorylated STAT (pSTAT) and total STAT are shown in Fig. 6**A**. H_2O_2 dose-dependently caused a significant increase of both STAT1 and STAT3 phosphorylation in astrocyte. Time course study revealed the peak of STAT1 and STAT3 activation at 60 and 30 min after H_2O_2 stimulation, respectively (Fig. 6**B**), that precedes the induction of GFAP observed in Fig. 4. Densitometric analysis of band intensities revealed a significant ($p < 0.01$) increase of p-STAT1 at 60 mins (355.3% ± 56.6%) and p-STAT3 at 30 mins (427.5% ± 54.9%) after H_2O_2 treatment (Fig. 6**B**). No significant change of total STAT1

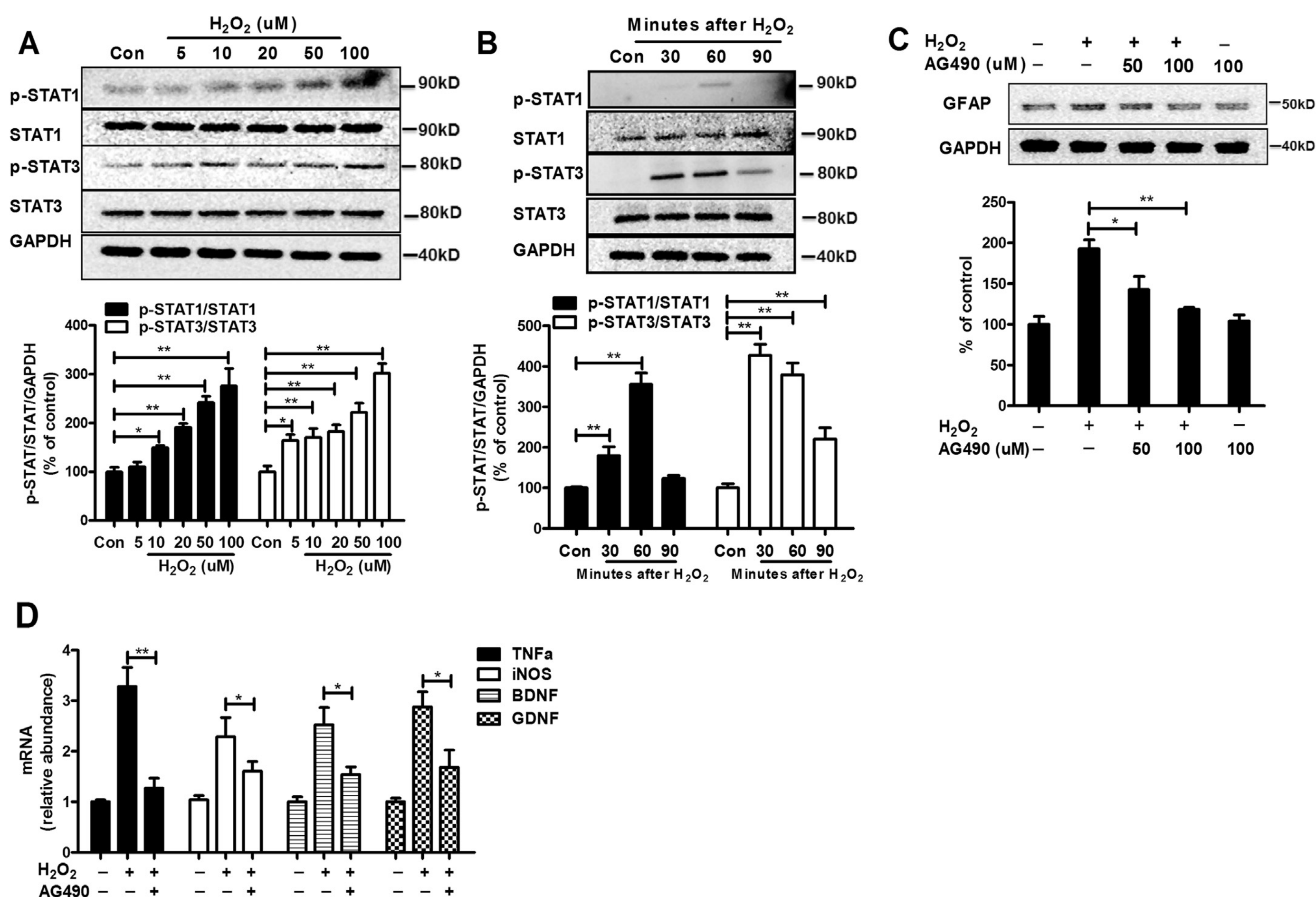

Fig. 6. H_2O_2 regulates the immunological functions of astrogliosis in a STAT1/3-dependent manner. (A) The levels of phosphorylated and nonphosphorylated STAT1 and 3 were determined in H_2O_2-treated astroglia cultures at 1 h of treatment by Western blot and the density of the blot was quantified. (B) The levels of phosphorylated and nonphosphorylated STAT1 and 3 were determined in astroglia culture at 30, 60 and 90 mins of H_2O_2 treatment by Western blot and the density of the blot was quantified. (C) The expressions of GFAP were determined in H_2O_2-treated astroglia cultures with or without AG490 pre-treatment by Western blot and the density of the blot was quantified. (D) The gene expressions of TNFα, iNOS, GDNF and BDNF were determined H_2O_2-treated astroglia cultures with or without AG490. Results are expressed as the percentage of controls from three experiments performed in duplicate. *$p < 0.05$. **$p < 0.01$.

and STAT3 was observed in H_2O_2-treated cells during the experiments.

To characterize the role of STAT1/3 in astrogliosis induced by H_2O_2, we examined the effects of AG490, an inhibitor of STAT1/3, on H_2O_2-induced astroglial activation. Pretreatment of AG490 significantly inhibited the expressions of GFAP in H_2O_2-treated astrocyte, indicating attenuation of astroglial activation (Fig. 6**C**). Quantitative analysis revealed that H_2O_2 caused a 192.8% (± 11.1%) increase of GFAP expression that was reduced to 142.9% (± 16.1%) and 118.5% (± 2.3%) in the presence of 50 and 100 μM AG490, respectively (Fig. 6**C**). Meanwhile, RT-PCR analysis showed that the elevation of TNFα, iNOS, GDNF and BDNF mRNA levels in H_2O_2-treated cultures was also suppressed by AG490 (Fig. 6**D**).

