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Inhibition of NOX2-NLRP1 signaling pathway protects against chronic glucocorticoids exposure-induced hippocampal neuronal damage

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ABSTRACT

Glucocorticoids (GCs) exposure has deleterious alteration on the structure and function in hippocampal neurons. NADPH oxidase 2 (NOX2) is a major contributor to oxidative stress in neurological diseases, and NLRP1 inflammasome can be activated in response to oxidative stress. We hypothesize that inhibition of NOX2-mediated NLRP1 inflammasome activation may protect against chronic GCs exposure-induced neuronal injury. In this study, the lentivirus with NLRP1-siRNA was injected into the hippocampus of male mice which were then treated with dexamethasone (DEX, 5 mg/kg) for 28 d. The data indicated that NLRP1-siRNA treatment down-regulated the NLRP1 expression and significantly improved the exploratory behavior and spatial memory deficits in open field tests and Morris water maze which were deteriorated by chronic DEX treatment in mice. Additionally, inhibition of NLRP1 expression significantly alleviated neuronal degeneration and increased MAP2 expression in the hippocampus in mice. Meanwhile, the results showed that DEX exposure increased NOX2, p22phox and p47phox expression in hippocampus tissue in mice. We further examined the effect of tempol (ROS scavenger) and apocynin (NOX inhibitor) treatment on NLRP1 inflammasome activation in chronic DEX-treated hippocampal neurons. The results revealed that the tempol (50 μM) and apocynin (50 μM) treatment significantly decreased generation of ROS, expression of NOX2 and NLRP1-related protein in DEX-treated hippocampal neurons. These data indicate that NOX2-mediated NLRP1 activation involves in chronic GCs exposure-induced neuronal injury and inhibition of NOX2-NLRP1 signaling pathway protects against GCs-induced neuronal damage.

1. Introduction

Glucocorticoids (GCs) are important adrenal steroid hormones that are closely associated with almost all tissues of the body, regulating metabolism, development, immune and stress response [1]. GCs have been used in the treatment of a variety of diseases, such as autoimmune diseases, inflammation and allergies. However, long-term treatment with GCs also brings many adverse reactions. Growing evidences have shown that chronic stress and stress-level of GCs exposure may induce frontal cortex and hippocampus atrophy and functional impairments, which is closely related to the development of Alzheimer's disease (AD) [2,3]. And clinical studies also indicate that plasma level of GCs is correlated with the rate of dementia progression of AD [3]. Our previous studies showed that chronic stress-level of GCs treatment significantly induced learning and memory impairment and hippocampal neurons damage [4]. These studies suggest that chronic GCs exposure may result in hippocampal neurons damage, but the precise mechanism of these alterations have not been fully elucidated.

It has been reported that neuroinflammation plays important roles in the pathogenesis of neurodegenerative diseases such as AD. The neuroinflammation is considered to be reaction and contributor to the neuronal injury [5]. The inflammasome is a large macromolecular complex and plays critical roles in regulating the inflammatory response and contributes to the pathogenesis of various neurologic diseases, such as AD and traumatic brain injury [6]. The formation of inflammasome activates caspase-1 and subsequently mediates the release of pro-inflammatory cytokines [7]. NLRP1 is the first reported and seems to be expressed rather extensively. High-level of NLRP1 was found to be expressed in central nervous system, especially in neurons [8]. Tan et al. reported that inhibition of NLRP1 expression could ameliorate age-related cognitive deficits [9]. In our latest study, we found that chronic dexamethasone (DEX) treatment activated NLRP1

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inflammasome and subsequently induced hippocampal neurons damage both *in vitro* and *in vivo* [10,11]. However, the precise mechanism underlying this phenomenon remains unclear.

Oxidative stress is due to the imbalance of oxidation and reduction in the body, resulting in excessive production of reactive oxygen species (ROS). ROS oxidative stress is considered to be the main cause of neurodegenerative diseases [12]. NADPH oxidase (NOX) is the main family of enzyme solely dedicated to the generation of ROS that can damage cellular components and induce functional abnormalities in many cell types [13,14]. Growing evidences showed that ROS was closely related to the activation of NLRP inflammasome and progression of neurological diseases [15,16]. ROS generation has been heavily implicated as an upstream event in the activation of the NLRP3 inflammasome [17]. Furthermore, it has been reported that treatment with GCs increased ROS both *in vitro* and in the brains of animals, and also down-regulated expression of various antioxidant enzymes [18,19]. The above studies suggest that NOX can potentially regulate NLRP1 inflammasome activation. Inhibition of NOX2-mediated NLRP1 inflammasome activation may become an important therapeutic target for chronic GCs exposure-induced neuronal injury.

In this study, we hypothesize that inhibition of NOX2-mediated NLRP1 inflammasome activation may protect against chronic GCs exposure-induced neuronal injury. We firstly observed the protective effects of NLRP1-siRNA intrahippocampal injection on chronic DEX (5 mg/kg) treatment induced learning and memory impairment and neuronal damage. Furthermore, we examined the effect of apocynin (50 μM) and tempol (50 μM) treatment on ROS generation and NLRP1 inflammasome activation in DEX (5 μM)-treated hippocampal neurons. Our study suggests that inhibition of NOX2-mediated NLRP1 inflammasome activation alleviates chronic GCs exposure-induced neuronal damage.

2. Materials and methods

2.1. Animals and treatment

Adult male ICR mice (25–30 g) were purchased from the Center of Laboratory Animals of Anhui Medical University and maintained in 12-h light/dark cycle, temperature (22–24 °C) and humidity-controlled rooms. The mice were fed standard food with *ad libitum* access to water. All experiments were performed in accordance with protocols approved by the ethics committee of laboratory animals in Anhui Medical University.

Firstly, to confirm the down-regulation effect of NLRP1-siRNA (408, 883 and 1372) on NLRP1 expression, the mice were injected in both hemisphere of hippocampus with the PBS, negative control lentivirus (scramble control) with GFP or the NLRP1-siRNA lentivirus (408, 883 and 1372) for six weeks. The mice (n = 3) were sacrificed, half of brains were performed frozen sections to examine the expression of GFP under fluorescence microscope to show the transfection effect of lentivirus vector. Another half of brains hippocampus tissues were taken to extract the total protein to detect the expression of NLRP1 in hippocampus was detected by WB. The method of intrahippocampal injection was described as previous study [20]. The mice were anesthetized with 0.35% pentobarbital sodium (0.1 ml/10 g, i.p.). The lentivirus (1 μl) was injected stereotactically into the hippocampal CA1 region of both hemisphere of mouse (coordinates: 2.0 mm posterior to bregma; lateral, ± 2.0 mm; dorsoventral, 2.5 mm) with a 10 microsyringe at an injection speed of 0.2 μl/min for 5 min. The needle was kept in the injection site for another 5 min and then slowly withdrawn. The sequence of negative lentivirus vector is 5′ TTC TCC GAA CGT GTC ACG T 3′, NLRP1-siRNA (883) is 5′ GGG TCT TAG CAG ACC AGA ATC 3′, NLRP1-siRNA (408) is 5′ GCT CTT TAC CCT CTT CTA ACA 3′ and NLRP1-siRNA (1372) is 5′ GCC AAA GAA GGA CCC TGT TCA 3′ (1×10^7 TU/ml, Shanghai GenePharma Co., China). The lentivirus with NLRP1-siRNA (408) sequence was selected to perform subsequent experiments.

Then the mice were randomly divided into 4 groups (n = 8): control group (normal saline, NS), dexamethasone (DEX, 5 mg/kg) group, control lentivirus group (scramble control), and NLRP1-siRNA group (NLRP1-siRNA 408 + DEX). Mice were injected stereotactically into the both hippocampal CA1 regions with PBS, the control lentivirus or the NLRP1-siRNA (408) lentivirus. After injection of the lentivirus for 2 weeks, the DEX and NLRP1-siRNA groups mice were treated subcutaneously (s.c.) with DEX (Sigma, USA, 5 mg/kg/d, 0.05 ml/10 g body weight) for 28 days (d), and the control and scramble control groups mice were injected (s.c.) with equal volume of solvent for 28d. After behavioral tests, the mice were sacrificed and the brains were removed immediately and stored at −80 °C for immunoblot analysis or fixed with 4% paraformaldehyde for histological and immunohistochemistry examination. The DEX (Sigma, USA) solution was prepared by dissolving DEX in alcohol in concentrations of 500 mg/ml and diluted with NS in concentrations of 1 mg/ml.

2.2. Open field test (OFT)

The open field apparatus (Shanghai Biowill Co., Ltd., Shanghai, China) (60 × 60 × 50 cm) consists of a roofless box with plywood walls in which the floor is divided into 9 (3 × 3) smaller rectangular units. The OFT was often used for analysis of locomotion, exploration, and anxiety. A video camera was mounted centrally above the open field and connected to a computerized tracking system. Each mouse was placed in the apparatus for 2 min as a pre-adaption period, then permitted to move and explore freely for 3 min. The moving distance (m), the mean moving speed (m/s), the numbers of line crossing and standing up events (indicating exploratory behavior) were recorded by Any-maze tracking software (Stoelting Co., Wood Dale, IL, USA).

