Molecular Neurobiology (2019) 56:6293–6309
https://doi.org/10.1007/s12035-019-1512-7

Hesperetin Confers Neuroprotection by Regulating Nrf2/TLR4/NF-κB Signaling in an Aβ Mouse Model

Muhammad Ikram[1] · Tahir Muhammad[1] · Shafiq Ur Rehman[1] · Amjad Khan[1] · Min Gi Jo[1] · Tahir Ali[1] · Myeong Ok Kim[1]

Received: 5 December 2018 / Accepted: 24 January 2019 / Published online: 12 February 2019

Abstract

Hesperetin is a bioactive flavonoid in the body, produced from hesperidin. No comprehensive studies have shown its protective effects in neurodegenerative disorders. Here, we hypothesized that hesperetin may protect the mice brain against Aβ-induced neurodegeneration. Twenty-four hours after intracerebroventricular injection of Aβ1-42, the treated group was injected hesperetin. For in vitro experiments, HT22 and BV-2 cells were used. Immunoblot, immunofluorescence, and behavioral analyses were used to evaluate the different parameters. Our results indicated that hesperetin significantly attenuated oxidative stress, as assessed by the expression of Nrf2/HO-1 and LPO and ROS assays, in the hippocampus, cortex, and in vitro HT22 cells. Similarly, activated glial cells were regulated by hesperetin, as assessed by the expression of GFAP and Iba-1. Moreover, the expression of TLR4, p-NF-κB, and downstream targets was analyzed; the results showed that hesperetin reinstated the expression of these markers. The effects of hesperetin were further confirmed by using specific TLR4 and p-NF-κB inhibitors in BV-2 cells. Next, we evaluated Aβ pathology in the cortex, hippocampus, and HT22 cells, showing that hesperetin significantly reduced the Aβ pathology. Furthermore, the antiapoptotic effects of hesperetin were assessed, which showed strong antiapoptotic effects. Overall, the neuroprotective effect of hesperetin was found to be a multipotent effect, involving the inhibition of oxidative stress, neuroinflammation, apoptotic cell death, and cognitive consolidation. Given antioxidant, anti-inflammatory, and antiapoptotic potentials against Aβ-induced neurodegeneration and memory impairment, hesperetin may be a promising therapeutic agent for Alzheimer's disease–like neurological disorders.

Keywords Amyloid beta · Neuroinflammation · Neurodegeneration · Hesperetin · Neuroprotection

Introduction

Alzheimer's disease is a neurodegenerative disorder affecting a number of aging people [1, 2], has a preclinical phase of 20–30 years before clinical onset [3], and characterized by the progressive loss of memory and cognition [4]. Worldwide, 33.9 million people suffer from this condition, and it is hypothesized that there will be a threefold increase in the burden of the disease in the next 40 years [5]. The main pathological hallmarks of the AD (Alzheimer's disease) are neurofibrillary tangles [6], senile plaques, synaptic dysfunction, and neurodegeneration [7]. Senile plaques are extracellular aggregates composed of amyloid beta (Aβ) peptides, while neurofibrillary tangles are made of hyperphosphorylated tau protein [7].

Many hypotheses have been proposed so far, but the most popular one is the Aβ hypothesis. Aβ is continuously generated by the catalytic cleavage of the amyloid precursor protein (APP) by β-secretase and γ-secretase [8]. The formation of Aβ has been shown to be neurotoxic both in vivo [9] and in vitro, contributing to the pathogenesis of AD-like conditions [10, 11]. Aβ plaques promote the pathogenesis of AD by different mechanisms, such as through generation of ROS (reactive oxygen species) [12] and activation of astrocytes and microglia [13]. Nrf2 (nuclear factor erythroid 2-related factor 2) has been shown to regulate endogenous antioxidant mechanisms [14, 15]. When ROS levels are elevated, Nrf2 and HO-1 (heme oxygenase-1) are downregulated (as endogenous antioxidant regulators), which contributes to the pathogenesis of AD-like effects [16]. Activation of microglia by Aβ [17] may exert protective effects by enabling phagocytic clearance of Aβ [18], and it may also contribute to progressive

✉ Myeong Ok Kim
mokim@gnu.ac.kr

[1] Division of Life Science and Applied Life Science (BK21 plus), College of Natural Sciences, Gyeongsang National University, Jinju 52828, Republic of Korea

neurodegeneration [19], thus playing conflicting roles in the CNS (central nervous system). Microglia are tissue macrophages performing tissue maintenance and immune surveillance [20]. Activated microglia have a wide variety of pattern recognition receptors from the Toll-like receptor (TLR) group that detect microbial intruders. Thirteen types of TLRs have been identified that identify molecules that may be referred to as toxins. Activation of these TLRs exacerbates various signaling, leading to activation of inflammatory agents, such as cytokines, ROS, and nitric oxide [21]. Microglial expression of TLRs in the CNS is a vital first line of defense against endogenous and exogenous agents [22, 23]. TLRs are involved in Aβ signaling, in which they activate an intracellular mechanism resulting in activation of proinflammatory agents and clearance of Aβ [24–26]. TLR-mediated inflammatory responses induced by Aβ can lead to neurotoxic effects. TLRs 2, 4, and 9 have been suggested as therapeutic targets for AD treatment [27, 28].

Flavonoids are considered the most beneficial agents for neurodegenerative disorders because of their antioxidant [29], anti-inflammatory, and antiapoptotic effects [30, 31]. The flavonoid hesperetin (3′,5,7-trihydroxy-4-methoxy flavanone) is a flavanone, a member of a subclass of flavonoids, found in *Citrus* fruit species [32]. Although hesperetin, which is produced from hesperidin, is a bioactive molecule in the body, only a few in vitro studies have assessed hesperetin rather than hesperidin. The in vitro studies suggest that hesperetin is a powerful radical scavenger that promotes cellular anti-inflammatory activity [33]. Previously, hesperetin has been shown to be neuroprotective in different models [34, 35], but the protective role of hesperetin in AD models has not been explored to date. Therefore, here, for the very first time, we explored the basic neuroprotective mechanisms of hesperetin against amyloid beta–induced neurodegeneration, focusing on abnormal ROS production, activation of astrocytes and microglia, apoptotic cell death, neuroinflammation, and synaptic dysfunction in an in vivo Aβ-injected mouse model, and in vitro Aβ-treated cells. Based on our findings, we conclude that hesperetin may serve as a promising and reasonable neuroprotective agent against Aβ1-42–induced neurodegeneration and memory impairment.

Materials and Methods

Animal Handling

The animals were handled according to the protocols approved by the Ethical Committee of the Applied Life Sciences, Gyeongsang National University, South Korea. Thirteen healthy adult male mice (C57BL/6N, wild type) were included in each group, ages ranged from 8 to 10 weeks, and the body weights were up to 25–30 g. The mice were purchased from Samtako Bio Korea. All animals were acclimatized for 1 week under the same laboratory conditions with a 12-h light/dark cycle at 60 ± 10% humidity and at room temperature and were provided with standard food and water ad libitum.

Mice Groups and Treatments

The mice were randomly divided into 4 groups, control (saline-injected group), Aβ1-42 (5 μl/5 min/mouse), Aβ1-42 + hesperetin (50 mg/kg for 6 weeks), and hesperetin-alone (50 mg/kg for 6 weeks) group. The human-derived peptide Aβ1-42 was reconstituted in sterile saline solution as a stock solution at a concentration of 1 mg/ml, followed by incubation at 37 °C for 4 days. After that, the aggregated Aβ1-42 peptide was stereotaxically (i.c.v.) injected into the ventricles by using a Hamilton microsyringe (2.4 mm dorsoventral (DV), 0.2 mm anteroposterior (AP), 1 mm mediolateral (ML) to the bregma). Before the surgical procedures, the mice were anesthetized with 0.05 ml/100 g body weight Rompun (and 0.1 mL/100 g body weight Zolitil) [36]. The control mice were injected with 0.9% saline, and the Aβ1-42 + hesperetin mice were injected with hesperetin (50 mg/kg) for 6 weeks (alternate day injection). Hesperetin-alone group was included in some experiments to examine its effect on cell viability in MTT assay and in oxidative stress–related experiments.

Morris Water Maze Test

Cognitive dysfunction in experimental mice was assessed by the Morris water maze (MWM) test as described previously with modifications [37, 38]. The apparatus used for the MWM test consisted of a circular tank filled with water and made opaque with nontoxic white ink. A platform with a 10 cm diameter was placed hidden in the water (1 cm below the water surface) in one quadrant of the apparatus during the experiment. In each trial, the mice were allowed to explore the location of the hidden platform; if the mice failed to find the platform in 60 s, the mice were guided to the platform and given 30 s to remain there. For 4 consecutive days, the mice were trained, and the escape latency and swimming speed was recorded. Next, the hidden platform was removed, and a probe test was conducted. In the probe trial, the number of crossings, the time spent in the target quadrant, the latency to the platform area, and the swimming speed were calculated. The data were recorded using a video tracking system (SMART, Panlab Harvard Apparatus, Bioscience Company).