4. Discussion

Results from this study provide interesting insights into the mechanisms of how activated microglia regulates immunological functions of astrogliosis in experimental models of PD (Fig. 7). We found that the activation of astroglia was delayed compared with microglia in both LPS and MPTP-treated mice, which could be further suppressed by inhibiting microglia through inactivation of NOX2. For the mechanism of regulation, we discovered that H_2O_2 released from NOX2 was a key for the immunological regulation of astrogliosis by microglia. Whereby, extracellularly generated H_2O_2 diffused into the cytoplasm of astrocyte and subsequently stimulated activation of STAT1 and STAT3, resulting in GFAP expression and both proinflammatory and neurotropic factors production.

In response to multiple pathological conditions in the CNS, astrocyte become reactive. Importantly, the response of astroglia is heterogeneous and exhibits different functional states depending on the disease context, which inevitably influences disease progression. Therefore, it is crucial to unravel the signaling cascades controlling the reactivity of astrocyte. The importance of microglia for astrogliosis has been reported previously by using minocycline, an inhibitor of microglial activation. Raghavendra et al. provided experimental evidence in an animal model of neuropathic pain by showing that inhibition of microglial activation by minocycline was associated with suppression of astrogliosis [27]. The inhibitory effect of minocycline on astroglial activation was also observed in an inflammatory mouse PD model, demonstrated by low levels of GFAP expression compared with no minocycline treatment group [28]. However, minocycline fails to interfere with astroglial activation in acute MPTP PD model, although the activation of microglia is markedly mitigated [6]. The inconsistent reports prompted us to further explore the role microglia in the regulation of astrogliosis in PD models. In the present study, we found that microglial activation preceded astrogliosis in two experimental PD models and inhibition of microglial activation was associated with suppressed astrogliosis. Moreover, depletion of microglia by LME abolished astroglial activation induced by LPS-induced inflammation. Our findings favored that microglia are capable of regulating astro-