2.3. Morris water maze (MWM)

The Morris water maze (MWM) test is a well-established test of hippocampal dependent spatial learning and memory [21]. Briefly, a circular black pool (diameter 120 cm, height 50 cm) filled with water kept at temperature of 23 °C–25 °C. The pool was divided into four equal quadrants by 2 mutually perpendicular lines and the wall was marked with square, triangle, diamond and circle respectively. Moreover there was a circular escape platform (9 cm in diameter) that was submerged approximately 1 cm below the water surface at the midpoint in the third quadrant. Mice (n = 8) were performed four trials from four quadrants to locate the escape platform for 4 d. In each trial, the animal faced with the pool wall was allowed to swim within a maximum of 1 min to find the platform and the animal that failed to find the escape platform was gently guided to the platform by the experimenter. Animals were allowed to remain for 10 s on the platform at the end of every trial to familiarize the location of the platform. On the fifth day, the platform was removed from the pool, and each mouse was allowed to swim freely for 1 min from the first quadrant. The parameters of mean escape latency (MEL, s) in learning test, the swimming time in the quadrant of platform (STP, s), the number of crossing the platform (NCP) and the latency of first entry to the platform (LFP, s) were monitored with a video camera linked to a computer with ANY-maze tracking software for analysis.

2.4. Histological examination

After intraperitoneally anesthetized with 0.35% pentobarbital sodium (0.1 ml/10 g), the mice were perfused transcardially with NS and 4% paraformaldehyde (PFA) to wash out the blood cells and fix the brains. Then the brains (n = 4) were carefully removed and immersed into 4% paraformaldehyde for further fixation. Paraffin-embedded brain tissues were cut into at 5 μm transverse sections. After that, all the sections were stained with hematoxylin and eosin (H&E) reagents and

the neuronal morphology in the hippocampus CA1 and CA3 was observed using an optical microscope (Olympus BX52, Japan). The degenerating cells that displayed small soma and were deeply stained were counted in double-blind to indicate the neuronal pathological injury.

2.5. Hoechst 33258 staining

To further examine the hippocampal neurons damage, the Hoechst 33258 staining was used to evaluate the apoptosis of hippocampal neurons [4,22]. Briefly, paraffin slices (n = 4) were deparaffinized and hydrated, then stained with Hoechst33258 (5 μg/ml, Zhongshan Golden Bridge Biotechnology Co.) for 15 min. The section was washed with PBS three times, then mounted with anti-fade mounting medium (Beyotime Biotechnology Co.). The apoptosis cells were detected with a fluorescence microscope (Olympus BX52, Japan). The cells undergoing apoptosis appear smaller and the nucleus is condensed and deeply staining [11]. The normal cells and apoptosis cells were identified in double-blind from three fields (×400) of hippocampus CA1 and CA3 in each section. The apoptosis rate of neurons was used to evaluate the neuronal damage.

2.6. TUNEL assay

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay was used to detect apoptotic nuclei in hippocampus CA1 and CA3. TUNEL assay was performed according to the manufacturer's method of TUNEL Detection Kit (Beyotime Biotechnology Co.). The positive neurons were stained brown and three fields (×400) of hippocampus CA1 and CA3 in each section were analyzed by using Image-Pro Plus 6.0 automatic analysis system (n = 3). The apoptosis cells were expressed as the mean optical density over control group.

2.7. Immunohistochemistry

We further detected the expression of MAP2 in hippocampus by immunohistochemistry. The paraffin sections (n = 4) were deparaffinized and hydrated, then were treated by microwave in citric acid buffer (pH 6.0) for antigen retrieval. After washing three times in PBS, endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide for 30 min. Subsequently, the sections were blocked with 1% non-immune goat serum for 30 min. Then, the sections were incubated with the mouse anti-MAP2 antibody (1:200; ab32454; Abcam, UK) at 4 °C overnight. Then the sections were washed and incubated in the biotinylated secondary antibody for 60 min. After that, the sections were washed and visualized by using an ABC kit (Beijing Zhongshan Golden Bridge Biotechnology Co., China). The sections were mounted and observed by a microscope (Olympus BX52; Olympus). The positive neurons were stained brown and three fields (×400) of hippocampus CA1 and CA3 in each section were analyzed by using Image-Pro Plus 6.0 automatic analysis system. The mean optical density was counted to evaluate the expression of MAP2.

2.8. The hippocampal neurons primary culture and treatment

The hippocampal neurons were isolated from the hippocampus of neonatal rats (within 24 h). The neurons were planted on poly-L-lysine-coated (10 μg/ml) 6-well culture plates with 1×10^7 cells/well or 24-well culture plates with 1.5×10^5 cells/well. The Neurobasal mediums with B27 supplements (Invitrogen, USA) were used to culture the neurons at 37 °C with 5% CO_2 and changed every 3 d. After the neurons were cultured for 6 d, the neurons were divided into 4 groups: control group, DEX (5 μM), DEX (5 μM) + tempol (50 μM) and DEX (5 μM) + apocynin (50 μM) groups. Except the control group, the neurons were treated with DEX (5 μM), DEX (5 μM) + tempol (50 μM) and DEX (5 μM) + apocynin (50 μM) for 3 d. All experiments were repeated at least three times.

2.9. Neuronal apoptosis and intracellular ROS measurement

The Hoechst 33258 staining was used to evaluate the apoptosis of hippocampal neurons *in vitro*. The Hoechst 33258 staining is similar to the mouse experiment. The apoptotic neurons were examined in double-blind from five random fields (400×) per slide in triplicate by fluorescence microscopy (Olympus IX71, Japan). The neuronal apoptosis rate was determined to indicate the changes of neuronal apoptosis.

Intracellular ROS production was assessed by staining with DHE, which is the mostly used ROS fluorescent probe for labeling the living cells. For the ROS examination, the neurons were incubated in DHE assay reagent (5 μM) for 30 min at 37 °C in the dark. Then the cells were washed with PBS and mounted with anti-fade mounting medium (Beyotime Biotechnology Co.). The production of ROS was examined with a fluorescence microscope (Olympus IX71, Japan). The red mean optical density of five fields (×400) from three experiments was counted with the Image-Pro Plus 6.0 automatic analysis software (Media Cybernetics, Inc., USA) to indicate the ROS production in the neurons.

2.10. Western blot analysis

The mice hippocampus tissues and the primary hippocampal neurons were lysed in RIPA buffer (Beyotime Biotechnology, Shanghai) including protease and phosphatase inhibitors to extract the total protein. The BCA protein assay kit (Beyotime Biotechnology, Shanghai) was used to determine protein concentrations. Equal amounts (30 μg) of proteins were separated electrophoretically by 12–15% SDS-PAGE gels and then transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were soaked in blocking buffer (5% skimmed milk in TBST) for 1 h and then probed with primary antibodies of NLRP1 (Abcam, ab3683, 1:1000), ASC (Santa Cruz, SC-514414, 1:500), caspase-1 (Abcam, ab1872, 1:1000), IL-1β (Abcam, ab9722, 1:500), NF-κB (Abcam, ab16502, 1:1000), p-NF-κB (Wanleibio, WL02169, 1:500), NOX2 (Abcam, ab31092, 1:1000), P22phox (Bioworld Technology, BS60290, 1:500), P47phox (Bioworld Technology, BS4852, 1:1000), RAC (Bioworld Technology, BS71440, 1:1000), MAP2 (Abcam, ab32454, 1:1000), cleaved-caspase-3 (Bioworld Technology, BS90186, 1:1000), or β-actin (1:1000) overnight at 4 °C, followed by secondary antibody conjugated to HRP (ZSGB-BIO, ZF-2301, 1:10,000) for 1 h at room temperature. Following three washing in TBST, protein signal was visualized by the enhanced chemiluminescence reagents (Amersham Biosciences, UK). The results were visualized by using a Chemi Q4800 mini imaging system (Shanghai Bioshine Technology, Shanghai, China). The density of protein bands were measured with Image J 1.44 software (National Institutes of Health, USA), then normalised to its β-actin bands. The relative density over the control group was counted to present the expression of target proteins.

