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NADPH oxidase 2-mediated NLRP1 inflammasome activation involves in neuronal senescence in hippocampal neurons *in vitro*

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A R T I C L E I N F O

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A B S T R A C T

Oxidative stress and inflammation are closely related to neuron ageing. NADPH oxidase 2 (NOX2) is a major source of reactive oxygen species (ROS) generation in brain. The nucleotide-binding oligomerisation domain (NOD)-like receptor protein 1 (NLRP1) inflammasome is responsible for the formation of proinflammatory molecules in neurons. We hypothesize that NOX2-derived ROS accumulation mediates activation of NLRP1 inflammasome, which is involved in age-related neuronal damage. In the present study, we investigated the changes of NOX2-NLRP1 signaling pathway in primary hippocampal neurons cultured for different time (6, 9 and 12 days, d). Meanwhile, we further examined the effect of ROS inhibitor and NLRP1-siRNA on neuronal senescence. The results showed that, compared with 6 d group, the neuronal apoptosis and β-Galactosidase (β-Gal) expression were significantly increased, and the microtubule-associated protein 2 (MAP2) expression significantly decreased in primary hippocampal neurons cultured for 12 d. In addition, the results also showed that the production of ROS, the expressions of NOX2 and NLRP1 inflammasome were significantly increased with the prolongation of culture time in hippocampal neurons. Moreover, the NOX inhibitor (apocynin) and ROS scavenger (tempol) significantly decreased ROS production and alleviated neuronal damage. Meanwhile, the tempol and apocynin treatment significantly decreased the expression of NLRP1 inflammasome in hippocampal neurons. Furthermore, the NLRP1-siRNA and caspase-1 inhibitor treatment also alleviated neuronal damage. These results suggest that NOX2-derived ROS generation may induce brain inflammation *via* NLRP-1 inflammasome activation and lead to age-related neuronal damage. The NADPH oxidase and NLRP1 inflammasome may be important therapeutic targets for age-related neuronal damage.

1. Introduction

Brain ageing is characterized by the progressive accumulation of harmful changes, such as oxidative stress and neuronal inflammation, which increases risk of age-related cognitive decline and dementia [1,2]. With ageing of the population, the brain pathological ageing and age-related neurodegenerative diseases are gradually increasing and have become a major public health concern [3]. At present, the possible mechanisms of the brain ageing are related with oxidative stress, neuronal inflammation, excitotoxicity, energy depletion and neuronal apoptosis et al. However, the detailed mechanisms remain unknown.

Increasing evidence showed that accumulation of ageing-associated reactive oxygen species (ROS) might cause neuronal oxidative stress, inflammation and neuronal damage [4,5]. There are several enzymes and metabolic processes that can generate intracellular ROS. Among them, the NADPH oxidase 2 (NOX2) is constitutively expressed in many cells in the brain, especially in neurons. NOX2 is a major source of excessive ROS generation and closely involves in the development of ageing-related neuronal senescence and neurodegenerative diseases, such as AD [6]. The expression of NOX2 has been found to be increased in the brain of ageing mice [7]. Moreover, the expressions of the NOX2 subunits, p47phox and p67phox, were found to be increased in the cortex of mild cognitive impairment patients, which correlated closely with a decline in cognition [8]. These findings strongly support a substantial role for NOX2-derived ROS oxidative stress in the development of brain ageing. However, the roles and mechanisms of NOX2 in brain

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ageing have not yet been fully elucidated.

It has been reported that neuronal inflammation plays important roles in the pathogenesis of neurodegenerative diseases. And the inflammatory response is both a reaction to the disease process and a contributor to the neuronal damage [9,10]. The inflammasome is a multiprotein complex in cytoplasm that is responsible for the formation of proinflammatory molecules. Growing data show that neuronal inflammation mediated by the inflammasome plays an important role in many neurological diseases [11,12]. The NOD-like receptor (NLR) family protein 1 (NLRP1) is the first reported member of the NLR family and is a critical component of the inflammasome [13]. NLRP1 inflammasome appears to be expressed ubiquitously, and high levels of NLRP1 was found in the brain, particular in neurons [12]. The activation of NLRP1 inflammasome can generate a functional caspase-1 to drive the inflammatory response and pyroptosis [14,15]. Moreover, down-regulation of the NLRP1 inflammasome could improve age-related cognitive deficits in different animal models [16,17]. However, it remains unclear whether NOX2-derived ROS oxidative stress can induce ageing-associated neuronal damage *via* activating NLRP1 inflammasome in hippocampal neurons.

At present, fewer studies are available to study the hippocampus neuronal age-associated damage and its mechanisms *in vitro*. And prolongation of culture time in primary hippocampal neurons is considered a model of neuronal ageing, since some of the changes occurring in the elderly *in vivo* are similar to prolonged culture of neurons *in vitro* [18,19]. In the study, we hypothesize that NOX2-derived ROS may activate the NLRP1 inflammasome and induce neuronal damage in prolonged culture of primary hippocampal neurons. So we firstly investigated the effects of different culture time (6, 9 and 12 d) on the neuronal damage and NOX2-NLRP1 signaling pathway in primary cultured hippocampal neurons. We further observed the effects of apocynin (50 μM), a NADPH oxidase inhibitor, and tempol (50 μM), a ROS scavenger, on neuronal inflammation and neuronal damage in hippocampal neurons cultured for 12 d. Moreover, we also examined the effects of NLRP1-siRNA and caspase-1 inhibitor on neuronal damage in hippocampal neurons cultured for 12 d. The study has the potential to contribute to a more complete understanding of the mechanisms of brain ageing and ageing-related neurodegenerative diseases.

2. Materials and methods

2.1. Culture of primary hippocampal neurons and experimental treatment

In this study, primary hippocampal neurons were prepared from hippocampus of postnatal Sprague Dawley rats (within 24 h) obtained from the Center of Laboratory Animals of Anhui Medical University as described previously [20]. The hippocampal neurons were planted onto poly-L-lysine (10 μg/ml)-coated 24-well culture plates (1.5×10^5 cells/well) or 6-well culture plates (1×10^7 cells/well), then cultured in neurobasal medium containing 2% B-27 supplement, which was recommended for primary neuronal cultures [21], and assessed by immunostaining for the neuronal marker microtubule-associated protein-2 (MAP2). Then the neurons were used to perform the following experiments: (1) The hippocampal neurons were cultured for 6 days (d), 9 d and 12 d respectively. (2) The neurons were cultured for 9 d and then were treated with apocynin (50 μM, Merck Millipore) and tempol (50 μM, Merck Millipore) for 3 d. (3) The neurons were cultured for 6 d and then were treated with scramble lentivirus vector (LV control), NLRP1-siRNA (1×10^7 TU/ml) and caspase-1 inhibitor (5 μM, Merck Millipore) for 6 d. The sequence of scramble lentivirus vector (Shanghai GenePharma Co., China) is 5′ TTC TCC GAA CGT GTC ACG T 3′, NLRP1-siRNA (408) is 5′ GCT CTT TAC CCT CTT CTA ACA 3′, NLRP1-siRNA (883) is 5′ GGG TCT TAG CAG ACC AGA ATC 3′ and NLRP1-siRNA (1372) is 5′ GCC AAA GAA GGA CCC TGT TCA 3′. The medium was changed every 3 d. All experiments were repeated at least three times.