Y-Maze Test

The Y-maze was made from black-painted wood, having three arms (50 cm in length, 20 cm in height, and 10 cm in width) [38]. Briefly, the mice were placed in the center of the

apparatus and allowed to explore the apparatus for 8 min. The arm entries were observed visually. Spontaneous alternation behavior was defined as successive entry of the mice into the three arms in overlapping triplet sets. The alternation behavior percentage (%) was calculated as [successive triplet sets (entries into three different arms consecutively)/total number of arm entries-2] × 100. A higher percentage of spontaneous alternation behavior was considered improved cognitive performance, and vice versa.

Extraction of Proteins and Homogenization

The expression of the proteins was assessed by western blot, as described previously [39]. Briefly, the mice were anesthetized, decapitated, and the cortex and hippocampus of the brain were separated. Next, the sections were homogenized in PRO-PREP extraction solution (iNtRON Biotechnology) and centrifuged at a speed of 13,000 RPM for 25 min at 4 °C. After centrifugation, the supernatant was collected and stored at − 70 °C for further studies. For morphological analysis, the mice were perfused with saline and 4% neutral buffered paraformaldehyde, fixed in NBP and sucrose for 48 h each [40], and frozen in OCT compound, cut into uniform sections (14 μm) by using a microtome (Leica, Germany). The sections were thaw-mounted on ProbeOn Plus charged slides (Thermo Fisher) and stored at − 70 °C.

Western Blot Analysis

The protein concentrations were quantified by using a Bradford assay (Bio-Rad Protein Assay kit, Bio-Rad Laboratories, CA, USA), as described previously [41, 42]. Briefly, equal amounts of samples were electrophoresed using 10–12% SDS-PAGE with a broad-range prestained protein marker (GangNam-STAIN, iNtRON Biotechnology, South Korea) as a molecular size control. The protein bands from the gels were transferred to PVDF membranes. To reduce the nonspecific bindings, the membranes were blocked for 1 h in skim milk (5% *w*/*v* skim milk in 1× TBST). After blocking, the membranes were incubated with the respective primary antibodies at 4 °C (1:1000 dilutions, as optimized) for 16 h, washed with 1× TBST (10 min, 3 times), and blocked with horseradish peroxidase-conjugated secondary antibodies as appropriate. The bands were detected using an ECL detection reagent (EzWestLumiOne, ATTO, Tokyo, Japan) according to the manufacturer's instructions. The films were scanned, and the expression of the proteins was quantified via densitometry using computer-based ImageJ software.

Immunofluorescence (In Vivo)

The slides were washed twice for 15 min with 0.01 M PBS, blocked for 1 h with 5% normal goat serum, and incubated with appropriate antibodies (diluted 1:100 in 0.01 M PBS) for 24 h. After washing, the sections were incubated with FITC/TRITC-goat anti-mouse/goat anti-rabbit secondary antibodies for 2 h (1:100 in 1% PBS) (Santa Cruz Biotechnology, Santa Cruz, USA). After incubation, the slides were washed with PBS and treated with 4,6′-diamidino-2-phenylindole (DAPI) for 10 min, rinsed and covered with fluorescent mounting medium DAKO [43].

Images were captured by using a confocal scanning laser microscope (Fluoview FV1000 MPE, Olympus, Japan), and the intensity of the immunoreactivity was quantified by using ImageJ, and the graphs and statistical analyses were made with GraphPad Prism 6.

Antibodies and Reagents

The antibodies used in western blot and immunofluorescence studies were anti-Nrf2 (sc-722), anti-HO-1 (sc-136,961), anti-PSD-95 (sc-71,933), anti-Syntaxin (sc-12,736), anti-synaptosomal-associated protein 23 (SNAP-23) (sc-374,215), anti-Aβ (sc-28365), anti-BACE-1 (sc-33711), anti-Caspase 3 (sc-7272), anti- PARP-1, anti-TLR4 (sc-16240), Synaptophysin (sc-17750), anti-Bax (sc-7480), anti-Bcl2 (sc-7382), anti-TNF-α (sc-52,746), anti-IL-1β (sc-32,294), anti-p-NF-κB (sc-136,548), anti-Iba-1 (sc-32,725), anti-GFAP (sc-33,673), and anti-β- actin (sc-47,778) from Santa Cruz Biotechnology (Dallas, TX, USA). In addition, anti-Cleaved Caspase-3 (#9664) and anti-APP (#2452S) antibodies were obtained from Cell Signaling Technology (Massachusetts, USA). The primary antibodies were diluted in TBST (1:1000) (Santa Cruz Biotechnology), and secondary anti-mouse HRP conjugated (Promega Ref# W402), anti-rabbit HRP conjugated (Promega Ref# W401) diluted in 1:10,000 in 1× TBST were purchased from Promega, USA. For confocal microscopic studies, the secondary fluorescent antibodies used were Goat anti-mouse (Ref# A11029), and Goat anti-rabbit (Ref# 32732) diluted in 1× PBS, BAY 11-7082 (BAY, the inhibitor of p-NF-kB (sc-200615), and TAK242, resatorvid (CAS 243984-11-4), the specific inhibitor of TLR4.

ROS Assay

The assay was performed to analyze the levels of ROS in the brain and in the cells of the experimental groups ($n = 7$ mice/group). The ROS assay was based on the oxidation of DCFH-DA to 2′7′-dichlorofluorescein (DCF) [44]. The conversion of DCFH-DA to DCF was assessed by a spectrofluorimeter at an excitation wavelength of 484 nm and an emission wavelength of 530 nm. To measure the conversion of DCFH-DA to DCF in the absence of homogenate (background fluorescence), parallel blanks were used to standardize DCF. The

ROS levels were quantified from a DCF standard curve and expressed as relative pmol DCF/mg protein.

Lipid Peroxidation Assay

The lipid peroxidation assay was performed for the different experimental groups ($n = 7$ mice/group) by analyzing the levels of malondialdehyde (MDA), for which the commercially available lipid peroxidation assay kit (catalog #K739-100 from BioVision, USA) was used, as performed previously [40, 45].

Cell Viability Assay

Cell viability (HT22 cells, from the Korean Cell Bank, Korea) was assessed with an MTT assay according to the manufacturer's instructions, and as performed previously [46]. Briefly, cells were cultured in 96-well plates (Thermo Fisher Scientific 75 Panorama Creek Drive Rochester, NY14625-2385, USA) at a density of 1 × 10 cells per well in 100 μl of Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies, USA). After 24 h, the medium was replaced with fresh medium containing Aβ1-42 (5 μM) alone or with different concentrations of hesperetin (10, 20, or 50 μM), and the cells were incubated for another 24 h. For the control group, only DMEM was added. The cells were incubated with MTT solution for 2–4 h at normal body temperature. The medium was replaced with DMSO, and the absorbance in each well was measured at 580 nm using an ApoTox (Promega) instrument. The results were obtained in triplicate.

In Vitro Western Blot and Immunofluorescence

For in vitro western blot and immunofluorescence studies, the HT22 and BV-2 cells were cultured in four-well chambers and divided into 4 groups: the control group (treated with DMEM), the Aβ1-42–treated (5 μM) group, the Aβ1-42 plus hesperetin–treated (50 μM) group, and the hesperetin-alone (50 μM) group. The dose of hesperetin was selected on the basis of the MTT assay results, while the Aβ1-42 dose was selected on the basis of previous literature [36]. Doses of BAY 11-7082 were used 15 μM and TAK242 was used 2 μM. The cells were washed with 0.01 M PBS and mixed with PROPREP extraction solution for immunoblot analysis. For immunohistological analysis, the cells were incubated and treated in a similar way and then fixed with ice-cold 4% paraformaldehyde (NBP) for 30 min.

In Vitro Oxidative Stress (ROS) and Lipid Peroxidation (LPO) Assays

As described previously, the experimental groups of cells cultured in 96-well plates were incubated with a 600 μM solution of DCFDA (20, 70-dichlorofluorescein diacetate) dissolved in DMSO/PBS for 30 min. Readings were taken by using an ApoTox-Glo (Promega) instrument at 488/530 nm. The lipid peroxidation assay was performed by analyzing the levels of malondialdehyde (MDA), for which a commercially available lipid peroxidation assay kit (catalog #K739-100, BioVision, USA) was used.