2.11. Statistical analysis

All results were expressed as mean ± SD. Statistical differences were performed by one-way ANOVA and Bonferroni's *post hoc* test for between-group comparisons with SPSS 17.0 software. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. NLRP1-siRNA treatment inhibits NLRP1 expression in hippocampus in mice

We first observed the transfected effect of intrahippocampal injection of lentivirus with GFP for 6 weeks (w). The results showed that

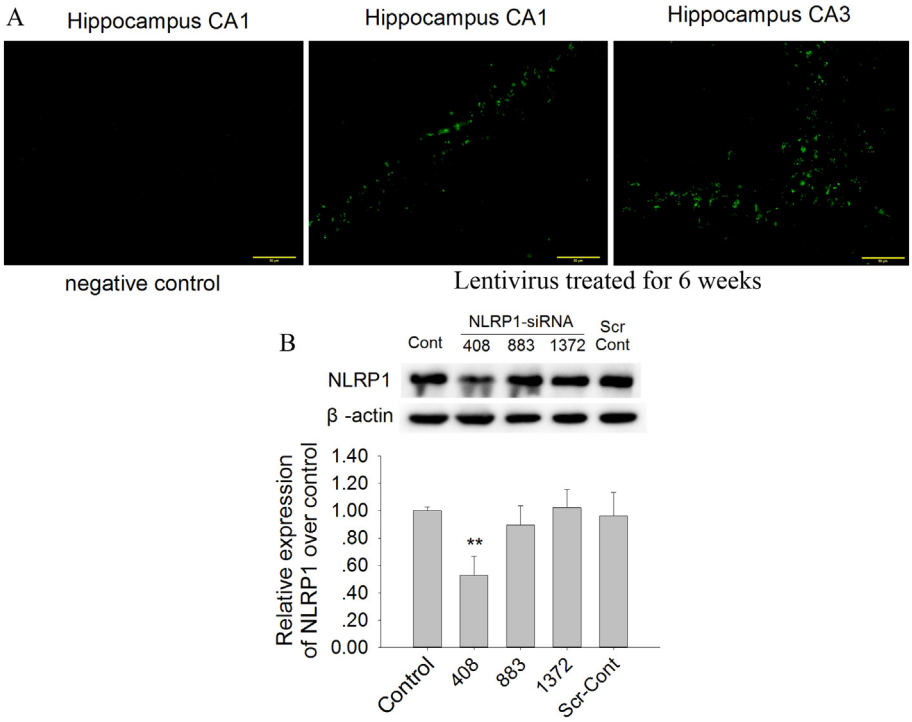

Fig. 1. Effects of lentivirus-mediated NLRP1-siRNA intrahippocampal injection on NLRP1 expression in mice. (A) The results of lentivirus treatment for 6 weeks on GFP expression in the hippocampus CA1 and CA3 (× 400, n = 3). (B) The results of lentivirus-mediated NLRP1-siRNA (408, 883 and 1372) treatment for 6 weeks on NLRP1 expression in the hippocampus (immunoblot). Results are expressed as mean ± SD, n = 3, $^{**}P < 0.01$ compared to control group.

treatment with lentivirus with GFP for 6 weeks showed obvious expression of GFP in hippocampus CA1 and CA3, indicating successful lentivirus infection in hippocampus in mice (Fig. 1A). We further observed the interference effect of NLRP1-siRNA (408, 883 and 1372) treatment for 6w on NLRP1 expression in hippocampus in mice. The results indicated that the lentivirus with NLRP1-siRNA (408) treatment could down-regulate NLRP1 expression in hippocampus (Fig. 1B, $P < 0.01$), and this provided a strong foundation for the following experiments.

3.2. Effects of NLRP1-siRNA treatment on motor and cognitive behavior in chronic DEX-treated male mice

The open field test (OFT) was used to measure the motor and exploratory behavior in mice [23]. The results indicated that, compared with control group, the control lentivirus with scramble RNA treatment had no significant influence on the motor and exploratory behavior in male mice. While chronic DEX treatment for 28 d significantly decreased the total moving distance (m), the mean moving speed (m/s), the number of line crossing and the number of standing up events (Table 1, $P < 0.05$ or $P < 0.01$). Compared with DEX-treated group, intrahippocampal injection of lentivirus-mediated NLRP1-siRNA (408) significantly increased the total moving distance (m), the mean moving speed (m/s), the number of line crossings and the number of standing up events which were decreased by chronic DEX exposure (Table 1, $P < 0.05$).

We further examined the learning and memory capabilities by Morris water maze (MWM) test. In the navigation test, the results showed that chronic DEX treatment significantly increased the escape latency (s) on the second day and forth day compared to control group mice (Table 2, $P < 0.05$ or $P < 0.01$). While compared with DEX-treated group, NLRP1-siRNA (408) treatment significantly decreases the escape latency (s) on the fourth day in the DEX-induced mice (Table 2, $P < 0.05$). In the space exploration experiment on fifth day, the platform was removed from the pool to measure spatial memory capability. The results showed that chronic DEX treatment significantly increased the latency of first entry to the platform (LFP, s), decreased the swimming time in the quadrant of platform (STP, s) and the number of crossing the platform (NCP) (Table 3, $P < 0.05$ or $P < 0.01$). While compared with DEX-treated group, NLRP1-siRNA treatment could reduce the latency of first entry to the platform (LFP, s), increased the swimming time in the quadrant of platform (STP, s) and the number of crossing the platform (NCP) (Table 3, $P < 0.05$ or $P < 0.01$). And compared with control group, the control lentivirus treatment had no significant difference in both learning and memory test in male mice.

3.3. Effects of NLRP1-siRNA treatment on neuronal damage and apoptosis in the hippocampus in DEX-induced mice

We investigated the morphological changes of neuronal

Table 1
Effects of NLRP1-siRNA treatment on motor and exploratory behavior in chronic DEX-treated male mice (open field test).

Group	Moving distance (m)	Mean speed (m/s)	Line crossing	Number of stand up
Control	13.71 ± 3.40	0.076 ± 0.019	63.00 ± 15.30	48.13 ± 8.68
DEX	9.08 ± 2.87*	0.050 ± 0.016*	42.25 ± 15.09*	27.75 ± 9.82**
Scr-RNA	12.45 ± 1.91	0.069 ± 0.011	59.25 ± 10.19	41.38 ± 12.45
NLRP1-siRNA	12.26 ± 2.51#	0.068 ± 0.014#	58.75 ± 12.19#	40.00 ± 7.48#

Results are expressed as mean ± SD, n = 8.
* $P < 0.05$.
** $P < 0.01$ *vs* control.
# $P < 0.05$ *vs* DEX group.

Table 2
Effects of NLRP1-siRNA treatment on the mean escape latency in chronic DEX-treated male mice (navigation test, Morris water maze).

Group	Mean escape latency (MEL, s)			
	d1	d2	d3	d4
Control	38.38 ± 15.34	21.94 ± 10.07	25.11 ± 14.02	18.57 ± 6.26
DEX	49.90 ± 10.26	43.99 ± 11.85**	35.55 ± 10.41	36.36 ± 16.34*
Scr-RNA	39.62 ± 15.04	23.35 ± 12.07	20.88 ± 8.83	19.27 ± 11.45
NLRP1-siRNA	39.23 ± 14.42	34.33 ± 15.38	30.06 ± 15.42	21.71 ± 7.45#

Results are expressed as mean ± SD, n = 8.
* $P < 0.05$.
** $P < 0.01$ *vs* control.
# $P < 0.05$ *vs* DEX group.

Table 3
Effects of NLRP1-siRNA treatment on memory capability in chronic DEX-treated male mice (the space exploration experiment, Morris water maze).

Group	Latency of first entry to the platform (LFP, s)	Swimming time in the quadrant of platform (STP, s)	Number of crossing the platform (NCP)
Control	11.51 ± 6.23	23.03 ± 7.88	2.63 ± 0.92
DEX	49.00 ± 17.92**	13.41 ± 5.87*	1.14 ± 0.90**
Scr-RNA	19.51 ± 8.65	23.98 ± 9.15	1.88 ± 0.83
NLRP1-siRNA	22.60 ± 14.32##	21.28 ± 7.20#	2.38 ± 1.19#

Results are expressed as mean ± SD, n = 8.
* $P < 0.05$.
** $P < 0.01$ *vs* control.
# $P < 0.05$.
$P < 0.01$ *vs* DEX group.

histopathology of hippocampus CA1 and CA3 and counted the average numbers of degenerating cells which displayed small soma, karyopyknosis and were deeply stained. As shown in Fig. 2, there were less degenerating neurons in the hippocampus CA1 and CA3 in control group and scramble control group. Compared with control group, DEX treatment significantly increased the degenerating neurons in hippocampus CA1 and CA3 (Fig. 2A, B and C, $P < 0.01$). While compared with DEX-treated group, NLRP1-siRNA treatment dramatically decreased the degenerating neurons in hippocampus CA1 and CA3 (Fig. 2A, B and C, $P < 0.05$).