2.2. Neuronal LDH release and apoptosis assay

To detect the effect of different culture time (6 d, 9 d and 12 d) on hippocampal neurons damage, we detected the activity of LDH released in the medium according to the protocol of LDH kit (Nanjing Jiangcheng Bioengineering Institute, China). Briefly, the supernatant was reacted with nicotinamide adenine dinucleotide (NAD) and lactate solution. Colorimetric absorbance was measured at 490 nm with a microplate reader (Thermo Fisher Scientific, USA). To confirm the neuronal damage, the neuronal apoptosis was examined by using Hoechst 33258 nucleus staining as described previously [11,22]. For Hoechst 33258 staining, the hippocampal neurons were incubated with Hoechst 33258 (5 μg/ml, Zhongshan Golden Bridge Biotechnology Co., China) for 15 min. After washed 3 times with PBS the neurons were sealed onto slides using anti-fade mounting medium (Beyotime Biotechnology Co., China). Then the apoptotic neurons were examined from three slides and five random fields (400×) per slide by fluorescence microscopy (Olympus IX71, Japan). The total neurons and apoptotic neurons were counted and the neuronal apoptosis rate was determined to indicate the changes of neuronal apoptosis.

2.3. Senescence-associated β-galactosidase (β-gal) staining

To detect the effect of different culture time on senescence marker of hippocampal neurons, the β-Gal activity was detected by β-Gal staining kit (Beyotime Institute of Biotechnology, China). In brief, the neurons were washed three times with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. The slides were washed 2 times in PBS, and then stained with fresh β-Gal staining solution (X-Gal) as a substrate overnight at 37 °C. After staining, cells were washed twice with PBS. The β-Gal activity was increased in ageing cells and the positive cells were stained blue/green. The β-Gal positive neurons were examined from three slides and five random fields (400×) per slide by microscopy (Olympus IX71, Japan). The mean density of positive cells was quantified by using Image-Pro Plus analysis system to indicate the changes of β-Gal activation.

2.4. Measurement of ROS production

ROS production was detected by dihydroethidium (DHE) microfluorography method described previously [23]. DHE is a cell permeable dye and is oxidized by ROS to ethidium bromide, which is associated with ROS production. Ethidium bromide is trapped intracellularly into DNA and can be identified using red fluorescence [23]. Briefly, the DHE (10 μM, Beyotime Institute of Biotechnology, China) was added in the medium and incubated at 37 °C for 30 min. After incubation, neurons were washed with PBS and detected by using fluorescence microscope (Olympus IX71, Japan). The mean fluorescence density from three slides and five random fields (400×) per slide was performed by using Image Pro Plus software to indicate changes of ROS in the hippocampal neurons.

2.5. Immunofluorescence

The hippocampal neurons grown on glass coverslips were fixed with 4% paraformaldehyde for 30 min at room temperature and washed with PBS three times. The neurons were permeabilized with Triton X-100 (0.25%) for 30 min, and blocked with 1% BSA in PBS for 1 h. Then the neurons were incubated with primary antibodies of MAP2 (1:200, Abcam, ab11267) overnight at 4 °C. Secondary antibody conjugated to FITC (1:200, Zhongshan Golden Bridge Biotechnology Co., China) was incubated at for 1 h. Then slides were mounted with anti-fade medium and examined by using fluorescence microscope (Olympus IX71, Japan). The mean fluorescence density from three sections and five random fields (400×) per section was quantified by using Image-Pro Plus analysis system to indicate the changes of MAP2 expression.

2.6. Immunoblotting assay

The protein concentration was determined by BCA Protein Assay Kit (Shanghai Sangon Bio-Telch, China). Equal amount of protein (30 μg) was transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk for 1 h. Then the membranes were reacted with antibodies for β-Gal, NOX1, NOX2, p22phox, p47phox, RAC1, NLRP1, ASC, caspase-1, IL-1β and β-actin (1:1000) overnight at 4 °C. The primary antibodies of NOX1(Cat. ab131088), NOX2 (Cat. ab31092), NLRP1 (Cat. ab3683), caspase-1 (Cat. ab1872) and IL-1β (Cat. Ab9722) were from Abcam. The ASC (Cat. SC-514414) was from Santa Cruz Technology Company. The β-Gal (Cat. BS71412), p22phox (Cat. BS60290), p47phox (Cat. BS4852) and RAC1 (Cat. BS71440) were from Bioworld Technology Company. The dilution of β-Gal, NOX1, NOX2, NLRP1, p47phox and RAC1 was 1:1000. The dilution of ASC, caspase-1, IL-1β and p22phox was 1:500. Then the membranes were extensively washed and incubated with anti-rabbit IgG antibody conjugated to HRP (1:10000) for 1 h. After extensively washed, the protein bands were detected by chemiluminescence reagents (ECL kit, Amersham Biosciences, UK). The Chemi Q4800 mini Imaging System (Shanghai Bioshine Technology, China) was used to visualize protein bands. The density of protein bands was performed with Image J software (National Institutes of Health, Bethesda, MD, USA) and normalised to the corresponding β-actin bands. The relative density of each target protein over control was used to represent the changes in expressions of target proteins.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The supernatants were collected and the ELISA kit was used for the quantitative determination of IL-1β and IL-18 (Cloud-Clone Corp., USA). Briefly, the samples and IL-1β, IL-18 standards were added to the assay plates and incubated at 37 °C for 1 h. Then the HRP conjugated reagent (100 μl) was added into each well and incubated at 37 °C for 1 h. The plate was washed 4 times with PBS, and the chromogen solution (100 μl) was added into each well. The plate was gently mixed and incubated for 15 min at 37 °C. Then the stop solution (50 μl) was added into each well and the absorbance was examined at 450 nm with a microplate reader (Thermo Fisher Scientific, USA).

2.8. Statistical analysis

Data are presented as mean ± SD. The SPSS 17.0 software was used to perform the statistical analyses. The data were analyzed by one-way ANOVA and then subjected to between-group comparisons using the Bonferroni's *post-hoc* test. The $P < 0.05$ was considered statistical difference.

3. Results

3.1. Effects of culture for 6 d, 9 d and 12 d on neuronal damage and MAP2 expression in primary hippocampal neurons

When the hippocampal neurons cultured for 6 d, we assessed the neuronal marker MAP2 by immunofluorescence (Fig. 1A). The results showed that, consistent with previous reports, most of the cells expressed the neuronal marker MAP2. We then examined the LDH release in the primary cultured hippocampal neurons for 6 d, 9 d and 12 d. The results showed that, compared with 6 d group, the LDH release was significantly increased in hippocampal neurons for 12 d (Fig. 1B, $P < 0.01$). We further measured the neuronal apoptosis by staining with Hoechst 33258. The results showed that there were few apoptotic neurons in 6 d group (Fig. 1C and D, the apoptosis rate is 18.44 ± 3.32%), while in 9 d and 12 d groups, the apoptotic neurons were significantly increased (Fig. 1C and D, 26.67 ± 3.89% for 9 d, $P < 0.05$; 34.49 ± 1.65% for 12 d, $P < 0.01$). The results showed that prolongation of cultured time significantly increased neuronal damage in hippocampal neurons.

The MAP2 is a cytoskeletal protein localized in the neuronal dendritic compartment. It has been reported that the expression of MAP2 in the hippocampus and cortex significantly decreased in old rats [24]. We investigated the expression of MAP2 in the hippocampal neurons by immunofluorescence. The results showed that the expression of MAP2 had no significant difference in hippocampal neurons cultured for 6 d and 9 d (Fig. 1E and F). Compared with 6 d group, the expression of MAP2 was significantly decreased in the hippocampal neurons cultured for 12 d (Fig. 1E and F, $P < 0.05$).

3.2. Effects of culture for 6 d, 9 d and 12 d on ROS production and β-Gal expressions in primary hippocampal neurons

ROS oxidative stress plays an important role in the senescence of hippocampal neurons. So we detected the ROS production in the hippocampal neurons. The results showed that the ROS production significantly increased with the prolongation of culture time. Compared with 6 d group, the ROS production was significantly increased in hippocampal neurons cultured for 9 d and 12 d (Fig. 2A and C, $P < 0.05$). Senescent cells display increased β-Gal activity when measured *in situ* [25]. In order to observe the effect of prolonged culture on senescence, the β-Gal was detected in hippocampal neurons. The β-Gal staining results showed that the expression of β-Gal increased with the prolongation of culture time in hippocampal neurons. Compared with 6 d group, the expression of β-Gal significantly increased in hippocampal neurons cultured for 12 d (Fig. 2B and D, $P < 0.01$). Meanwhile, the immunoblot results also showed that the expression of β-Gal was significantly increased in hippocampal neurons cultured for 12 d (Fig. 2E, $P < 0.05$).