Statistical Analysis

The intensity of the bands was quantified and analyzed through densitometry using computer-based ImageJ software. The data has been presented as the mean ± standard error of the mean (SEM). For statistical analysis, one-way analysis of variance (ANOVA) followed by Student's *t test* was used for comparisons among the different groups. Calculations were performed and graphs were generated via Prism 6 software (San Diego, CA). *P* values less than 0.05 were considered to indicate significant differences, number sign indicates a significant difference from the vehicle-treated control group, while asterisk indicates a significant difference from the Aβ1-42–treated groups ($^{*}p < 0.05$ and $\#p < 0.05$) (Fig. 1).

Results

Hesperetin Regulates the Oxidative Stress in Aβ1-42–Treated Mice Cortex, Hippocampus, and in HT22 cells

To validate the protective effects of hesperetin against Aβ1-42–induced toxicity in HT22 hippocampal cells, we tested cell viability by treating HT22 cells with Aβ1-42 (5 μM) and different concentrations of hesperetin (10, 20, and 50 μM) for 24 h. The results showed that after 24 h, Aβ1-42 negatively affected cell viability compared with the control treatment. Hesperetin was nontoxic to HT22 cells at all the three concentrations (50, 100, and 200 μM), and hesperetin administration with Aβ1-42 significantly increased cell viability. To explore the antioxidant potential of hesperetin against Aβ1-42–induced oxidative stress, we conducted a ROS assay in vitro. Aβ1-42 (5 μM) resulted in increased ROS levels compared with the control group, which was reduced with hesperetin (50 μM). Similarly, there was an enhanced expression of LPO in the Aβ1-42–treated group, which was significantly attenuated with hesperetin (50 μM). Similarly, there was a significant reduction in the level of ROS and LPO in the in vivo–treated group. Moreover, the antioxidant potential of hesperetin was further assessed by analyzing the relative expression of Nrf2 and HO-1 in the mouse

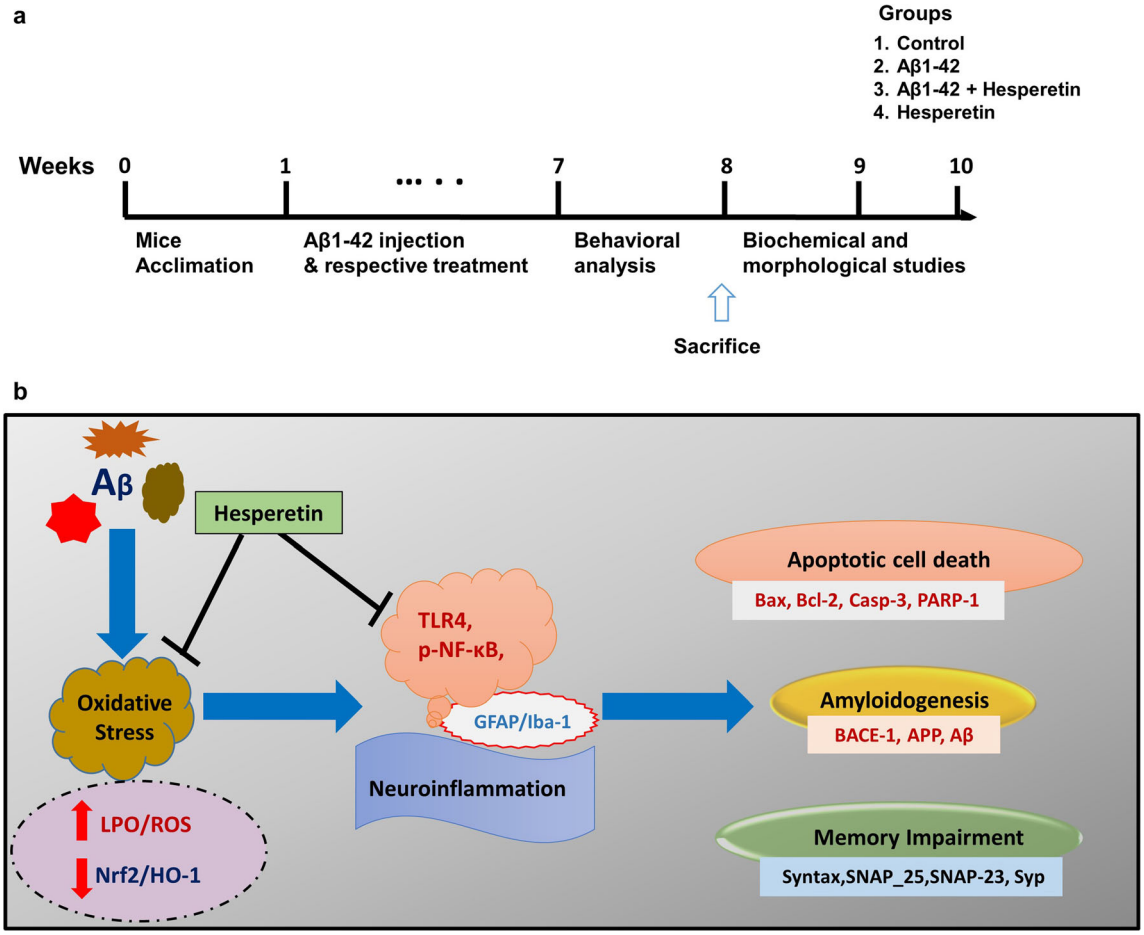


Fig. 1 Study design and schematic diagram of the possible neuroprotective mechanisms of hesperetin in the Aβ1-42–induced AD models. **a** Schedule of the experiment conducted in the current study. **b** Graphical abstract, showing the involvement of the Nrf2/TLR4/NF-κB signaling mechanisms, by which hesperetin protects the mouse brain against Aβ-induced neurodegeneration. The injection of Aβ into the mouse brain triggers AD-like pathology by inducing APP, BACE-1, and Aβ. The accumulated Aβ induces abnormal ROS production, thus disturbing the endogenous antioxidant genes Nrf2 and HO-1, and elevating the abnormal ROS. The elevated ROS level may promote the induction of the innate immune response by inducing TLR4 and the phosphorylation of NF-κB, which leads to the activation of apoptotic and inflammatory signaling. According to our hypothesis, hesperetin regulates the expression of Aβ pathology by regulating ROS, TLR4/NF-κB, and Bax/Bcl-2, thereby rendering neuroprotection to Aβ-treated in vivo and in vitro models

brain. Our results showed that the expression of Nrf2 and HO-1 was significantly reduced in Aβ1-42–injected mice brain, but was upregulated by hesperetin. The western blot results were further confirmed by the results of the immunofluorescence studies, which showed that hesperetin significantly upregulated the expression of Nrf2 and HO-1 in the of Aβ1-42–injected mice brains (Fig. 2).

Hesperetin Regulates the Activated Astrocytes and Microglia in the Cortex and Hippocampus of an Amyloid Beta–Injected Mouse Model

Previous studies have extensively shown that there is increased activation of astrocytes and microglia, the prime pathological hallmarks of neurodegeneration, upon administration of Aβ [47]. To determine whether hesperetin could reverse Aβ-induced activation of astrocytes and microglia in mouse brains, we performed western blots for the different experimental groups. According to our results, there was an increased expression of GFAP and Iba-1 in brain homogenates from the experimental groups; however, this increase was significantly attenuated by the administration of hesperetin, showing that hesperetin might be a novel and effective compound that could reverse Aβ-induced astrogliosis. The western blot results were further confirmed by the results of immunofluorescence analysis, which indicated that hesperetin significantly reversed the toxic effects of Aβ on astrocytes and microglia in the mouse brain, as shown by the relative immunofluorescence of GFAP and Iba-1 respectively (Fig. 3).