We further performed Hoechst 33258 staining and TUNEL assay to evaluate the neuronal apoptosis in the hippocampus CA1 and CA3. The results of Hoechst 33258 staining showed that the neurons undergoing apoptosis were smaller and brighter than normal cells, and the chromatin appeared condensed and deeply stained. There were less apoptotic cells in the hippocampus CA1 and CA3 in control group and scramble control group (Fig. 3A). Compared with control group, DEX treatment significantly increased the apoptotic cells in hippocampus CA1 and CA3 (Fig. 3A, B and C, $P < 0.05$ or $P < 0.01$). While compared with DEX-treated group, NLRP1-siRNA treatment dramatically reduced the apoptotic cells in hippocampus CA1 and CA3 (Fig. 3A, B

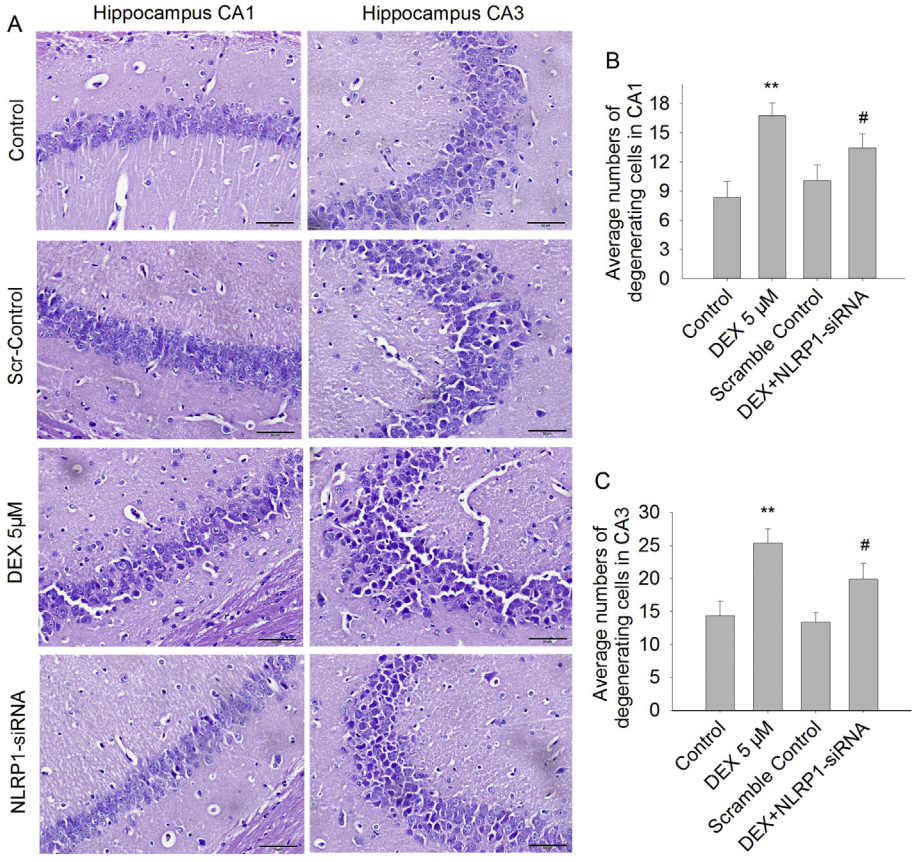

Fig. 2. Effects of NLRP1-siRNA treatment on neuronal damage in the hippocampus CA1 and CA3 induced by chronic DEX exposure (H&E staining, ×400). (A) The pathological changes of neurons in hippocampus CA1 and CA3. The degenerating cells showed smaller and were deeply stained. (B) The average numbers of degenerating cells in hippocampus CA1. (C) The average numbers of degenerating cells in hippocampus CA3. Results are expressed as mean ± SD, n = 4. $^{**}P < 0.01$ compared with the control group; $^{\#}P < 0.05$ compared with the DEX-treated group.

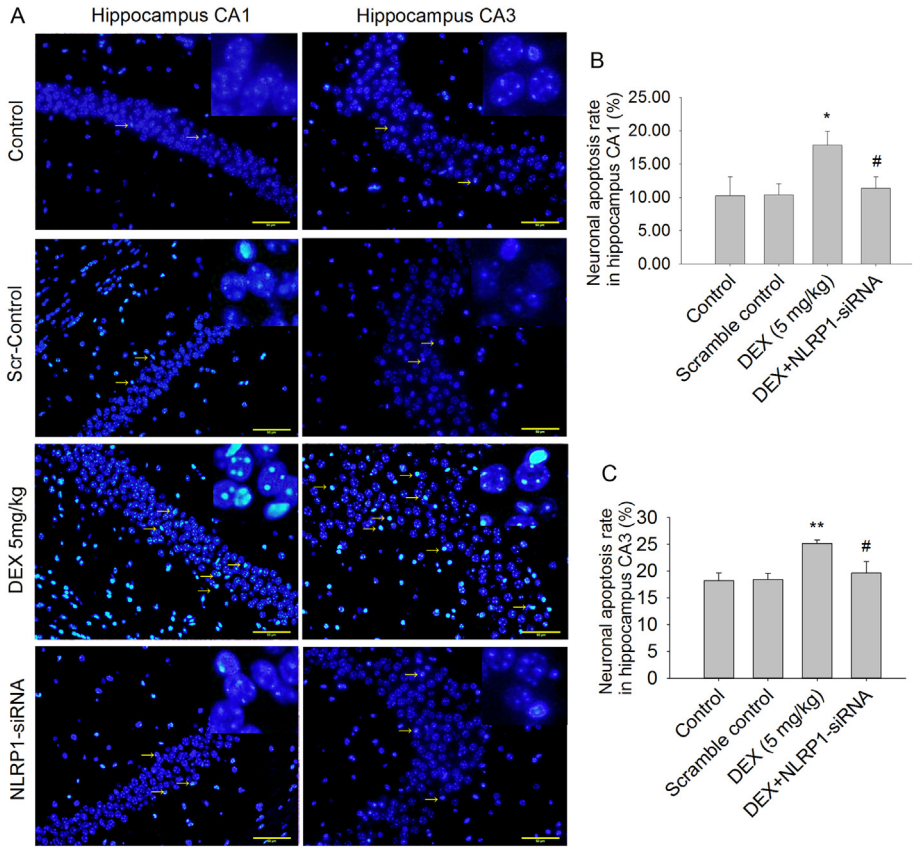

Fig. 3. Effects of NLRP1-siRNA treatment on neuronal apoptosis in the hippocampus CA1 and CA3 induced by chronic DEX exposure (Hoechst 33258 staining, ×400). (A) The morphological changes of neurons in hippocampus CA1 and CA3. The apoptosis cells are smaller and brighter than normal cells (indicated by arrows). (B) The neuronal apoptosis rate in hippocampus CA1. (C) The neuronal apoptosis rate in hippocampus CA3. Results are expressed as mean ± SD, n = 4. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group; $^{\#}P < 0.05$ compared with the DEX-treated group.

and C, $P < 0.05$). The TUNEL assay showed similar results that DEX treatment significantly increased the apoptotic cells in hippocampus CA1 and CA3 (Fig. 4A–D, $P < 0.01$). And NLRP1-siRNA treatment significantly reduced the apoptotic cells in hippocampus CA1 and CA3 (Fig. 4A–D, $P < 0.01$). Meanwhile, we detected the expression of cleaved-caspase-3 in hippocampus by immunoblot. The results also showed that DEX treatment significantly increased the expression of cleaved-caspase-3 (Fig. 4E, $P < 0.01$), while NLRP1-siRNA treatment significantly reduced the expression of cleaved-caspase-3 (Fig. 4E, $P < 0.01$).

3.4. Effects of NLRP1-siRNA treatment on MAP2 expression in hippocampus in DEX-induced mice

Immunohistochemistry was performed to examine the MAP2 expression. As shown in Fig. 5A and C, the immunoreactive neurons are stained brown. The results showed that the MAP2 expression was abundant in the hippocampus CA1 and CA3 in control group and scramble control group. Compared with control group, DEX treatment for 28 d significantly reduced the expression of MAP2 in hippocampus CA1 and CA3 (Fig. 5A–D, $P < 0.05$ or $P < 0.01$). While compared with DEX-treated group, NLRP1-siRNA treatment dramatically up-regulated MAP2 expression in hippocampus CA1 and CA3 in DEX-induced mice (Fig. 5A–D, $P < 0.05$). Meanwhile, we detected the expression of MAP2 in hippocampus by immunoblot (Fig. 5E). The results were similar to the immunohistochemistry that DEX treatment significantly decreased the expression of MAP2 (Fig. 5E, $P < 0.01$), while NLRP1-siRNA treatment significantly increased the expression of MAP2 in hippocampus in mice (Fig. 5E, $P < 0.05$).