3.3. Effects of culture for 6 d, 9 d and 12 d on expression of NOX1, NOX2, p22phox, p47phox and RAC1 in primary hippocampal neurons

NADPH oxidase (NOX) is one of the main enzyme systems for the generation of ROS in the neurons. We further detected the expression of NOX1, NOX2, p22phox, p47phox and RAC1 in hippocampal neurons. The results showed that there was no significant alteration of NOX1 in hippocampal neurons cultured for 6 d, 9 d and 12 d (Fig. 3A, $P > 0.05$). Meanwhile, there were less expressions of NOX2, p22phox, p47phox and RAC1 in hippocampal neurons cultured for 6 d. While with the prolongation of cultured time, the expressions of NOX2, p22phox, p47phox and RAC1 were significantly increased in hippocampal neurons cultured for 9 d and 12 d (Fig. 3B–E, $P < 0.05$ or $P < 0.01$).

3.4. Effects of culture for 6 d, 9 d and 12 d on expressions of NLRP1, ASC, caspase-1, IL-1β in primary hippocampal neurons and IL-1β, IL-18 release in supernatant

NLRP1 inflammasome plays an important role in the pathogenesis of neurodegeneration diseases. We further detected the expressions of NLRP1, ASC, caspase-1 and IL-1β in hippocampal neurons cultured for 6 d, 9 d and 12 d. Our results showed that the expressions of NLRP1, ASC, caspase-1 and IL-1β in hippocampal neurons had an increase trend with prolongation of culture time. Compared with 6 d group, the expressions of NLRP1, ASC, caspase-1 and IL-1β were significantly increased in hippocampal neurons cultured for 9 d and 12 d (Fig. 4A–D, $P < 0.05$ or $P < 0.01$). To further confirm the effect of long-term culture on activation of NLRP1 inflammasome in hippocampal neurons, we measured the amount of IL-1β and IL-18 released in supernatant. The results showed that, compared with 6 d group, the levels of IL-1β

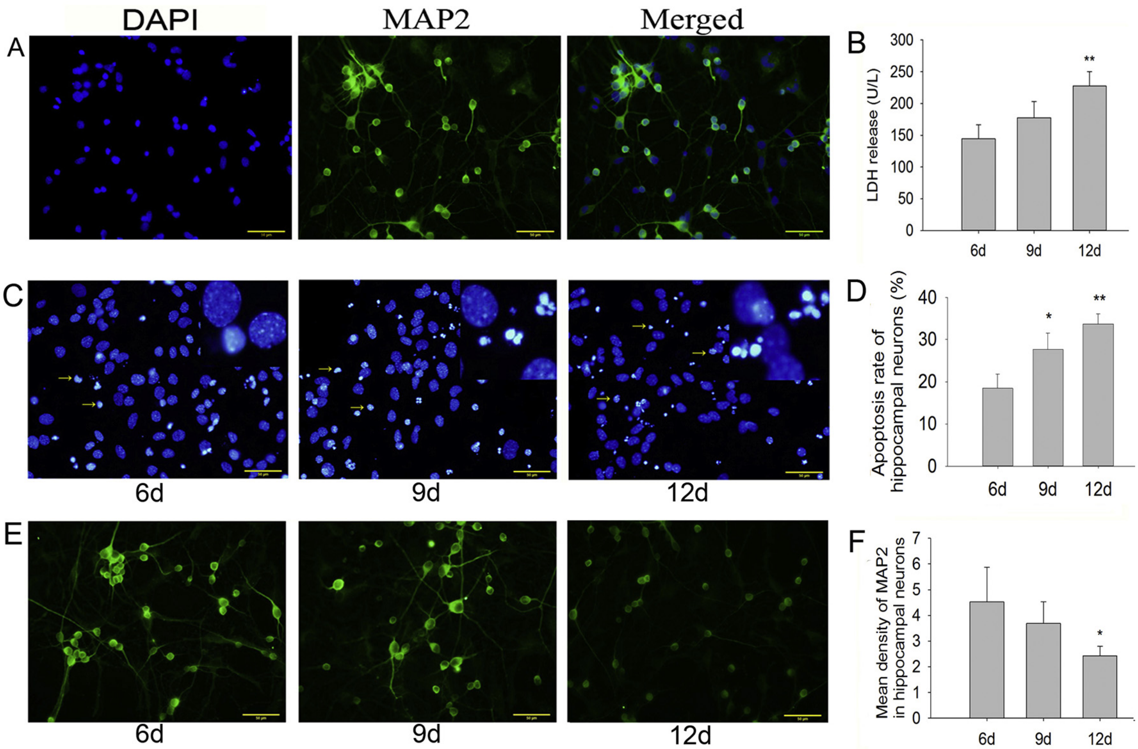

Fig. 1. The effects of different culture time for 6 d, 9 d and 12 d on neuronal damage and MAP2 expression in hippocampal neurons. (A) The expression of neuronal marker of MAP2 in primary hippocampal neurons cultured for 6 d (immunofluorescence, 400×). (B) The LDH release from hippocampal neurons cultured for 6 d, 9 d and 12 d. (C) The neuronal apoptosis of hippocampal neurons cultured for 6 d, 9 d and 12 d (Hoechst 33258 staining, 400×). The apoptotic cells appear smaller than normal and deeply stained (yellow arrow). (D) Quantitative analysis of apoptosis rate in hippicampal neurons. (E) The MAP2 expression in hippocampal neurons culture for 6 d, 9 d and 12 d (immunofluorescence, 400×). (F) Quantitative analysis of MAP2 expression in hippocampal neurons. Results are expressed as mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to 6 d control group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