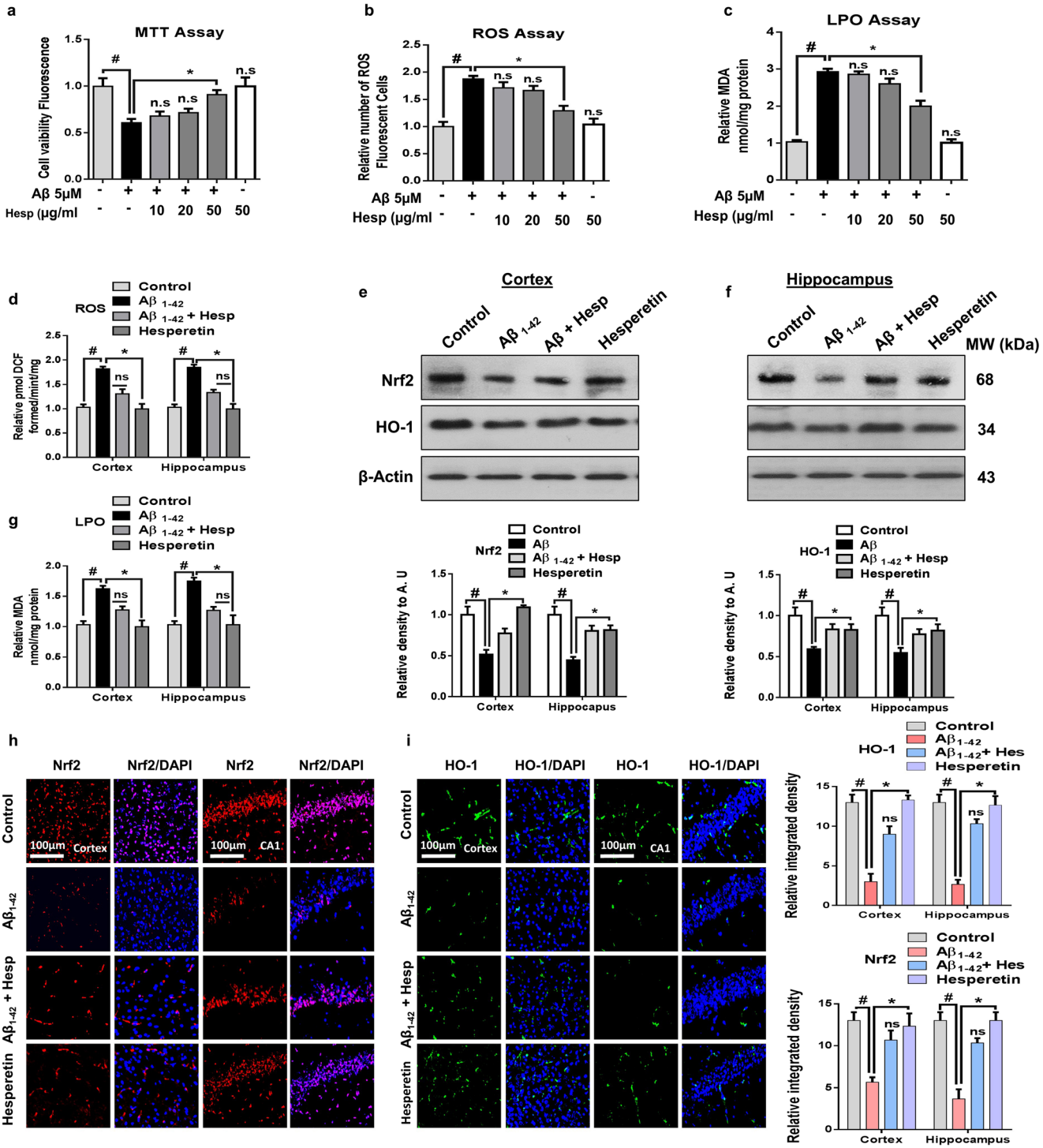


Fig. 2 Hesperetin regulates the oxidative stress in Aβ1-42–treated in vivo and in vitro models. **a** MTT assay in vitro HT22 cells. **b** and **c** Bar graphs showing the results of ROS and LPO assays in vitro, respectively. **d** and **g** Bar graphs showing the results of ROS and LPO assays in vivo, respectively. **e** and **f** Immunoblots and bar graphs of Nrf2 and HO-1 expression in the cortex and hippocampus of experimental mice. **d** The densities of the bands were quantified by ImageJ software, and the differences are given in the bar graphs, which were generated with GraphPad Prism 6 software. β-Actin was used as a loading control. The density values are shown in arbitrary units (A.U.) as the mean ± SEM for the indicated proteins ($n = 8$ mice/group). **h** and **i** Confocal microscopic images and graph bars showing the expression of Nrf2 and HO-1 in the cortex and hippocampus of the experimental mice. ImageJ software was used for immunohistological analysis. The values show the mean ± SD. Asterisk indicates significantly different from the control group; number sign indicates significantly different from the treated group. Significance $^{*}P < 0.05$ and $\#\ P < 0.05$

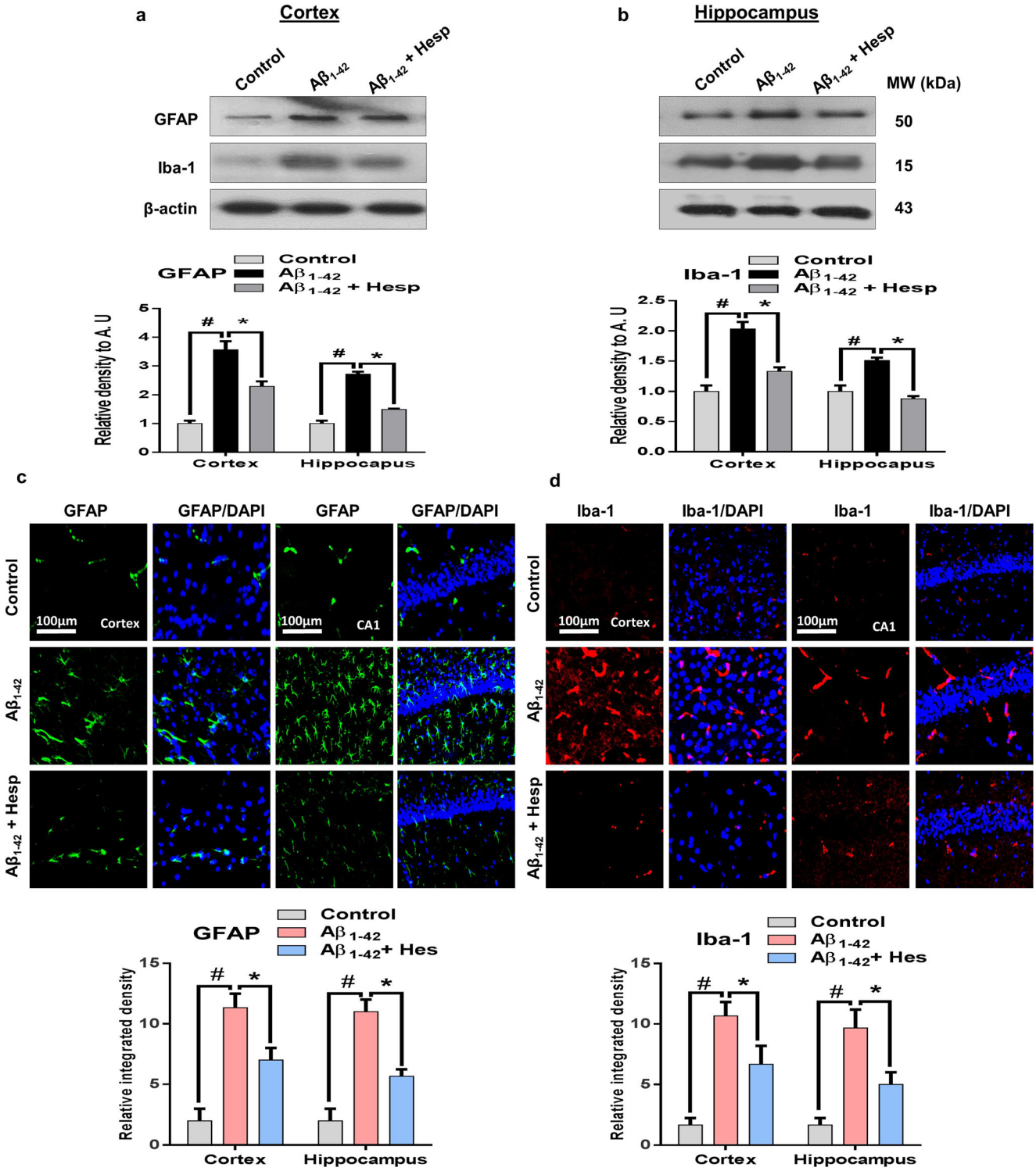


Fig. 3 Hesperetin regulates the activation of microglia and astrocytes in Aβ1-42–treated mice cortex and hippocampus. **a** Immunoblots and bar graphs for GFAP and Iba-1 expression in the cortex of the experimental mice. **b** Immunoblots and bar graphs for GFAP and Iba-1 expression in the hippocampus of the experimental mice. The differences are shown in the bar graphs. **c** and **d** Confocal microscopic images of the expression of GFAP and Iba-1 in the cortex and hippocampus of the experimental groups, and their bar graphs showing their expressions in the different experimental groups ($n = 4$). ImageJ software was used for immunohistological analysis. The values show the mean ± SD. Asterisk indicates significantly different from the control group; number sign indicates significantly different from the treated group. Significance $^{*}P < 0.05$ and $\#\ P < 0.05$

Hesperetin Regulates the Expression of TLR4 and Its Downstream Targets in the Aβ1-42–Treated Mice Cortex, Hippocampus, and in Microglial Cells

Previous literature has shown that there is enhanced expression of TLR4 with Aβ1-42 due to activation of astrocytes and microglia [48]. To analyze the effects of Aβ1-42 on TLR4 and its downstream inflammatory markers in the mouse hippocampus and cortex, we performed western blotting for TLR4, p-NF-κB, TNF-α, and IL-1β. According to our results, there was increased expression of TLR4, p-NF-κB, TNF-α, and IL-1β with Aβ1-42 administration, which was significantly attenuated by hesperetin. The in vivo findings were further confirmed by in vitro studies. In microglial cells (BV-2), there was an enhanced expression of TLR4, p-NF-κB65, and TNF-α in Aβ-treated cells, which was significantly downregulated with hesperetin. For more confirmation, specific TLR4 and p-NF-κB inhibitors were used, which showed that hesperetin downregulated the expression of TLR4 and p-NF-κB in the same manner as the specific inhibitors. These beneficial effects were further confirmed by fluorescent microscopy, using TNF-α and p-NF-κB antibodies, showing elevated immunoreactivity in the hippocampal and cortical regions in the Aβ1-42–injected groups that were significantly attenuated with hesperetin, suggesting that hesperetin may inhibit the upregulation of TLR4 and p-NF-κB, which may aid in the neuroprotective mechanisms of hesperetin (Fig. 4).