3.5. Effects of NLRP1-siRNA treatment on expressions of NLRP1, ASC, caspase-1, IL-1β and NF-κB in hippocampus in DEX-induced mice

To confirm whether NLRP1 inflammasome activation is related to chronic DEX-induced neuronal damage, we further investigated the expressions of NLRP1, ASC, caspase-1, IL-1β, NF-κB and p-NF-κB in the hippocampus tissue in mice. The immunoblot results showed that DEX exposure for 28 d significantly increased the expressions of NLRP1, ASC, caspase-1 and IL-1β compared to control group (Fig. 6A–E, $P < 0.05$ or $P < 0.01$). While the NLRP1-siRNA treatment significantly reduced the expressions of NLRP-1, ASC and IL-1β in hippocampus which increased by chronic DEX treatment (Fig. 6A–E, $P < 0.05$). Meanwhile, we detected the expression of NF-κB and p-NF-κB in hippocampus by immunoblot. The results showed that DEX treatment significantly increased the expressions of NF-κB and p-NF-κB compared to control group (Fig. 6F–H, $P < 0.05$). NLRP1-siRNA treatment had no influence on the expressions of NF-κB which increased by chronic DEX treatment (Fig. 6F and G), but it could decrease the expression of p-NF-κB which increased by chronic DEX treatment (Fig. 6F and H, $P < 0.05$).

3.6. Effects of chronic DEX treatment on expressions of NOX2, p22phox and p47phox in the hippocampus in male mice

We further investigated the effects of DEX exposure on the expressions of NOX2-related proteins in the hippocampus. The immunoblot results showed that DEX exposure for 28 d significantly increased the expressions of NOX2, p22phox and p47phox in hippocampus compared to control group (Fig. 7, $P < 0.05$ or $P < 0.01$).

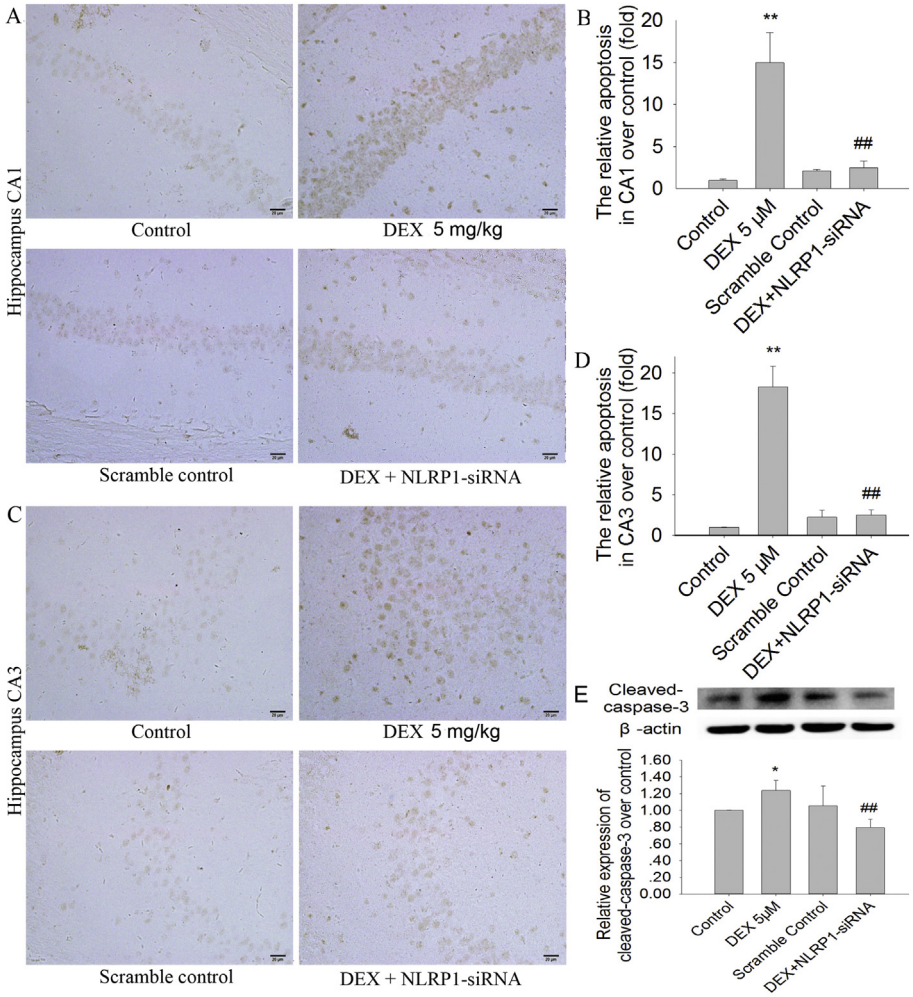

Fig. 4. Effects of NLRP1-siRNA treatment on neuronal apoptosis and cleaved-caspase-3 expression in the hippocampus induced by chronic DEX exposure. (A and C) The morphological changes of neurons in hippocampus CA1 and CA3 (TUNEL, ×400). (B) The relative neuronal apoptosis ratio in hippocampus CA1. (D) The relative neuronal apoptosis ratio in hippocampus CA3. (E) The relative expression of cleaved-caspase-3 over control (immunoblot). Results are expressed as mean ± SD, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group; $^{\#\#}P < 0.01$ compared with the DEX-treated group.

3.7. Effects of tempol or apocynin treatment on neuronal apoptosis and ROS production in chronic DEX-induced primary hippocampal neurons in vitro

To observe the effects of tempol and apocynin treatment on DEX-induced neuronal injury, we further examined the neuronal apoptosis *in vitro* by staining with Hoechst 33258. The results showed that there were few apoptotic neurons in control group, while DEX treatment for 3 d, the apoptotic neurons were significantly increased (Fig. 8A and B, $P < 0.01$). Compared with DEX-treated group, treatment with tempol or apocynin significantly reduced the apoptotic neurons (Fig. 8A and B, $P < 0.01$ or $P < 0.05$). To confirm whether ROS oxidative stress is involved in DEX-induced neuronal NLRP1 inflammasome activation, we further detected the generation of ROS in hippocampal neurons by DHE staining. As showed Fig. 8, chronic DEX treatment for 3 d significantly increased production of ROS in hippocampal neurons compared to control group (Fig. 8C and D, $P < 0.01$). Whereas compared with DEX-treated group, treatment with tempol or apocynin significantly reduced production of ROS in hippocampal neurons induced by DEX (Fig. 8C and D, $P < 0.01$).

3.8. Effects of tempol or apocynin treatment on expressions of NOX2 and NLRP1-related proteins in chronic DEX-induced hippocampal neurons in vitro

To confirm whether NOX2 is involved in ROS-mediated NLRP1 inflammasome activation in chronic DEX-treated hippocampal neurons. We firstly examined the expressions of NOX2, p22phox, p47phox and RAC in the hippocampal neurons induced by DEX (5 μM) exposure for 3 d. The results indicated that DEX treatment for 3 d could increase the expressions of NOX2, p47phox, p22phox and RAC (Fig. 9, $P < 0.05$ or $P < 0.01$). While compared with DEX-treated group, treatment with tempol and apocynin for 3d could reduce the expressions of NOX2, p47phox, p22phox and RAC in the DEX-induced hippocampal neurons (Fig. 9, $P < 0.05$ or $P < 0.01$).

We next examined the expressions of NLRP1, ASC, caspase-1 and IL-1β in chronic DEX-treated hippocampal neurons. Similarly to the study *in vivo*, DEX (5 μM) treatment for 3d significantly increased the expressions of NLRP1, ASC, caspase-1 and IL-1β in hippocampal neurons (Fig. 10, $P < 0.05$ or $P < 0.01$). Furthermore, treatment with tempol and apocynin for 3 d significantly reduced the expressions of NLRP1, ASC, caspase-1 and IL-1β, which increased by DEX treatment (Fig. 10, $P < 0.05$).