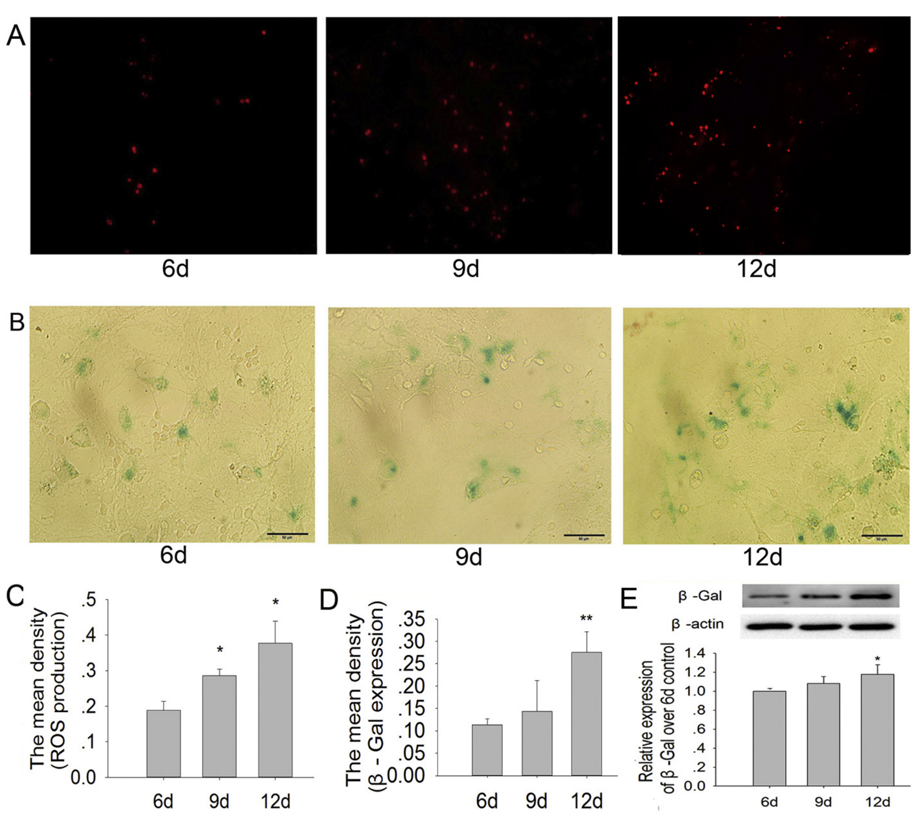

Fig. 2. The effects of different culture time for 6 d, 9 d and 12 d on ROS production and β-Gal expression in hippocampal neurons. (A) The effect of different culture time for 6 d, 9 d and 12 d on ROS production in hippocampal neurons (DHE staining, 400×). (B) The effect of different culture time for 6 d, 9 d and 12 d on β-Gal expression in hippocampal neurons (β-Gal staining, 400×). (C) Quantitative analysis of ROS production in hippocampal neurons. (D) Quantitative analysis of β-Gal expression in hippocampal neurons. (E) The relative expression of β-Gal over 6 d control (immunoblot). Results are expressed as mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to 6 d group.

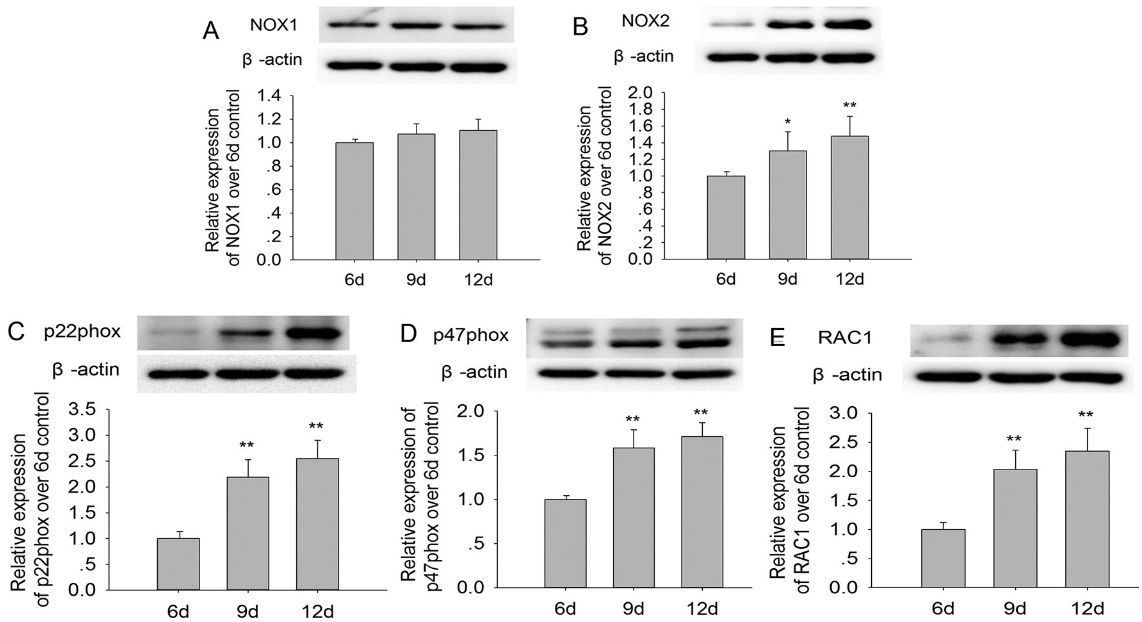

Fig. 3. The effects of different culture time for 6 d, 9 d and 12 d on the expressions of NOX1, NOX2, p22phox, p47phox and RAC1 in hippocampal neurons (immunoblot). (A) The relative expression of NOX1 over 6 d control. (B) The relative expression of NOX2 over 6 d control. (C) The relative expression of p22phox over 6 d control. (D) The relative expression of p47phox over 6 d control. (E) The relative expression of RAC1 over 6 d control. Results are expressed as mean ± SD, $n = 3$, *$P < 0.05$, **$P < 0.01$ compared to 6 d control group.

and IL-18 in the supernatants were significantly increased in hippocampal neurons cultured for 12 d (Fig. 4E and F, $P < 0.05$ or $P < 0.01$).

3.5. Tempol and apocynin attenuated age-associated damage in prolonged cultured hippocampal neurons

The primary hippocampal neurons were cultured for 9 d, then treated with tempol and apocynin for 3 d. The results showed that, compared with 12 d control group (apoptosis rate 32.09 ± 3.27%), both the tempol and apocynin treatment could significantly decrease neuronal apoptosis in hippocampal neurons (Fig. 5A and B, 17.52 ± 1.61% for tempol, $P < 0.01$; 18.28 ± 2.71% for apocynin, $P < 0.01$). We then examined the effects of tempol and apocynin on expression of MAP2 in hippocampal neurons. The results showed that, compared with 12 d control group, tempol and apocynin treatment significantly increased MAP2 expression in hippocampal neurons (Fig. 5C and D, $P < 0.05$ or $P < 0.01$).

We further examined the effects of tempol and apocynin on ROS production and β-Gal expression in hippocampal neurons. The results of ROS production showed that, compared with 12 d control group, the tempol and apocynin could significantly decrease ROS production in hippocampal neurons (Fig. 6A and B, $P < 0.05$). The immunoblot results also showed that tempol and apocynin treatment significantly decreased β-Gal in hippocampal neurons (Fig. 6C and D, $P < 0.05$).

3.6. Effects of tempol and apocynin on expression of NLRP1, ASC, caspase-1, IL-1β in hippocampal neurons

We further detected the effects of tempol and apocynin treatment for 3 d on the expression of NLRP1, ASC, caspase-1 and IL-1β in hippocampal neurons. Compared with 12 d control group, both the tempol and apocynin significantly decreased the expressions of NLRP1, ASC, caspase-1 and IL-1β in hippocampal neurons (Fig. 7A–D, $P < 0.05$ or $P < 0.01$). The results suggested that both scavenging ROS and inhibiting NOX could down-regulate NLRP1 inflammasome.

3.7. Effects of NLRP1-siRNA on the apoptosis and β-Gal in hippocampal neurons

We first cultured hippocampal neurons for 6 d, then treated with Lentivirus vector for 3 d and 6 d to observe the transfected effects. We found that the Lentivirus vector with GFP treatment for 3 d showed weak green fluorescence expression and treatment for 6 d showed obvious green fluorescence expression, indicating high GFP expression and confirming successful Lentivirus infection (Fig. 8A). So we detected the effect of Lentivirus-mediated NLRP1-siRNA treatment for 6 d on NLRP1 expression in hippocampal neurons by immunoblot. The results showed that the NLRP1-siRNA (408) significantly decreased the expression of NLRP1 in hippocampal neurons (Fig. 8B, $P < 0.01$).

To confirm the effects of NLRP1 inflammasome on hippocampal neurons senescence and injury, we further measured the effects of NLRP1-siRNA (408) and caspase-1 inhibitor treatment for 6 d on apoptosis and β-Gal in primary cultured hippocampal neurons. The hoechst 33258 staining results showed that caspase-1 inhibitor and NLRP1-siRNA treatment for 6 d significantly decreased the apoptosis in hippocampal neurons compared with control group (Fig. 9A and B, $P < 0.05$ or $P < 0.01$). The β-Gal staining results showed that, compared with control group, caspase-1 inhibitor and NLRP1-siRNA treatment significantly decreased the expression of β-Gal in hippocampal neurons (Fig. 9C and D, $P < 0.05$ or $P < 0.01$). Meanwhile, the immunoblot results also showed that caspase-1 inhibitor and NLRP1-siRNA treatment significantly decreased the expression of β-Gal in hippocampal neurons (Fig. 9C, $P < 0.01$).