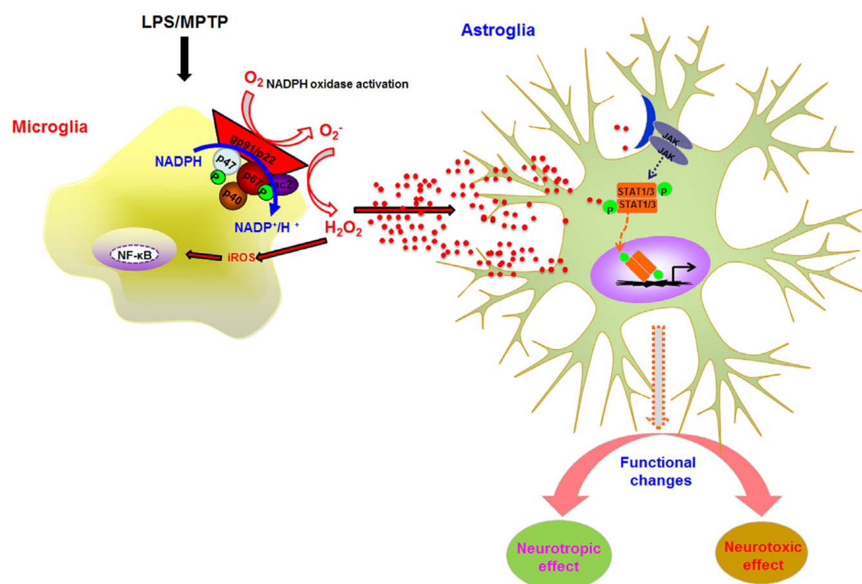

Fig. 7. Proposed mechanism showing how microglial NOX2-derived H_2O_2 regulates astrogliosis. Microglial activation induced by LPS or MPTP precedes astrogliosis. NOX2, a key superoxide-producing enzyme in microglia, is previously reported to play a pivotal role in the initiation of microglial activation leading to neuroinflammation. Pharmacological inhibition or genetic deletion of NOX2 attenuates microglial activation, which is associated with suppression of astrogliosis. NOX2-derived H_2O_2 directly activates astroglia via a process in which extracellularly generated H_2O_2 diffuses into the cytoplasm and subsequently enhances phosphorylation of transcription factor, STAT1 and STAT3. Activation of STATs regulates of the gene expressions of both pro-inflammatory and neurotropic factors, therefore, regulating the immunological functions of astrogliosis.

gliosis in experimental PD.

Mechanistically, the most critical question to address is how microglia regulates astrogliosis. Our studies suggested that H_2O_2 released from NOX2 could account for the regulation of astrogliosis by microglia. This conclusion was supported by the following findings: 1) pharmacological inhibition or genetic deletion of NOX2 attenuated LPS or MPTP-induced astroglial activation; 2) the levels of H_2O_2 in astrocyte were increased in LPS-induced inflammatory conditions, which could be further reduced by NOX2 inhibitor; and 3) H_2O_2 was able to directly stimulate astroglial activation. Consistent with our findings, the elevated GFAP expression by H_2O_2 was also observed in human glioblastoma cell line, A172 and human astrocytes cell line derived from the spinal cord [29]. Since pro-inflammatory factors are capable of inducing astroglial activation [12,13], we believed that exacerbating release of proinflammatory factors from activated microglia by NOX2-generated H_2O_2 could also contribute to astrogliosis. As an important secondary messenger, microglial NOX2-generated H_2O_2 can penetrate into microglia and enhance the activation of multiple immune-regulating pathways, such as mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB), inevitably elevating the gene expressions of a variety of inflammatory factors from activated microglia [7,30]. In agreement, lack of functional NOX2 markedly mitigates LPS-induced production of TNFα, PGE2 and IL-1β from microglia [31]. Notably, although NOX2 is recognized as the main source of H_2O_2 in regulating astrogliosis in the current study, we still cannot exclude the possibility that H_2O_2-generated from other enzymes may also contribute to the stimulation of astrogliosis, especially in *in vivo* conditions. It is well known that mitochondrial dysfunction is involved in the pathogenesis of PD [32]. Impairment of oxidative phosphorylation due to mitochondrial deficits is associated with increased free radical production and the resultant oxidative damage [33]. In MPTP-treated mice, 1-methyl-4-phenylpyridinium (MPP^+), the active metabolite of MPTP, accumulates within SNpc dopaminergic neurons and subsequently inhibits mitochondrial complex I to stimulate the production of superoxide radical. Superoxide can be changed into H_2O_2, resulting in elevation of H_2O_2 in microenvironment. Therefore, investigating the role of mitochondrial dysfunction in mediating astrogliosis should be guaranteed in the future.