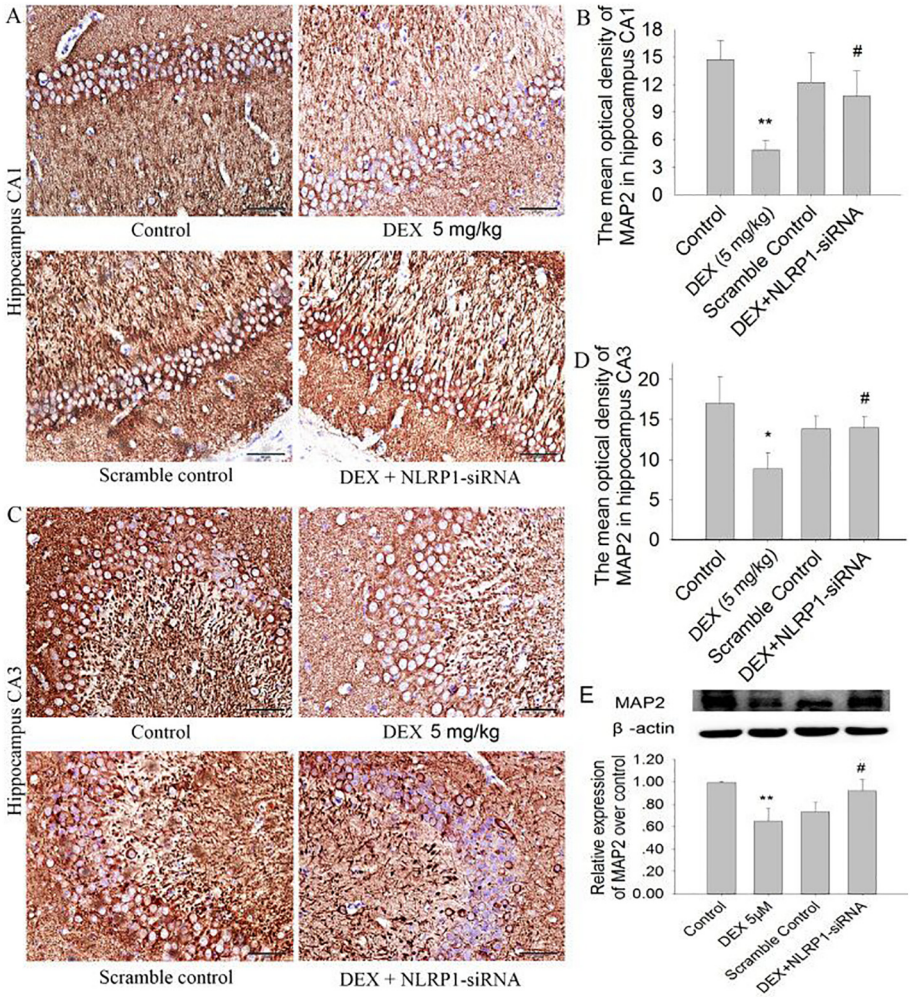

Fig. 5. Effects of NLRP1-siRNA treatment on MAP2 expression in the hippocampus CA1 and CA3 in DEX-induced mice. (A and C) The expression of MAP2 in hippocampus CA1 and CA3 (immunohistochemistry, ×400). (B) Quantitative analysis of MAP2 expression in hippocampus CA1. (D) Quantitative analysis of MAP2 expression in hippocampus CA3. (E) The relative expression of MAP2 over control (immunoblot). Results are expressed as mean ± SD, n = 4.*$P < 0.05$, **$P < 0.01$ compared with the control group; $^{\#}P < 0.05$ compared with the DEX-treated group.

4. Discussion

Our previous study showed that chronic DEX exposure could activate the NLRP1 inflammasome and induce neuronal damage in the hippocampal neurons [4]. However, the exact mechanisms of GCs-induced activation of NLRP1 inflammasome and neuronal damage in the hippocampal neurons are still unclear. In the present study, we demonstrate that chronic DEX exposure for 28d significantly induced NLRP1 inflammasome activation and hippocampal neurons damage in male mice. While treatment with NLRP1-siRNA could decrease the activation of NLRP1 inflammasome and alleviate neuronal injury in DEX-treated mice. Further study showed that chronic DEX exposure for 3 d significantly increased ROS generation, NOX2 and NLRP1 expressions in hippocampal neurons *in vitro*. Meanwhile, tempol and apocynin treatment significantly reduced ROS generation and decreased NOX2 and NLRP1 expressions in DEX-treated hippocampal neurons. These findings indicate the functional importance of NADPH oxidase and NLRP1 inflammasome in chronic GCs-induced neuronal damage, and inhibition of NOX2-NLRP1 signaling pathway has protective effect on chronic GCs-induced neuronal damage.

Accumulating studies have been reported that stress level GCs exposure is closely related with neurodegeneration diseases such as depression vulnerability and AD [2,24]. Sato et al. [19] reported that high-level corticosterone in the serum induces ROS generation, leading to oxidative damage in the hippocampus. And high doses of DEX significantly increased the production of ROS, lipid peroxidation and cell apoptosis in human neuroblastoma SH-SY5Y cells [25]. However, excessive productions of ROS are closely related to inflammasome activation and cells apoptosis. Our previous study showed that chronic DEX exposure for 28 d significantly induced NLRP1 inflammasome activation and neurodegeneration in frontal cortex and hippocampus in mice [4]. These data suggest that chronic GCs exposure may activate NLRP1 inflammasome *via* increase ROS generation. Inflammasomes are intracellular multiprotein complexes involved in inflammation. The neuroinflammation plays important roles in many neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease [26]. The NLRP1 inflammasome activation is closely related to the neurodegenerative diseases and patients with epilepsy [27]. Recent studies suggested that down-regulation of NLRP1 could decrease H_2O_2-induced apoptosis in primary cultured spinal cord neurons [28], and NLRP1-siRNA notably increased myocardial cell viability inhibited by Ischemia/Reperfusion (I/R), suppressed I/R-induced myocardial cells apoptosis and damage [29]. However, whether down-regulation of NLRP1 can alleviate GCs-induced neuronal damage is still unknown. In this work, intrahippocampal injection of lentivirus-mediated NLRP1-siRNA was used to observe the protective effect of down-regulation of NLRP1 on DEX-induced neuronal damage in hippocampus. Our results suggested that the GFP was significantly expressed in hippocampus and

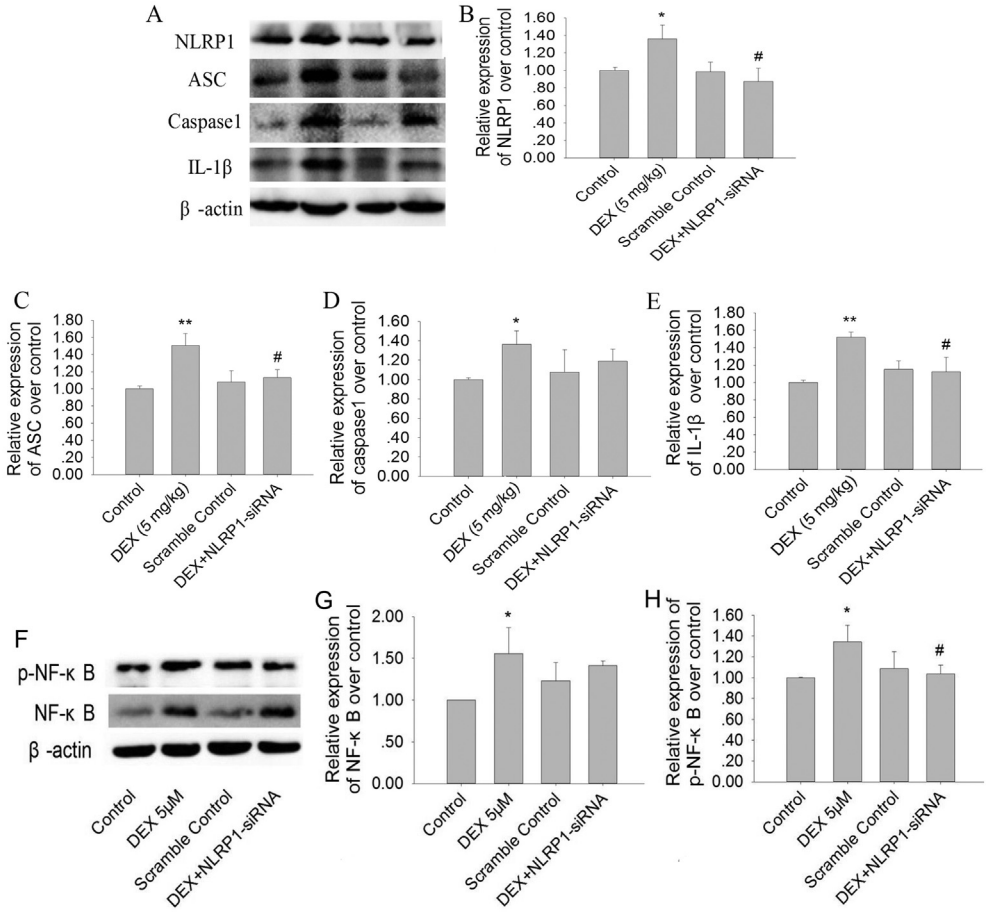

Fig. 6. Effects of NLRP1-siRNA treatment on the expressions of NLRP1, ASC, caspase-1, IL-1β, NF-κB and p-NF-κB in the hippocampus in chronic DEX-induced mice (immunoblot). (A and F) The bands of NLRP1, ASC, caspase-1, IL-1β, NF-κB and p-NF-κB in the hippocampus. (B) Quantitative analysis of the relative expression of NLRP1 over control. (C) The relative expression of ASC over control. (D) The relative expression of caspase-1 over control. (E) The relative expression of IL-1β over control. (G) The relative expression of NF-κB over control. (H) The relative expression of p-NF-κB over control. Results are expressed as mean ± SD, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control group; $^{\#}P < 0.05$ compared to DEX-treated group.

confirmed successful lentivirus infection. Meanwhile, the NLRP1-siRNA (408)-treated for 6 weeks significantly reduced the expression of NLRP1 in hippocampus tissue in mice. The open field test (OFT) is common used to measure locomotor activity and exploratory behavior, and the Morris water maze (MWM) test is the most preferred behavioral study to assess spatial learning and memory in mice [23]. The present results suggested that DEX treatment for 28d exhibited significant impairment in the activities of motor and exploration as well as the abilities of learning and memory in male mice, while treatment with NLRP1-siRNA significantly alleviated the cognitive dysfunction which induced by chronic DEX exposure.