3.8. Effects of NLRP1-siRNA treatment on expression of NLRP1 and release of IL-1β and IL-18 in the supernatants

We further measured the effects caspase-1 inhibitor and NLRP1-siRNA treatment for 6 d on expression of NLRP1 by immunoblot. The results showed that, compared with control group, caspase-1 inhibitor and NLRP1-siRNA treatment significantly decreased the expression of NLRP1 in hippocampal neurons (Fig. 10A, $P < 0.01$). Furthermore, we detected the effects caspase-1 inhibitor and NLRP1-siRNA treatment on release of IL-1β and IL-18 by ELISA. The results showed that caspase-1

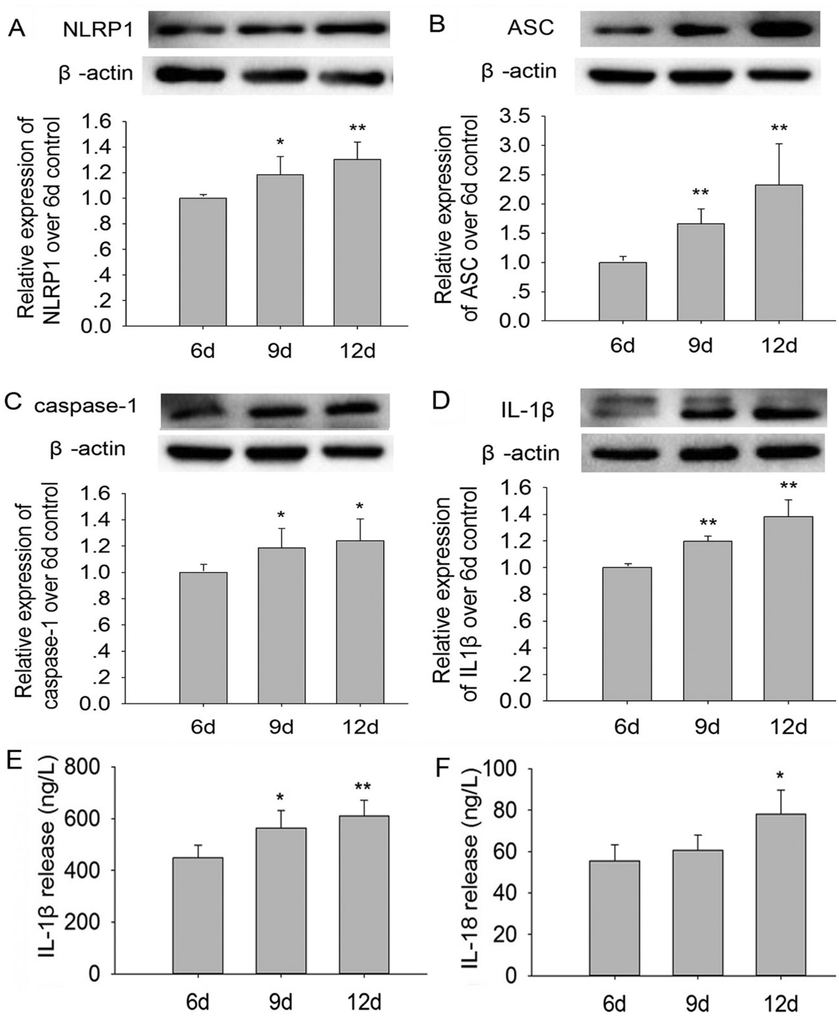

Fig. 4. The effects of different culture time for 6 d, 9 d and 12 d on the expressions of NLRP1, ASC, caspase-1 and IL-1β in hippocampal neurons (immunoblot) and the release of IL-1β and IL-18 in the supernatants (ELISA). (A) The relative expression of NLRP1 over 6 d control. (B) The relative expression of ASC over 6 d control. (C) The relative expression of caspase-1 over 6 d control. (D) The relative expression of IL-1β over 6 d control. (E) The release of IL-1β in the supernatants. (F) The release of IL-18 in the supernatants. Results are expressed as mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to 6 d control group.

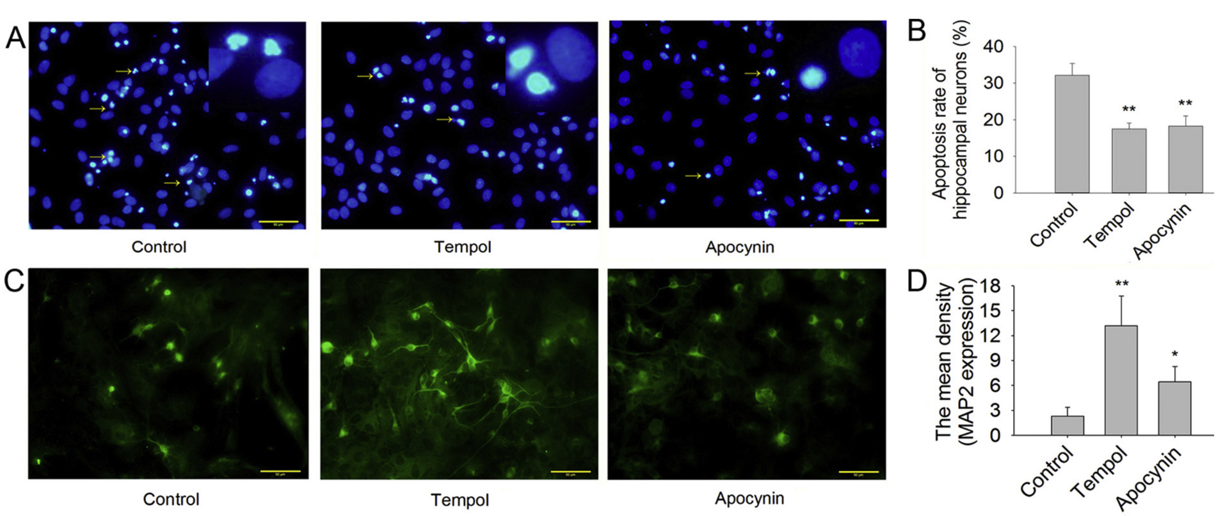

Fig. 5. The effects of tempol and apocynin treatment for 3 d on neuronal apoptosis and MAP2 expression in hippocampal neurons. (A) The effect of tempol (50 μM) and apocynin (50 μM) on neuronal apoptosis (Hoechst 33258 staining, 400 ×). The arrows point to the apoptotic cells. (B) Quantitative analysis of neuronal apoptosis rate. (C) The effect of tempol (50 μM) and apocynin (50 μM) on MAP2 expression in hippocampal neurons (immunofluoresence, 400 ×). (D) Quantitative analysis of MAP2 expression. Results are expressed as mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control group.