The JAK-STAT pathway is the predominant signaling pathway used by certain oxidative stress stimuli and cytokines, and is critical for initiating innate immunity. Although the critical role of JAK-STAT pathway in microglial activation is well documented, it is still remains unclear whether JAK-STAT pathway is involved in H_2O_2-induced astroglial activation. In this study, we found that H_2O_2 dose-dependently induced activation of both STAT1 and STAT3 in primary astrocyte cultures. Moreover, AG490 suppressed H_2O_2-induced elevation of GFAP expression, which was associated with reduction of gene expressions of TNFα, iNOS, BDNF and GDNF. These results suggest that H_2O_2 regulates astroglial activation through a STAT-dependent manner. In brains of scrapie-infected mice, immunostaining revealed that the activation of STAT1 was mainly localized in astrocyte and strongly associated with reactive astrogliosis [34]. Similarly, the activation of JAK-STAT pathway was also involved in prolactin-induced astroglial activation and proinflammatory cytokine TNFα production in cultured astrocyte [35]. In agreement, deletion of Yes-associated protein (YAP), an inducer of the suppressor of cytokine signaling 3, results in hyperactivation of both STAT1 and 3, and reactive astrogliosis in cultured astrocyte and in developing mice brain [36]. The mechanism responsible for JAK-STAT-mediated astroglial activation might be related to enhanced gene expression of gfa gene that encodes GFAP. A potential STAT-binding site (TTCCGAGAA) in both the rat and human GFAP promoters was found previously [37,38]. Furthermore, in a model of gliogenesis [26], a mutation in the STAT DNA-binding sequence within GFAP promoter that disrupted the binding of STAT1 and STAT3 blocked completely the ability of ciliary neurotrophic factor to activate GFAP promoter-driven reporter gene expression.

Previous studies reported that beyond controlling activation, different members of STATs are involved in regulating microglial phenotype (M1/M2) [39,40]. It is reported that STAT1 activation induced by IFN-γ leads to an increase in the production of proinflammatory cytokines and, therefore, programs microglia/macrophage to the M1 phenotype. In contrast, activation of STAT3 and STAT6 contributes to release of anti-inflammatory and neurotrophic factors from IL-4, IL-13, and IL-10-treated microglia/macrophage (M2 phenotype) [41]. However, whether the detrimental and beneficial functions of astrogliosis are also governed by different STATs remains unknown. Although our current study does not address this question,

related experiments are designed and undergoing in our lab.

Altogether, our results indicated that H_2O_2 derived from microglial NOX2 plays an important role in the initiation and maintenance of astrogliosis in experimental models of PD through a STAT1/3-dependent pathway. This study, at least partially, permits us to re-evaluate the immunomodulatory role of microglia-to-astrocyte communication in the pathogenesis and therapeutic studies of PD. Future studies should attempt to enhance the neurotrophic functions of astrogliosis once over-activated microglia were inhibited in attempting to interrupt progressive pathogenic cascade in neurodegenerative diseases.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2017.02.016.

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