Hesperetin Regulates Alzheimer's Disease–Like Pathology by Regulating APP, BACE-1, and Aβ in the Aβ1-42–Treated Mice Cortex, Hippocampus, and in HT22 Cells

Studies have shown that injection of Aβ1-42 into the mouse brain produces amyloid beta–like pathology [36] by elevating the expression of APP, BACE-1, and Aβ. To evaluate the protective effects of hesperetin against Aβ, we performed western blot for APP, BACE-1, and Aβ in cortical and hippocampal homogenates from the different experimental groups. Our results showed increased expression of APP, BACE-1, and Aβ in the model mouse brains that was significantly downregulated with the administration of hesperetin. The effects of hesperetin against AD-like pathology were further confirmed by confocal microscopic studies, which showed that there was an increased expression of Aβ in the model group that was significantly attenuated with the administration of hesperetin. The in vivo findings were further confirmed by in vitro studies, which showed that hesperetin reversed the deposition of amyloid beta in the mouse hippocampal cells, as evaluated by the relative expression of Aβ in HT22 cells, as shown in Fig. 5.

Hesperetin Regulates Apoptotic Cell Death in Aβ1-42–Treated Mice Cortex, Hippocampus, and in HT22 Cells

Previous studies have shown that there is an increase in apoptotic cell death and neurodegeneration with the deposition of amyloid beta [49]. To determine the effects of hesperetin against apoptotic neurodegeneration, we performed western blotting for Bax, Bcl-2, Caspase-3, and PARP-1 among the experimental groups. According to our western blot results, there was an increased expression of proapoptotic markers (Bax, Caspase-3, and PARP-1) and downregulation of antiapoptotic marker (Bcl-2) with the administration of Aβ. However, it was interesting to observe that hesperetin treatment regulated the mitochondrial apoptotic signaling molecules in the cortex and hippocampus regions of the treated mice. Furthermore, our Caspase-3 immunoreactivity analysis indicated that Aβ administration elevated the expression of Caspase-3 in the hippocampus and cortex of the model group. However, Caspase-3 expression was significantly decreased in the hesperetin-treated group. Same was in the case of PARP-1, while in the Aβ model group, there was an increased expression of PARP-1, which was inhibited with hesperetin. The in vivo findings were further supported by in vitro studies, which showed that in HT22 cells, hesperetin may reverse the apoptotic neurodegeneration associated with Aβ administration. From these observations, we concluded that hesperetin could reduce the intensity of neuronal apoptosis in the Aβ-treated mice group (Fig. 6).

Hesperetin Regulates Synaptic Markers in Aβ1-42–Treated Mice Brain

Studies have shown that administration of Aβ deregulates synaptic markers and causes behavioral changes in mice [36], which may be associated with hippocampal-dependent cognitive dysfunction. To determine whether hesperetin could reverse these effects, we analyzed the levels of synaptic markers, such as Syntaxin, SNAP-25, PSD-95, Syp, and SNAP-23, in the brains of the experimental group mice. According to our western blot results, the expression of synaptic markers was significantly reduced in the Aβ-treated group but was significantly upregulated in the hesperetin-treated group. The western blot results were further confirmed by confocal microscopic analyses. The results indicated that there was a decreased immunoreactivity of PSD-95 in the Aβ-treated group; however, it was significantly upregulated in the hesperetin-treated group (Fig. 7).

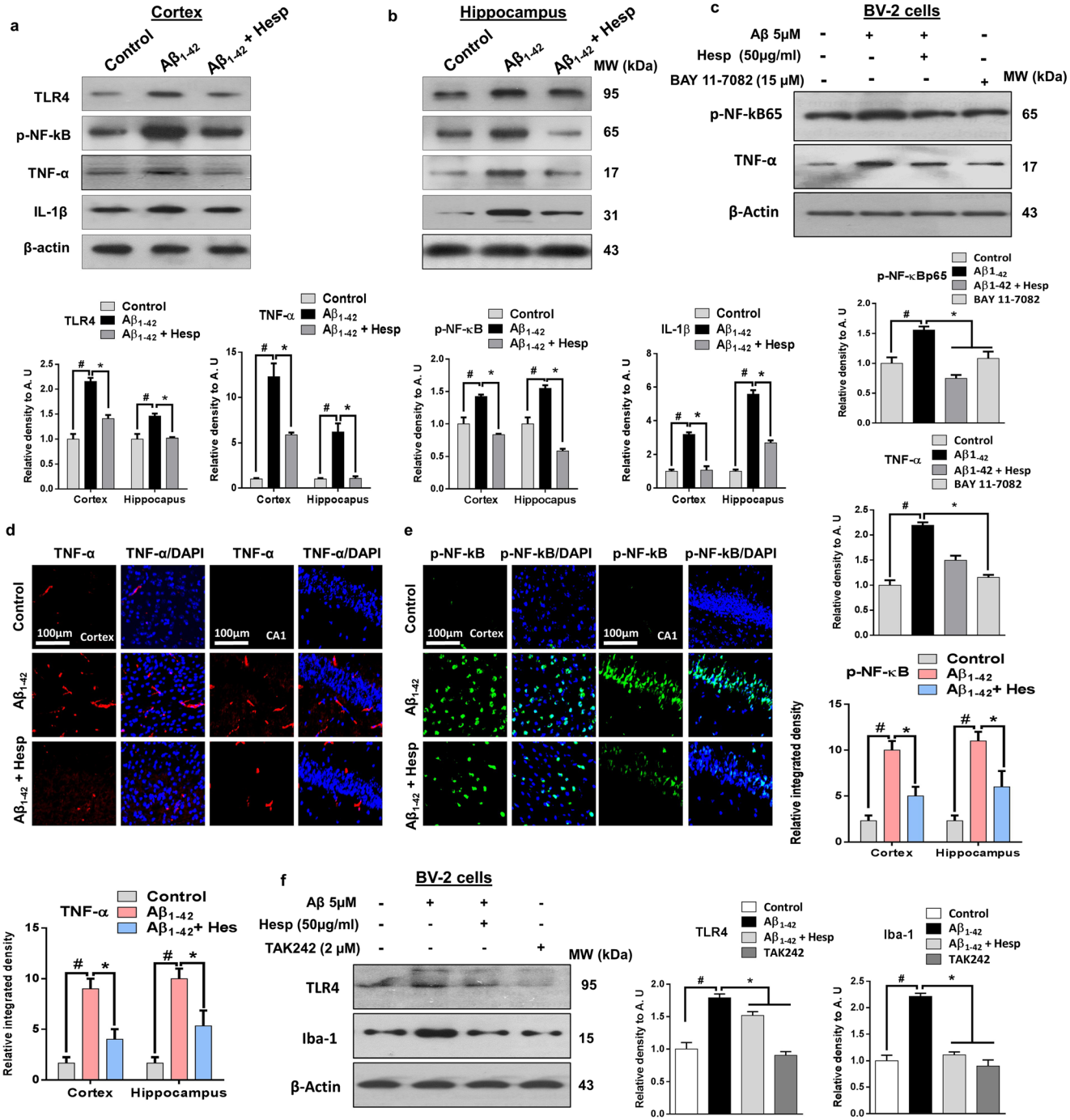


Fig. 4 Hesperetin regulates the expression of TLR4 and p-NF-κB in Aβ1-42–treated mice, and in vitro BV-2 cells. **a** and **b** Immunoblots and bar graphs of TLR4, p-NF-κB, TNF-α, and IL-1β expression in the cortex of the experimental groups. **c** Immunoblot results of the p-NF-κB and TNF-α expression in BV-2 cells, with bar graphs. The relative differences were quantified with ImageJ software and are shown graphically. **d** and **e** Confocal microscopic images and bar graphs of the expression of TNF-α and p-NF-κB in the cortex and hippocampus of the experimental groups. **f** Expression of TLR4 and IIba-1 in BV-2 cells, treated with hesperetin and TAK242, as a specific TLR4 inhibitor. The values are expressed as the mean ± SD for the indicated proteins ($n = 5$ mice/group). Number sign indicates significantly different from the vehicle-treated group; asterisk indicates significantly different from the Aβ-treated group. Significance * $P < 0.05$ and # $P < 0.05$