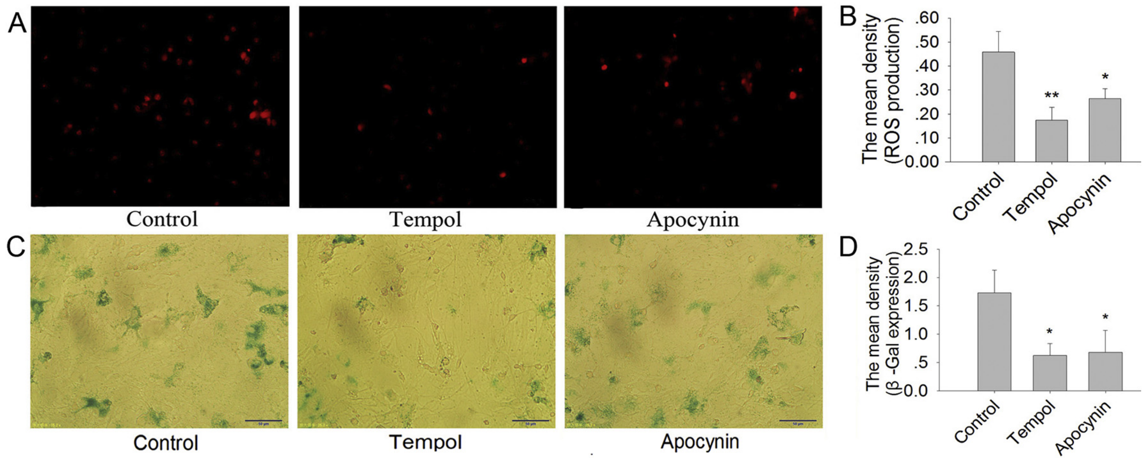

Fig. 6. The effect of tempol and apocynin treatment for 3 d on ROS production and β-Gal expression in hippocampal neurons. (A) The effect of tempol (50 μM) and apocynin (50 μM) on ROS production in hippocampal neurons (DHE staining, 400 ×). (B) Quantitative analysis of ROS production. (C) The effect of tempol (50 μM) and apocynin (50 μM) on β-Gal expression in hippocampal neurons (β-Gal staining, 400 ×). (D) Quantitative analysis of β-Gal expression. Results are expressed as mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control group.

inhibitor and NLRP1-siRNA treatment significantly decreased the release of IL-1β and IL-18 (Fig. 10B and C, $P < 0.05$).

4. Discussion

The present study demonstrated that primary hippocampal neurons developed characteristics of senescence with prolongation of culture time. And the expressions of NADPH oxidase 2 (NOX2) and NLRP1 inflammasome were significantly increased in primary hippocampal neurons cultured for 12 d compared with 6 d. Furthermore, our results indicated that the NADPH oxidase inhibitor, ROS scavenger, NLRP1-siRNA interference and caspase-1 inhibitor could reduce ageing-associated hippocampal neurons damage *in vitro*. Meanwhile, the NADPH oxidase inhibitor and ROS scavenger could down-regulate NLRP1 inflammasome in the prolonged culture of primary hippocampal neurons. Our results suggested that the NOX2 mediated NLRP1 inflammasome activation might play an important role in ageing-associated neuronal damage in hippocampal neurons.

With an ageing population, age-related cognitive decline will become an increasing problem for our society. However, the molecular mechanisms of brain ageing are largely unknown, which hinders development of the therapy to delay or prevent bran ageing. Both the ROS

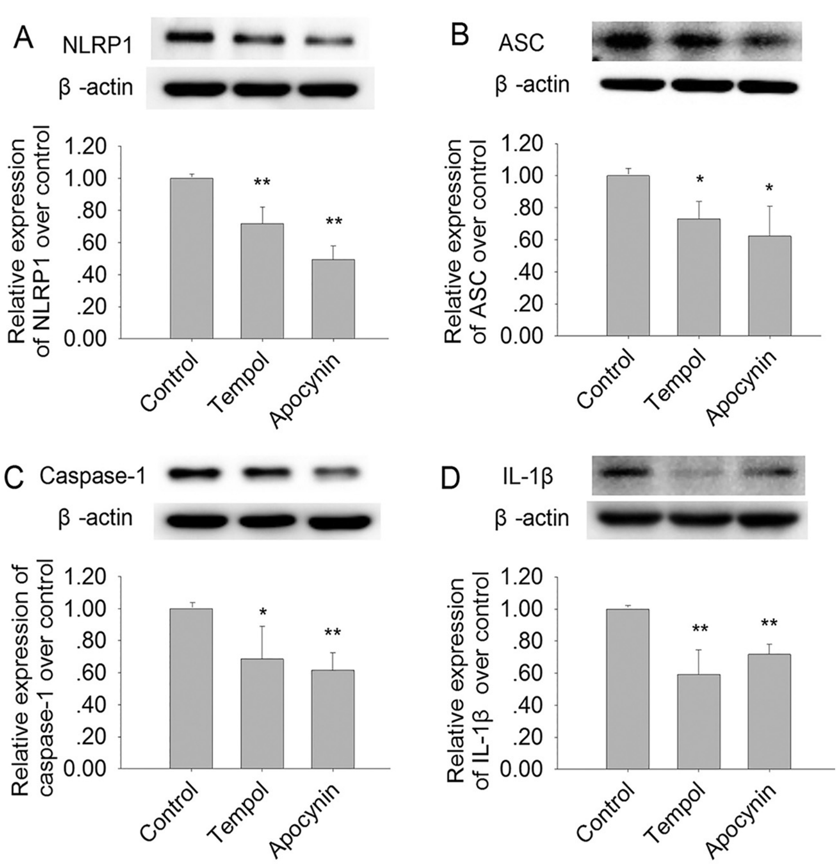

Fig. 7. The effects of tempol and apocynin treatment for 3 d on the expressions of NLRP1, ASC, caspase-1 and IL-1β in hippocampal neurons (immunoblot). (A) The relative expression of NLRP1 over control. (B) The relative expression of ASC over control. (C) The relative expression of caspase-1 over control. (D) 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.

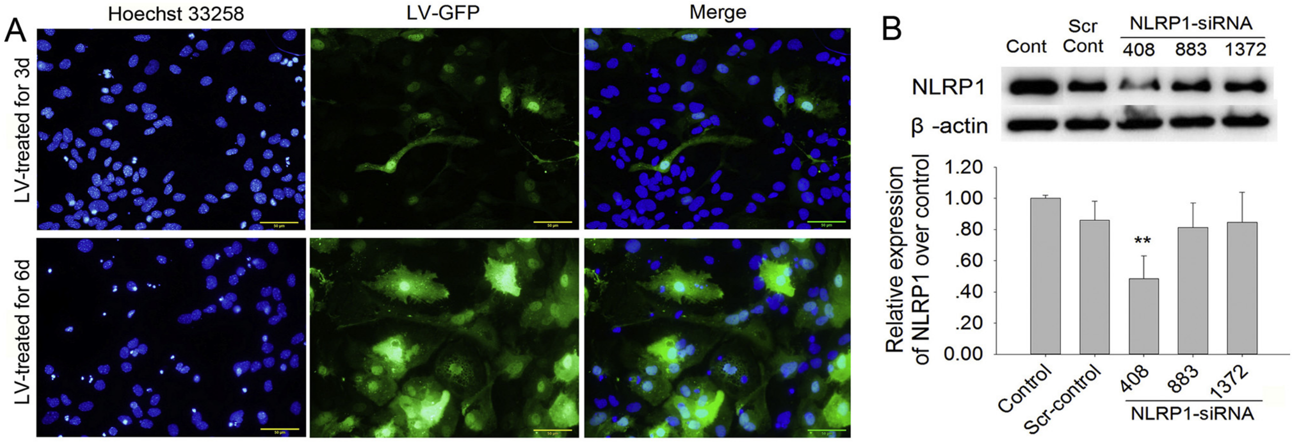

Fig. 8. The effect of NLRP1-siRNA on NLRP1 expression in primary culture hippocampal neurons. (A) The transfected effects of lentivirus vector treatment for 3 d and 6 d (GFP, 400×). (B) The effect of lentivirus-mediated NLRP1-siRNA treatment for 6 d on NLRP1 expression in hippocampal neurons (immunoblot). Results are expressed as mean ± SD, $n = 3$, $^{**}P < 0.01$ compared to control group.

oxidative stress and the neuronal inflammation are important events in brain ageing and senescence-associated neuronal damage [2,26]. The hippocampus is vulnerable in ageing processes, which has been linked to age-related memory loss [27,28]. However, the mechanisms of hippocampal neuronal damage in the process of brain ageing are still unclear.