The microtubule-associated protein (MAP2) is a cytoskeletal and neuronal marker protein. The expression of MAP2 coincides with dendritic outgrowth, branching, and postlesion dendritic remodeling [30]. And the MAP2 expression was significantly reduced in the hippocampus and cortex of old rats [31]. Our results showed that chronic DEX exposure significantly down-regulated the MAP2 expression in hippocampus CA1 and CA3. While NLRP1-siRNA (408) treatment could significantly increase the MAP2 expression in hippocampus CA1 and CA3 in DEX-treated mice. The results of histopathology and hoechst33258 staining showed that NLRP1-siRNA treatment significantly attenuated neuronal degenerative changes and apoptosis in hippocampus CA1 and CA3 regions in DEX-induced mice. Meanwhile, the results of TUNEL assay and cleaved-caspase-3 also showed that NLRP1-siRNA treatment significantly reduced neuronal apoptosis in hippocampus which increased in DEX-treated mice. Our data suggest that down-regulation of NLRP1 expression can attenuate chronic GCs-induced learning and memory impairment and neuronal damage in mice.

NLRP1 inflammasome is composed of NLRP1, ASC and caspase-1 and involves in the pathogenesis of many neurological disorders [9,32]. The maturation and production of IL-1β and IL-18 were controlled by the activation of caspase-1 [32,33]. The ASC is an important component of the inflammasome and correlates NLRP1 with caspase-1 [34]. The

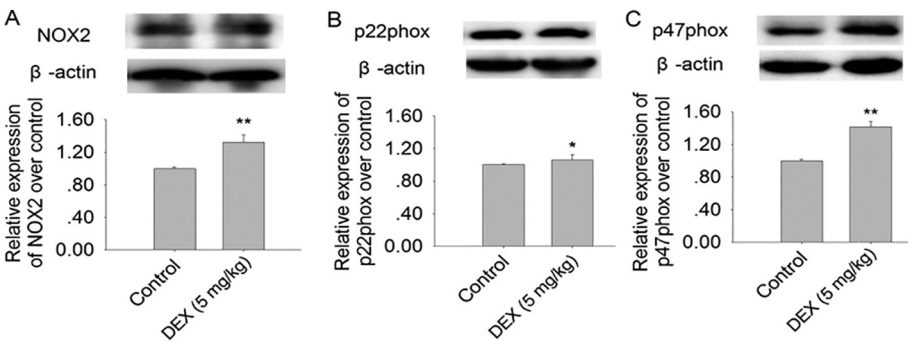

Fig. 7. Effects of chronic DEX treatment on expressions of NOX2, p22phox and p47phox in hippocampus in male mice (immunoblot). (A) The relative expression of NOX2 over control. (B) The relative expression of p22phox over control. (C) The relative expression of p47phox over control. Results are expressed as mean ± SD, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control group.

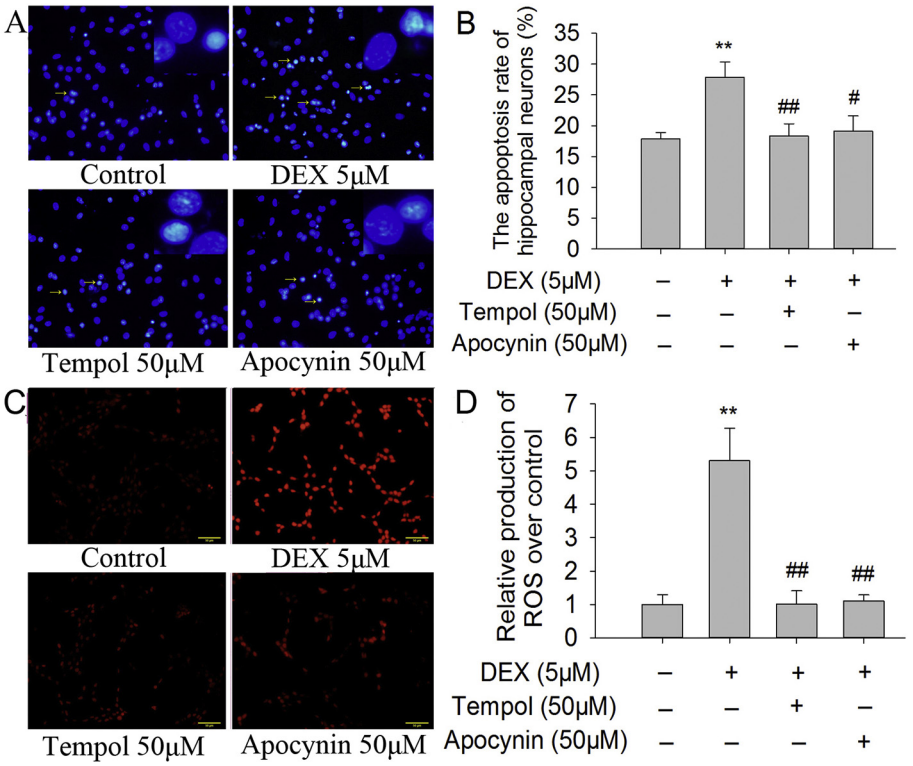

Fig. 8. Effects of chronic DEX exposure on neuronal apoptosis and ROS production in hippocampal neurons *in vitro*. (A) The neuronal apoptosis (indicated by yellow arrows) in hippocampal neurons (Hoechst 33258 staining, 400×). (B) Quantitative analysis of apoptosis rate. (A) The production of ROS in hippocampal neurons (DHE staining, ×200). (B) Quantitative analysis of ROS production over control. Results are expressed as mean ± SD, n = 3. $^{**}P < 0.01$ compared to control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to DEX-treated group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

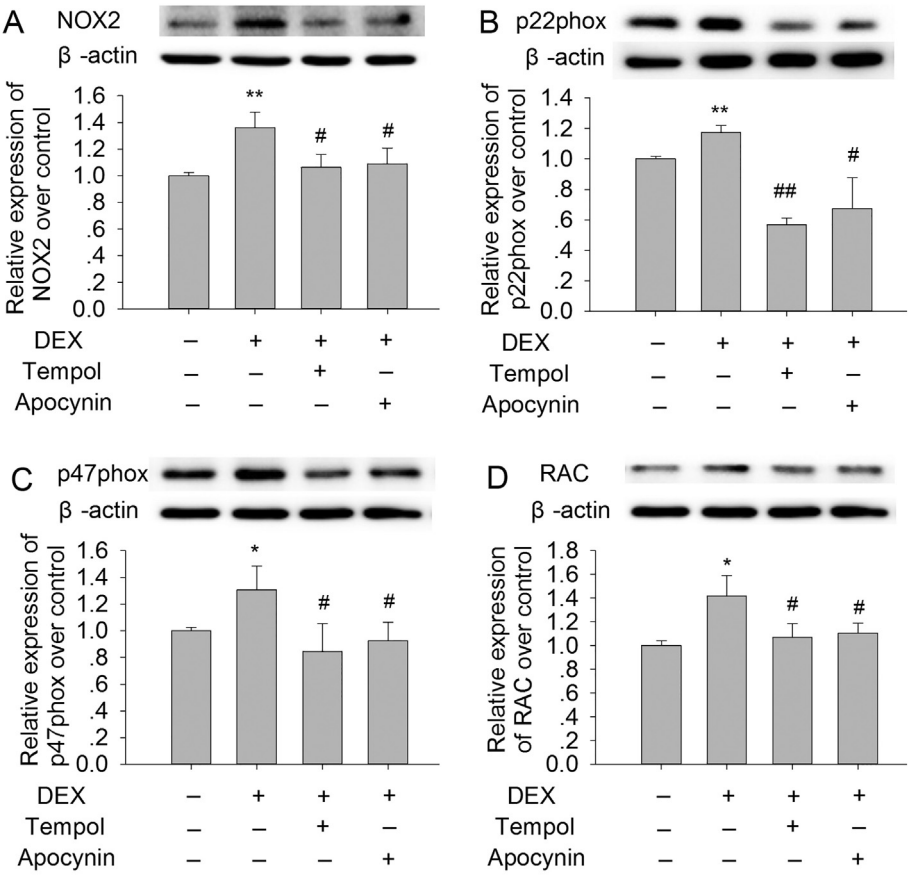

Fig. 9. Effects of chronic DEX treatment on expressions of NOX2, p22phox, p47phox and RAC in hippocampal neurons (immunoblot). (A) The bands and the relative expression of NOX2 over control. (B) The bands and the relative expression of p22phox over control. (C) The bands and the relative expression of p47phox over control. (D) The bands and the relative expression of RAC over control. Results are expressed as mean ± SD, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to DEX-treated group.