Hesperetin Improved Memory-Related Functions in an Aβ1-42–Treated Mouse Model of the AD

For behavioral studies, MWM and Y-maze tests [50] were performed. The MWM test was performed for 6 days, and the escape latency was recorded. As shown in, the mean escape latency was significantly increased in Aβ-treated mice compared to control mice. Hesperetin-treated mice (50 mg/kg i.p. for 6 weeks, on alternate days) showed significantly improved memory functions and reduced

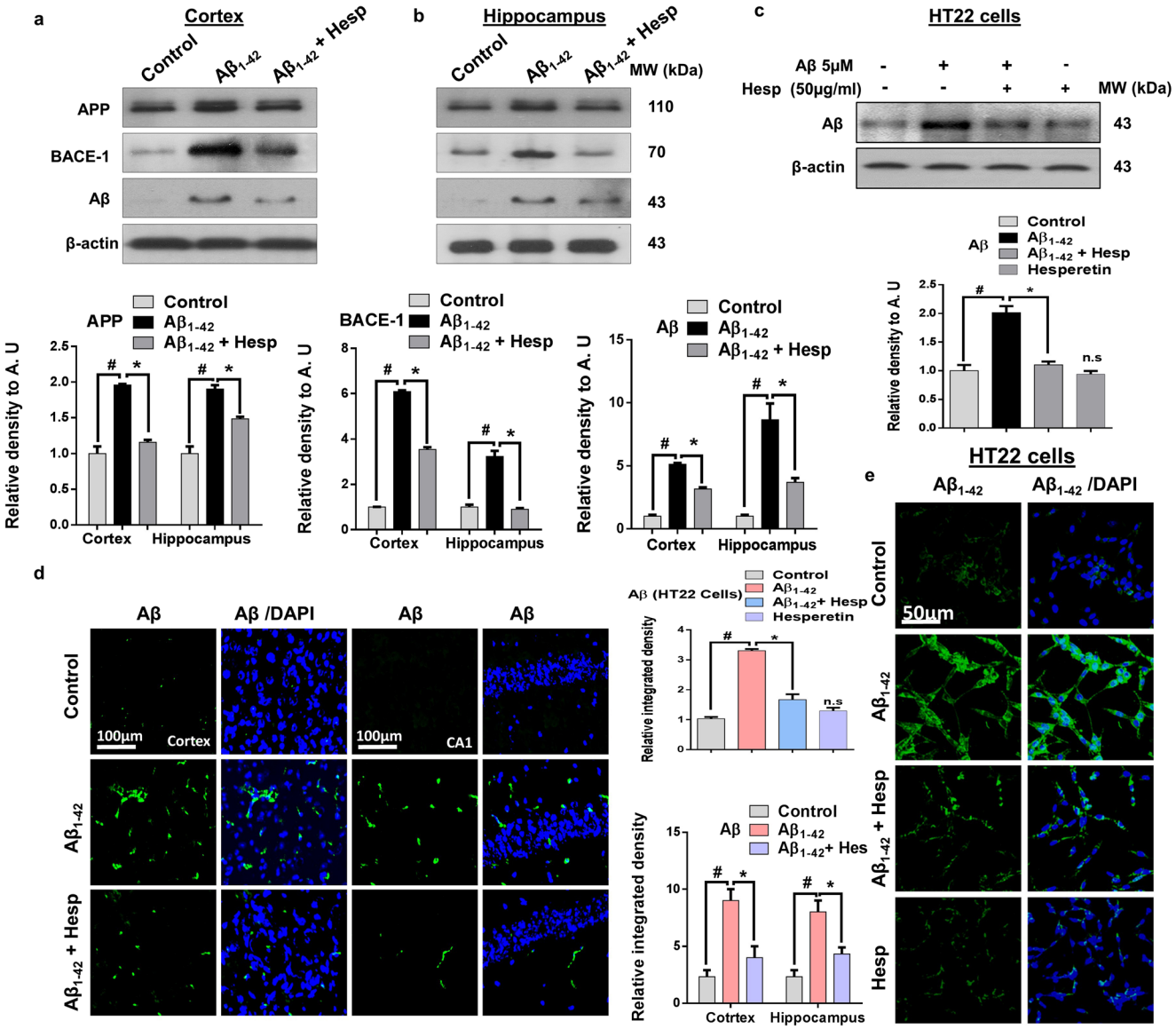


Fig. 5 Hesperetin regulates the Alzheimer-like pathology by regulating APP, BACE-1, and Aβ in the mouse brain and in vitro HT22 cells. **a** and **b** Immunoblot results and bar graphs of APP, BACE-1, and Aβ protein in the cortex and hippocampus of mice following Aβ and hesperetin treatment. **c** Immunoblots and bar graphs of Aβ in HT22 cells. The densities of the bands were quantified with ImageJ software, and the differences are shown in the bar graphs, which were generated with GraphPad Prism 6 software. β-Actin was used as a loading control. The density values are shown in arbitrary units (A.U.) as the mean ± SEM for the indicated proteins ($n = 8$ mice/group). **d** Immunofluorescence images and bar graphs of the Aβ expression in the brain (cortical and hippocampal regions) of the experimental mice ($n = 5$ mice/group). **e** Immunofluorescence of Aβ expression in mouse hippocampal HT22 cells in vitro. The values are expressed as the mean ± SD for the indicated proteins ($n = 5$ mice/group). Number sign indicates significantly different from the vehicle-treated group; asterisk indicates significantly different from the Aβ-treated group. Significance * $P < 0.05$ and # $P < 0.05$

escape latency compared with Aβ-treated mice (Fig. 8a and b). Moreover, the mean swimming speeds were also considered, but there were no significant effects of Aβ on mouse swimming speed in any of the experimental groups (Fig. 8c). On the seventh day, the number of platform crossings and time spent in the target quadrant were significantly increased in Aβ + hesperetin-treated mice compared to the Aβ-treated group (Fig. 7d and e), showing that hesperetin attenuated spatial memory deficits in Aβ-treated mice. Next, we performed a Y-maze test to analyze spatial working memory by using spontaneous alternation behavior. The results indicated that Aβ-treated mice had a lower percentage of alternation than control mice but that the alternation was significantly enhanced with hesperetin, showing that hesperetin alleviated short-term memory dysfunction in Aβ-treated mice (Fig. 7f).