There are many similarities between whole animal brain ageing and neuronal senescence in long-term culture of primary hippocampal neurons [29]. It has been reported that the prolonged culture for 21 d of primary hippocampal neurons present many of the typical hallmarks accompanying neuronal ageing *in vivo*, such as accumulation of ROS, lipofuscin granules, heterochromatic foci, and neuronal apoptosis [30]. While in the present study, we found that prolonged culture for 12 d significantly increased LDH release, β-Gal expression and neuronal apoptosis in primary hippocampal neurons. Furthermore, the results also showed that the ROS production and the level of IL-1β, IL-18 released in supernatants were significantly increased with the prolongation of culture time especially in 12 d. These data suggest that the

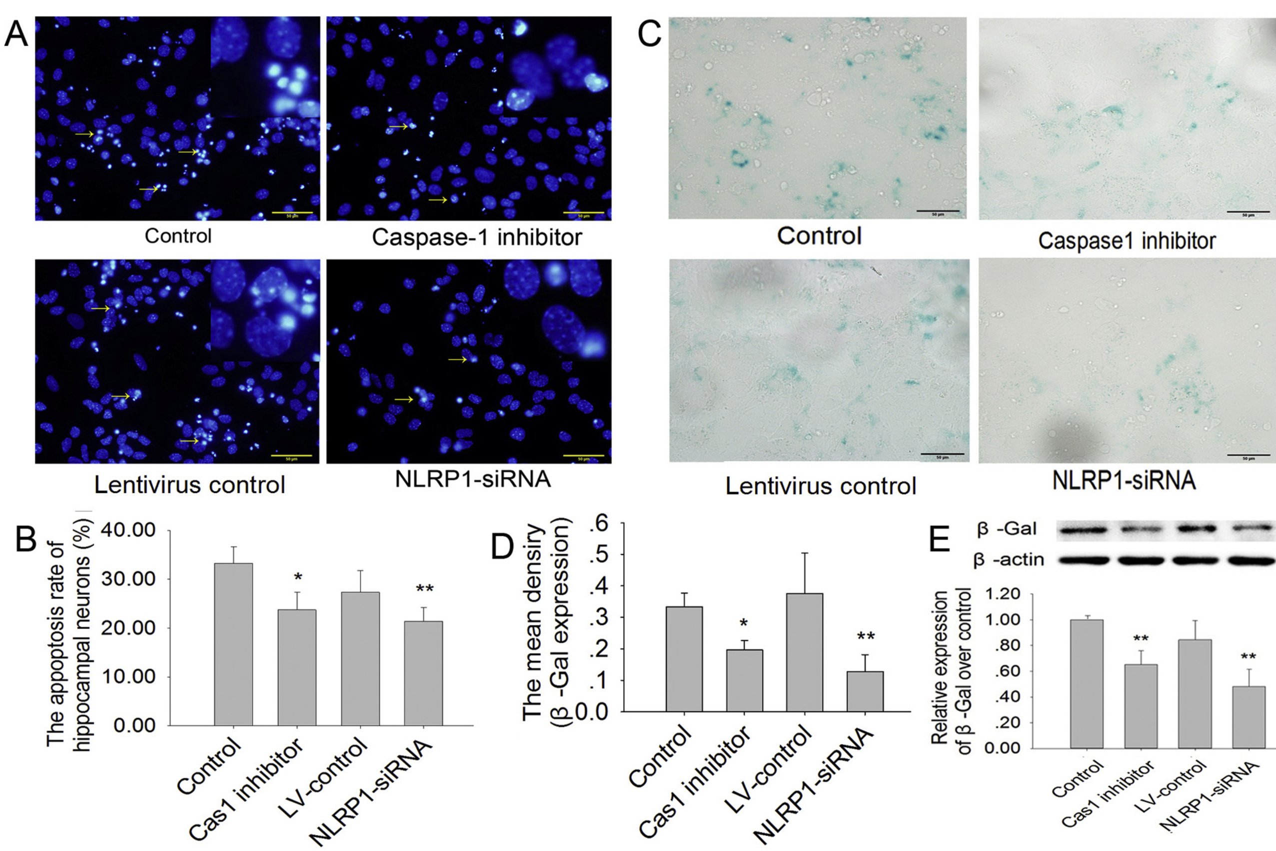

Fig. 9. The effects of NLRP1-siRNA and caspase-1 treatment for 6 d on neuronal apoptosis and β-Gal expression in hippocampal neurons. (A) The effect of NLRP1-siRNA and caspase-1 inhibitor (5 μM) on neuronal apoptosis (Hoechst 33258 staining, 400×). The arrows point to the apoptotic cells. (B) Quantitative analysis of neuronal apoptosis rate. (C) The effect of NLRP1-siRNA and caspase-1 inhibitor (5 μM) on β-Gal expression in hippocampal neurons (β-Gal staining, 400×). (D) Quantitative analysis of β-Gal expression. (C) The relative expression of β-Gal in hippocampal neurons over control (immunoblot). Results are expressed as mean ± SD, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control group.

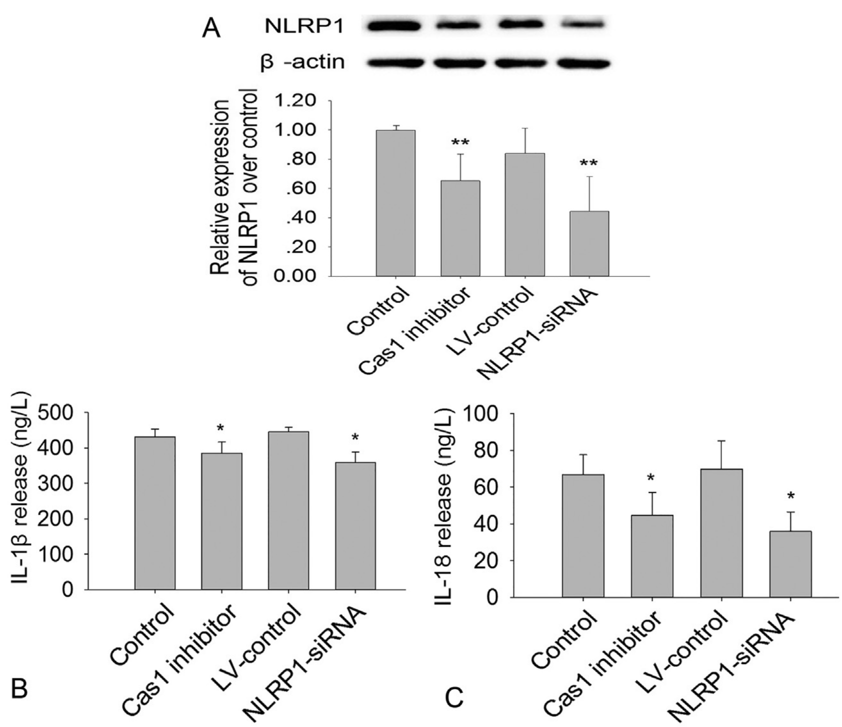

Fig. 10. The effects of NLRP1-siRNA and caspase-1 inhibitor treatment for 6 d on NLRP1 expression and the release of IL-1β, IL-18 in hippocampal neurons. (A) The relative expression of NLRP1 in hippocampal neurons over control (immunoblot). (B) The release of IL-1β (ELISA). (C) The release of IL-18 (ELISA). Results are expressed as mean ± SD, $n = 3$, $^*P < 0.05$, $^{**}P < 0.01$ compared to control group.

prolongation culture of primary hippocampal neurons for 12 d significantly increase the ageing-associated marker and can mimic the hippocampal neuron ageing *in vitro*. Both the oxidative stress and inflammation may play important roles in the age-related neuronal injury in primary cultured hippocampal neurons.