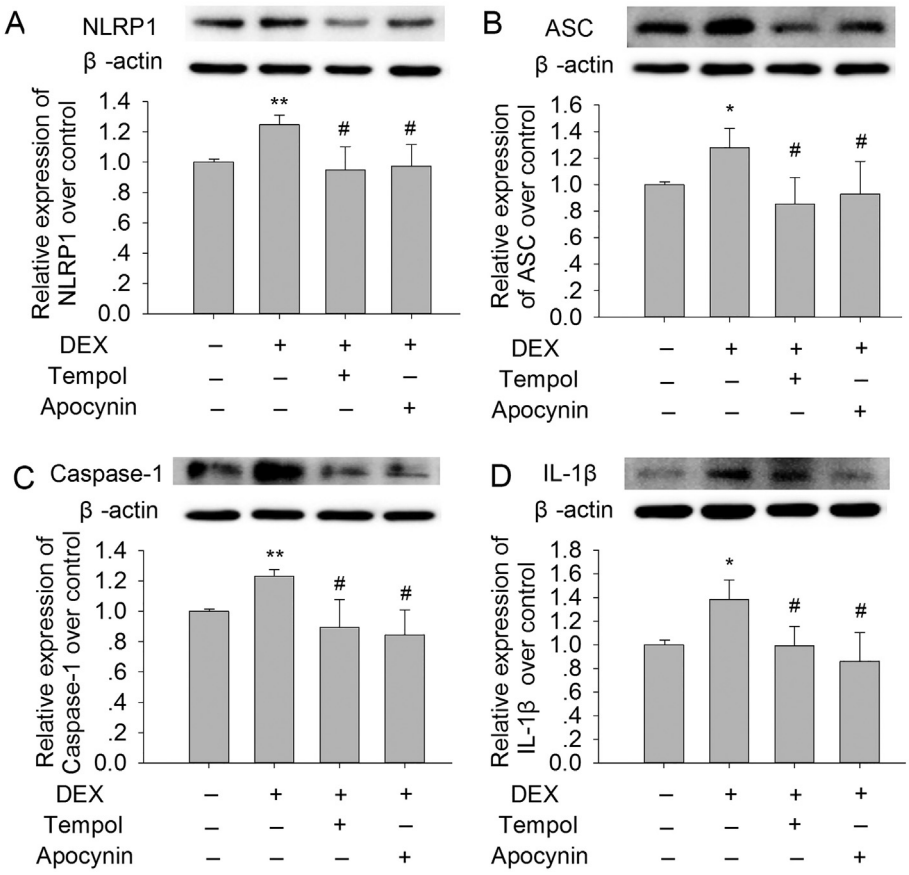

Fig. 10. Effects of chronic DEX treatment on expressions of NLRP1, ASC, caspase-1 and IL-1β in hippocampal neurons (immunoblot). (A) The bands and the relative expression of NLRP1 over control. (B) The bands and the relative expression of ASC over control. (C) The bands and the relative expression of caspase-1 over control. (D) The bands and the relative expression of IL-1β over control. Results are expressed as mean ± SD, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control group; $^{\#}P < 0.05$ compared to DEX-treated group.

present study indicated that treatment with NLRP1-siRNA (408) significantly reduced expressions of NLRP1, ASC and IL-1β which increased by DEX treatment in mice. NF-κB is one of the critical regulators involved in regulation of inflammation and innate immunity, and phosphorylation of NF-κB is associated with induction of proinflammatory genes [35]. It has been reported that selective inhibition of NF-κB activity could suppress glial-associated neuroinflammation and decrease dopaminergic neuron loss in a Parkinson's disease mouse model [36]. Our previous study revealed that chronic DEX exposure for 28d significantly increased the expression of NF-κB in hippocampus in mice [4]. The present results showed that NLRP1-siRNA treatment had no effect on expression of NF-κB, but it could decrease expression of p-NF-κB. These data suggest that the NLRP1 inflammasome may be an important target and closely involves in DEX exposure-induced neuronal injury.

It is well known that ROS oxidative stress is closely related to the neurodegenerative diseases such as AD [37]. Excessive ROS accumulation may even be one of the first pathogenic events during the progression of AD [38]. The NOX is one of important enzyme that is responsible for ROS generation in neurodegenerative diseases [39,40]. The NOX complex is composed of the membrane-associated heterodimers of gp91phox (NOX2) and p22phox, and four cytosolic proteins of p47phox, p67phox, p40phox and RAC [41]. It has been reported that the NOX2-mediated oxidative stress is closely related to the development of neurodegenerative diseases and aging [42,43]. Upon activation of NOX2, the cytosolic subunits (p47phox, p67phox, p40phox and RAC) translocate to the membrane and interact with the membrane subunits (gp91phox and p22phox) [44]. The latest study showed that the NOX2 was involved in DEX-induced ROS generation and apoptosis in cultured osteocytes [45]. Our previous study showed that chronic restrain stress significantly increased the expression of p47phox and RAC1 in cytoplasm, and NOX2, p47phox and RAC1 in cytomembranes [46]. However, it is still unknown whether chronic GCs exposure can activate NOX2 and induce NLRP1 inflammasome activation in hippocampal neurons. The present results showed that chronic DEX treatment for 28d markedly increased the expressions of NOX2, p47phox and p22phox in hippocampus tissue in mice. Additional, our results *in vitro* also showed that DEX (5 μM) treatment for 3d significantly increased ROS accumulation and NOX2, p22phox, p47phox and RAC expression, and promoted neuronal apoptosis in primary cultured hippocampal neurons. Furthermore, our results both *in vivo* and *in vitro* showed that chronic DEX exposure also increased the expressions of NLRP1, ASC, caspase-1 and IL-1β. These data suggest that NADPH oxidase 2 may play an essential role in activation of NLRP1 inflammasome induced by chronic GCs exposure.

Apocynin is commonly used as an inhibitor of NADPH oxidase, which can prevent the generation of ROS. It has been reported that apocynin can inhibit the NOX2 with an IC50 about 10 μM, while for NOX4, the IC50 > 200 μM [47]. It has been reported that apocynin treatment can decrease NOX2 and NOX4 expression, and ROS generation which increased by ischemia/reperfusion. Meanwhile, apocynin significantly decreased the expressions of NLRP3, ASC, caspase-1, IL-1β and IL-18 in the ischemic brain cortex [40]. Tempol is commonly used as a radical scavenger similar to catalase or superoxide dismutase. Tempol (50 μM) was often used to eliminate excessive ROS and recover the intracellular oxidative balance [48,49]. In this study, we detected the effect of tempol (50 μM) and apocynin (50 μM) on neuronal apoptosis and NOX2-related ROS generation in DEX-induced hippocampal neurons *in vitro*. The results revealed that treatment with tempol and apocynin significantly inhibited neuronal apoptosis, decreased the production of ROS and the expressions of NOX2, p22phox, p47phox and RAC, which were significantly increased by chronic DEX exposure in hippocampal neurons. Furthermore, our results showed that the expressions of NLRP1, ASC, caspase-1 and IL-1β were significantly down-

regulated by tempol and apocynin in chronic DEX-induced hippocampal neurons. These data suggest that the NOX2-derived ROS is closely related to activation of NLRP1 inflammasome in chronic GCs-induced neuronal damage. And both the NOX2 and NLRP1 inflammasome may be important therapeutic targets for chronic GCs-induced neuronal injury.

All in all, the mechanism of chronic GCs exposure-induced neuronal damage is complex. The study indicated that chronic DEX treatment significantly increased NOX2-mediated ROS generation, which further promoted NLRP1 inflammasome activation in hippocampal neurons and induced learning and memory impairment in mice. While treatment with NLRP1-siRNA significantly improved the learning and memory function and attenuated neuronal damage in DEX-treated mice. Meanwhile, both the ROS scavenger and NADPH oxidase inhibitor significantly reduced the production of ROS and down-regulate the expressions of NOX2 and NLRP1 inflammasome in DEX-treated hippocampal neurons. These findings suggest that NOX2-mediated ROS generation plays important roles in activation of NLRP1 inflammasome and promotes neuronal inflammatory damage, which induced by chronic GCs exposure in hippocampal neurons. This study suggests that NOX2 and NLRP1 inflammasome are important therapeutic targets in chronic GCs exposure-induced neuronal damage.

Declaration of Competing Interest

The authors report no conflicts of interest.

Acknowledgments

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