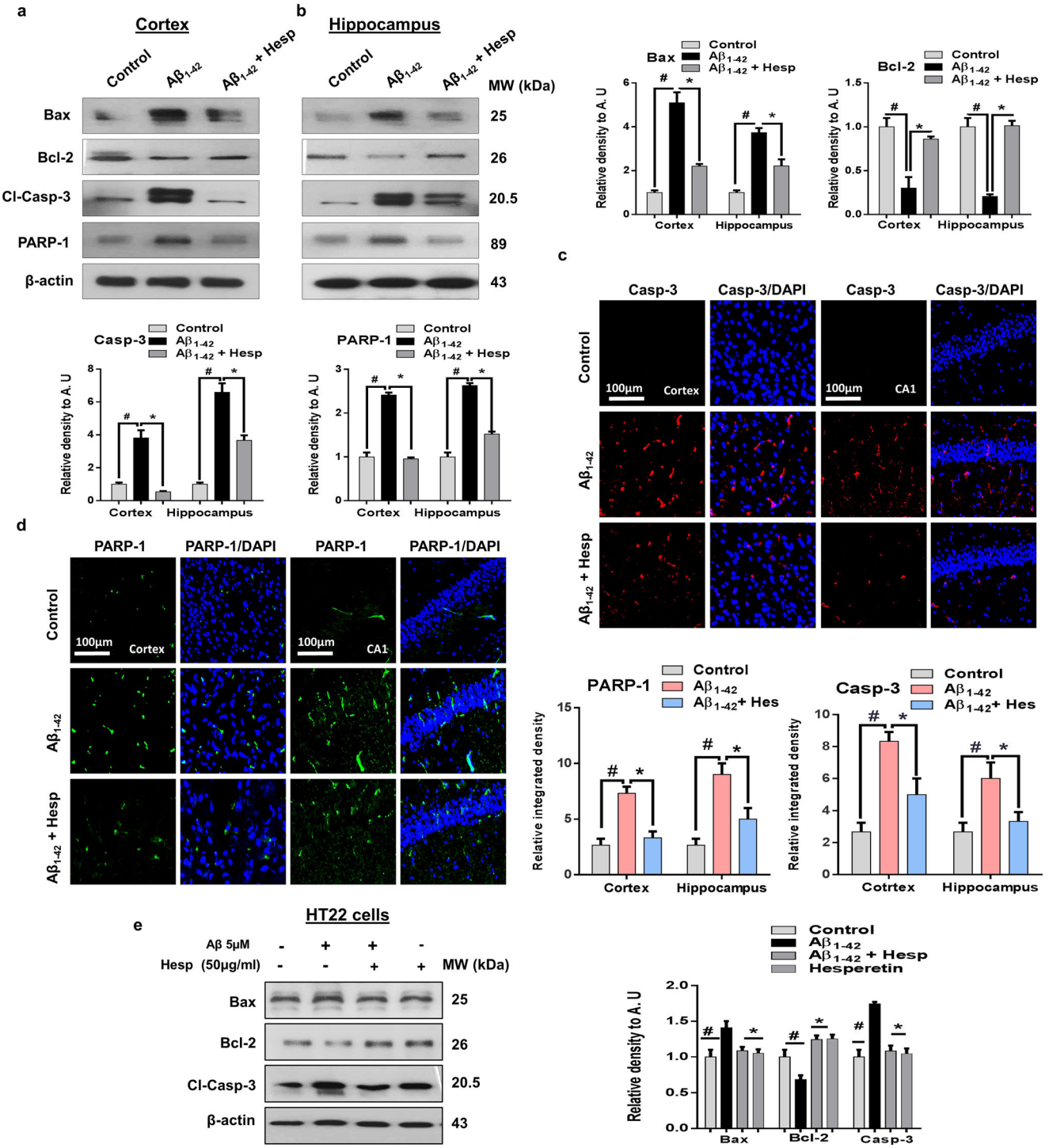
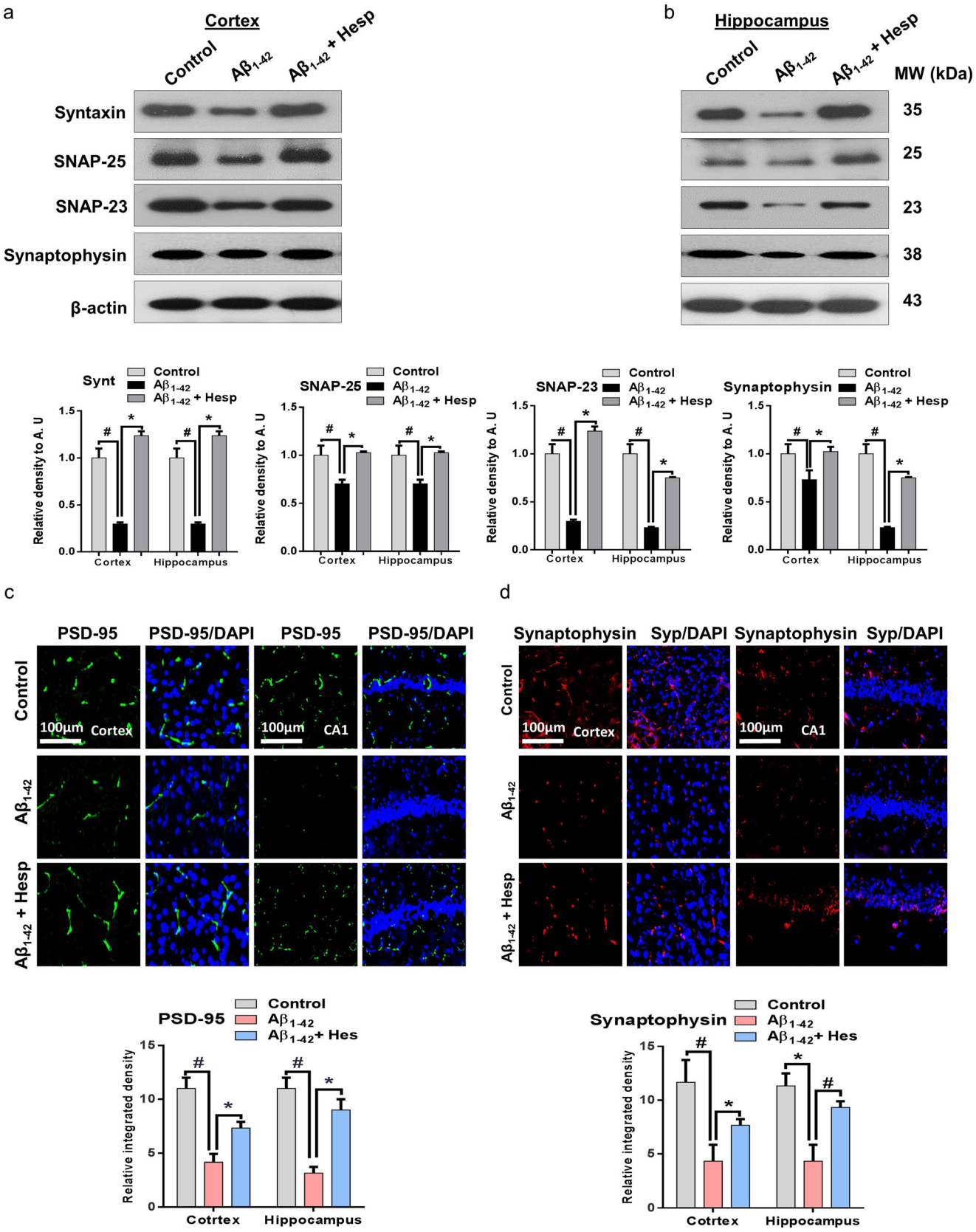


Fig. 6 Hesperetin regulates apoptotic cell death in the cortical and hippocampal regions of Aβ1-42–treated mice, and in vitro HT22 cells. **a** and **b** Immunoblots and graphs of Bax, Bcl-2, Cl-Caspase-3, and PARP-1 expression in the cortex and hippocampus of the experimental mice. **c** Immunofluorescence images of Caspase-3 in the cortex and hippocampus of the experimental groups, with graphs. **d** Immunofluorescence images of PARP-1 in the cortex and hippocampus of the experimental groups, with graphs. **e** Immunoblots and bar graphs of Bax, Bcl-2, and cl-Caspase-3 expression in the HT22 cells, in the absence and presence of hesperetin. The values are expressed as the mean ± SD for the indicated proteins (5 mice/group). Number sign indicates significantly different from the vehicle-treated group; asterisk indicates significantly different from the Aβ-treated. Significance * $P < 0.05$ and # $P < 0.05$


a
Cortex
Control
Aβ1-42
Aβ1-42 + Hesp
Syntaxin
SNAP-25
SNAP-23
Synaptophysin
β-actin
b
Hippocampus
MW (kDa)
35
25
23
38
43
Synt
SNAP-25
SNAP-23
Synaptophysin
Relative density to A. U
Cortex
Hippocampus
c
d
PSD-95
PSD-95/DAPI
Synaptophysin
Syp/DAPI
100μm
Cortex
CA1
Relative integrated density
Cotrtex
Hippocampus
Aβ1-42+ Hes

◀ **Fig. 7** Hesperetin regulates synaptic protein markers in the brains of Aβ1-42–treated mice. **a** and **b** Immunoblot analysis and bar graphs of Syntaxin, SNAP-25, SNAP-23, and Synaptophysin expression in the cortex and hippocampus of the experimental groups, with bar graphs. The bands were quantified with ImageJ software, and the differences have been given in the bar graphs. β-Actin was used as a loading control. The densities of the bands have been shown in arbitrary units (A.U.) as the mean ± SEM for the indicated proteins ($n = 8$ mice/group). **c** and **d** Representative immunofluorescence image of PSD-95 and Synaptophysin immunoreactivity in the cortex and hippocampus of the experimental groups ($n = 5$ mice/group), with their bar graphs. The values are expressed as the mean ± SD for the indicated proteins (5 mice/group). Number sign indicates significantly different from the vehicle-treated group; asterisk indicates significantly different from the Aβ-treated. Significance: * $P < 0.05$ and # $P < 0.05$

Discussion

Herein, we unveiled the critical protective role of hesperetin in vivo Aβ-injected mouse model, and in vitro HT22 and BV-2 cells. The pathological hallmarks of the AD are Aβ deposition, tau protein hyperphosphorylation [51], neurofibrillary tangle formation [52], and synaptic loss [53]. Intracerebroventricular injection of Aβ is the most accepted model for the AD, complementing transgenic mouse models [54] in the development and evaluation of different therapeutic approaches for the AD.