Oxidative stress has been reported to play an important role in the ageing process. During the normal ageing process, the multi-organ oxidative stress has been observed in numerous experimental studies, implicating that brain ageing may be associated with neuronal oxidative damage [31]. For example, the ROS production in the brain was found to increase in ageing mice (24 months old) in association with significant cognitive impairment [31]. In the present study, we found that the ROS production was significantly increased with the prolongation of culture time, especially at 12 d. However, it is still unclear how excessive ROS is formed in ageing neurons. It has been reported that activation of NADPH oxidases is an important contributor to the pathogenesis of numerous neurodegeneration diseases, such as Alzheimer's disease, Parkinson's disease and senescence associated cognitive dysfunction [32]. The NOX2 is a major source of ROS oxidative stress involved in the development of age-related cognitive dysfunction [32]. NOX2 expression has been found to be increased in the brain of ageing mice [33]. NOX2 consists of a membrane-bound catalytic core (including gp91phox and p22phox) and several cytosolic regulatory subunits (including p40phox, p47phox, p67phox and rac1) [34]. NOX2 is activated when the cytosolic subunits translocate to the membrane where multiple binding interactions in the subunits [35]. Aged mice over expressing the amyloid precursor protein had significantly increased levels of NOX2-derived superoxide production in the brain [36]. And there was a linear relationship between cognitive decline and NOX2 activity [37]. Moreover, NADPH oxidase may contribute to oxidative stress and neuronal apoptosis, which has been found that NADPH oxidase-deficient mice exhibit reduced injury after stroke [38]. However, whether excessive activation of NOX2 is involved in hippocampal neurons ageing *in vitro* needs further study. The present results showed that the expressions of NOX2 (pg91phox), p22phox, RAC1 and p47phox were significantly increased with the prolongation of culture time in primary hippocampal neurons. Like NOX2, NOX1 forms a heterodimer with p22phox. We also detected the expression of NOX1. The result showed that the expression of NOX1 had no significant difference with the prolongation of culture time in primary hippocampal neurons. These findings support an important role for NOX2-derived ROS oxidative stress in the senescence of hippocampal neurons.

Additional, the inflammatory response also plays an important role in age-related neurodegenerative diseases, such as AD, Parkinson's disease [39]. Growing studies indicated that the release of pro-inflammatory cytokines from microglia and astrocytes could cause direct neuronal damage and apoptosis [40,41]. Recent studies showed that, in addition to microglia and astrocytes, neurons also contribute to the inflammatory response in the brain by releasing cytokines, such as IL-1β and IL-18 [39]. Inflammasomes are intracellular multiprotein complexes. NLRP1 and NLRP3 inflammasomes are involved in the maturation of IL-1β and IL-18, and neuronal death in ischemic stroke [42]. The NLRP1 inflammasome is first reported member of the NLRP family. It has been reported that NLRP1 inflammasome plays an important role in neurodegeneration diseases. NLRP1 inflammasome is activated in patients with epilepsy and in amygdala kindling-induced rat model [14]. Recent studies showed that NLRP1 siRNA significantly reduced TUNEL-positive cells after H_2O_2 treatment in cultured primary spinal cord neurons and IL-1β exposure could significantly increase chondrocyte apoptosis [43,44]. However, whether prolonged culture of primary hippocampal neurons can activate NLRP1 inflammasome and induce neuronal injury and apoptosis is not fully understood.

The NLRP1 inflammasome is composed of NOD-like receptor protein 1 (NLRP-1) that recognizes danger signals or ligands, procaspase-1 which is central to inflammasome activation and the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) [45]. The ASC is also involved in the assembly or activity of inflammasomes, depending on the type of cell and stimulus [46,47]. Currently, the expressions of NLRP1 inflammasome-related proteins in senescence neurons are not extensively studied. The present results showed that the expression of NLRP1, ASC, caspase-1 and IL-1β significantly increased with the prolongation of culture time in primary hippocampal neurons. Meanwhile, our results also showed that the levels of IL-1β and IL-18 released in the supernatant were significantly increased with the prolongation of culture time, especially in 12 d. The data suggest that NLRP1 inflammasome is involved in the hippocampal neuronal damage in the process of brain ageing. We further detected the effects of NLRP1-siRNA and caspase-1 inhibitor on neuronal senescence in the hippocampal neurons. The results showed that NLRP1-siRNA and caspase-1 inhibitor could significantly down-regulate the expression of NLRP1, IL-1β, IL-18 and β-Gal in the long-term cultured hippocampal neurons. These data suggest that NLRP1 inflammasome participates in the ageing-related damage of hippocampal neurons, and inhibition of NLRP1 inflammasome may be an important target for delaying ageing of hippocampal neurons.

Several mechanisms were involved in inflammasome activation, including lysosome rupture, ion channel gating, and ROS activation [48,49]. The activation of NLRP3 inflammasome induced by excessive ROS is the most widely accepted and considered to be the most plausible mechanism [49]. Further study showed that the NLRP3 inflammasome could be activated by NADPH oxidase-derived reactive oxygen species (ROS) production [50]. NADPH oxidase inhibitors, diphenylene iodonium (DPI) and apocynin have been shown to reduce IL-1β production in a model of asbestos inhalation [50]. The latest research showed that both deleption and inhibition of NOX2 decreased the expression and activation of the NLRP3 inflammasome following traumatic brain injury. However, the NLRP3 is mainly expressed in microglia rather than neurons, and NLRP1 is mainly expressed in neurons [51]. In the present study, we think that the NADPH oxidase 2 may involve in activation of NLRP1 inflammasome in the process of senscence in hippocampal neurons. The present results showed that, with the prolongation of culture time, the production of ROS significantly increased and the NOX2 was also significantly increased in the hippocampal neurons. Meanwhile, the NLRP1 inflammasome was significantly activated in the ageing hippocampal neurons. The results suggest that the NOX2 may mediate the activation of NLRP1 inflammasome in the long-term culture hippocampal neurons. Apocynin is commonly used as a tool to inhibit NADPH oxidase activity, thereby preventing the production of oxygen radicals. Apocynin inhibits NOX2 with an IC50 of approx 10 μM and NOX4 with an IC50 of > 200 μM [52]. Resent study showed that apocynin attenuated ischemia/reperfusion-induced increases of NOX2, NOX4 and ROS. And apocynin significantly inhibited the expression of inflammasome proteins including NLRP3 ASC, caspase-1, interleukin (IL)-1β and IL-18 in the ischemic cortex [53]. Tempol, functions as a radical scavenger, similar to superoxide dismutase (SOD) or catalase. Tempol (50 μM) has been reported to restore the intracellular oxidative balance and reduce oxidative stress [54,55]. In the present study, we observed the effect of apocynin (50 μM) and tempol (50 μM) on NOX2-related ROS generation and NLRP1 inflammasome activation in ageing hippocampal neurons. Our results showed that both apocynin and tempol significantly reduced the ROS production, down-regulated the expression of NLRP1 inflammasome and attenuated the neuronal damage in the prolonged culture of hippocampal neurons. Our data suggest that NOX2-derived ROS is involved in activation of the NLRP1 inflammasome in the ageing neurons. And the NADPH oxidase and NLRP1 inflammasome may be important therapeutic targets for ageing-associated neuronal damage.

Overall, the neuronal ageing process is complex. The study demonstrated that the levels of ROS and inflammatory factors significantly increased with the prolongation of culture time in primary hippocampal neurons. NADPH oxidase inhibitor, the ROS scavenger, NLRP1-siRNA and caspase-1 inhibitor could significantly down-regulate the expression of NLRP1, IL-1β, IL-18 and β-Gal in the prolonged cultured hippocampal neurons. Our findings provide support for the hypothesis that NOX2-derived ROS generation may activate NLRP1 inflammasome and promote neuroinflammation and neuronal apoptosis which plays an important role in the brain ageing. However, the study only provided an experimental basis for NOX2-NLRP1 inflammasome signaling pathway on neuronal senescence in primary hippocampal neurons *in vitro*. Other related effects and mechanisms underlying the NOX2-NLRP1 inflammasome in brain ageing *in vivo* warrant further investigations.

Disclosure statement

The authors report no conflicts of interest.

Acknowledgments

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