Studies have shown that there are increased levels of oxidative stress in transgenic mouse models of AD [55], and experimental induction of oxidative stress leads to Aβ accumulation in primary neurons [44]. Abnormal ROS can destabilize proteins, lipids, and DNA in patients [12]. Thus, reducing oxidative stress and neuroinflammation in the brain may be a promising therapeutic intervention in AD [56]. Keeping in mind the role of oxidative stress in AD pathogenesis, we focused on the main endogenous antioxidant signaling mechanisms (involving Nrf2 and HO-1) that regulate oxidative stress [40]. The major exploration of the present study was the regulation of oxidative stress via Nrf2 and HO-1 signaling, inhibition of neuroinflammation via regulation of TLR4/p-NF-kB, and apoptotic cell death in the Aβ-mouse model and in vitro cells. The study provides evidence that hesperetin treatment has multipotent effects; it regulates oxidative stress via regulating Nrf2 and HO-1, inhibition of neuroinflammation via TLR4, and regulation of apoptotic cell death in the in vivo and in vitro AD models.

According to our western blot and confocal microscopy results, there was a decreased expression of Nrf2 and HO-1 in the brains of Aβ-injected mice, which was significantly upregulated with the administration of hesperetin. Our results are consistent with previous reports, showing decreased expression of Nrf2 and HO-1 in Aβ mouse brains [36]. NMD levels and ROS levels were also analyzed to evaluate the levels of oxidative stress in the mouse brains, which were significantly decreased in the hesperetin-treated mice brains.

ROS have been shown to be responsible for the activation of inflammatory signaling, which contributes to the pathogenesis of AD [57]. Inflammatory signaling is associated with astroglial activation in AD models, suggesting a link between activated astroglial cells and AD pathogenesis [58]; activated microglia secrete proinflammatory cytokines such as NO, TNF-α, and IL-1β [59], which aids in the pathogenesis of neurodegenerative conditions. Here, in Aβ-injected mouse brains, there was an increased expression of GFAP and Iba-1. Interestingly, this was significantly inhibited by hesperetin. Moreover, upon activation of glial cells, TLR4, NF-κB, and downstream targets, which are crucial players in boosting inflammatory signaling, are upregulated. Here, there was an increased expression of these markers in the brain in an AD animal model, consistent with the previous findings [60]. Interestingly, the elevation in TLR4 and phosphorylation of NF-κB was significantly attenuated by hesperetin, so the inflammatory signaling in Aβ pathology both in experimental animal models may be inhibited [44].

Another main hallmark of AD is the amyloid pathology, which is the deposition of amyloid beta, increased expression of amyloid precursor protein, and beta-amyloid–cleaving enzyme. To evaluate the effect of hesperetin against Aβ pathology, we performed western blot for APP, BACE-1, and Aβ. According to our findings, there was an increased expression of APP, BACE-1, and Aβ in the AD model, which is in accordance with the available literature [61]. Interestingly, hesperetin regulated the expression of APP, BACE-1, and Aβ in the mouse brain. The amyloidogenesis is responsible for apoptotic cell death and neurodegeneration in AD models [62]. According to our findings, there was an increased apoptotic cell death in the AD model group, which was significantly attenuated by hesperetin. To confirm the in vivo findings, the effects of hesperetin against AD-like pathological changes were further examined in vitro HT22 cells. According to the western blot results, there were increased expressions of Aβ, Bax, Caspase-3, and PARP-1 in the Aβ-injected model group. However, these markers were significantly attenuated by hesperetin, showing that hesperetin could reduce AD-like pathology and reduce apoptosis in neuronal cells.

Another main hallmark of AD is dementia, which arises due to synaptic dysfunctions, Aβ deposition, neurofibrillary tangle formation, and oxidative stress [36]. Consistent with previous reports, there was a significant loss in the expression of synaptic markers in the Aβ-treated mice brain, which was significantly upregulated to the baseline with hesperetin. The synaptic markers are the regulators of the behavioral changes [63]. For the assessment of behavioral alterations, we performed MWM and Y-maze tests, which indicated that there were significant alterations in behavioral outcomes as indicated by increased latency times, fewer crossings, and less time spent in the target quadrants in Aβ mouse model. Interestingly, these alterations were significantly attenuated with the administration of

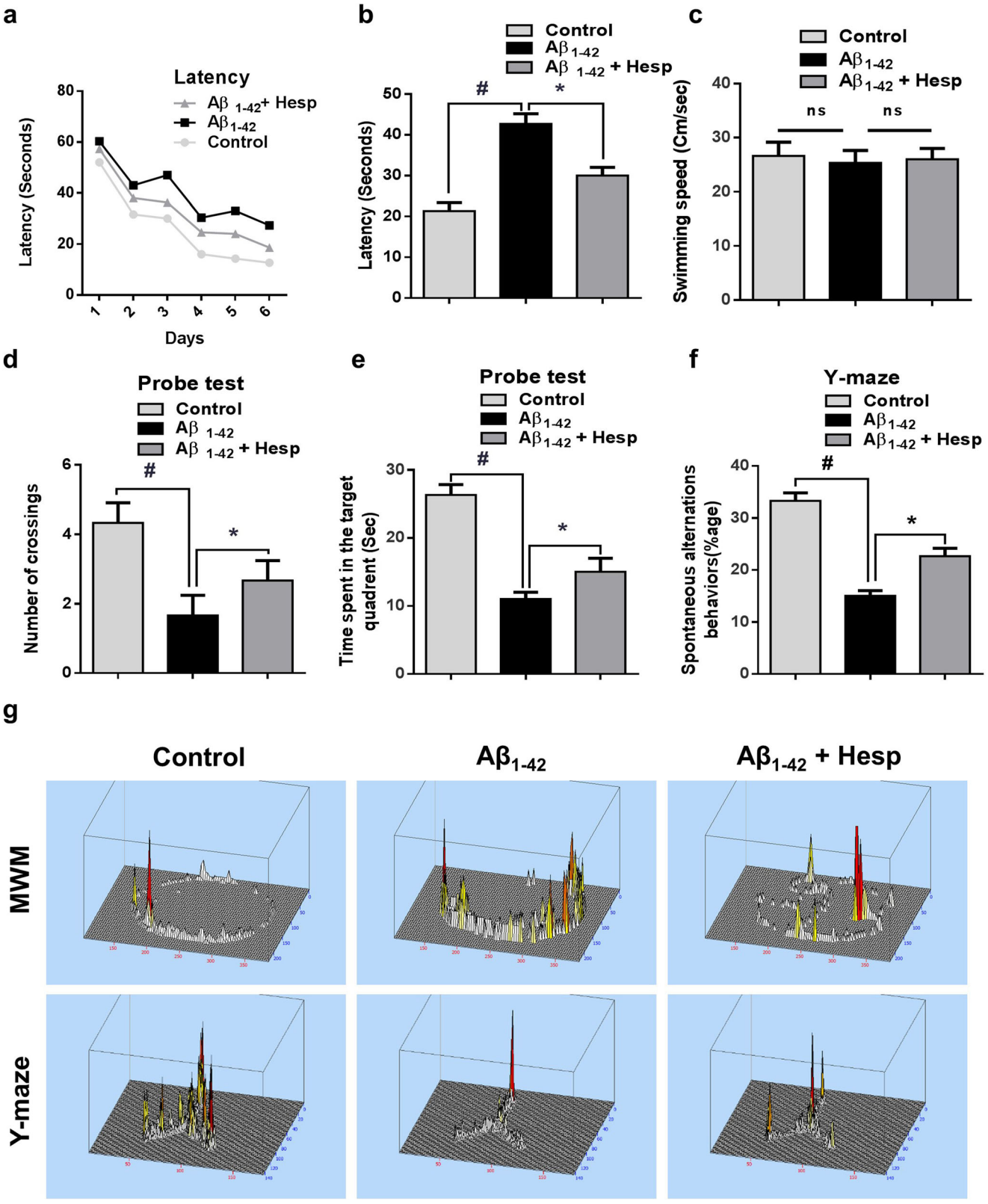


Fig. 8 Hesperetin regulates cognitive dysfunction and memory impairment in Aβ1-42–treated mice. **a** Bar graph showing the mean escape latency (sec), during the training days. **b** Final escape latency during the probe test. **c** Graphical representation showing the swimming speed (cm/s) of the mice on the seventh day of the training session before the probe test. **d** Number of platform crossings during the probe test. **e** Time spent in the target quadrant. **f** Spontaneous alternation behavior, given as a percentage (%). **g** Images of the trajectories, in the MWM and Y-maze test. The data are shown as the mean ± SEM ($n = 13$ mice/group). Significance $^*P < 0.05$, $\#P < 0.05$. ns, not significant

hesperetin, showing that hesperetin restores the memory impairment associated with neurodegeneration.

In conclusion, the current study provided the evidence that hesperetin efficiently reversed the pathological outcomes of Aβ treatment in mice and in cells, mainly by inhibiting oxidative stress via regulation of Nrf2/HO-1, neuroinflammation via regulation of TLR4/ NF-κB, and apoptotic cell death by regulating Bax/Bcl-2, Caspase-3, and PARP-1 in the Aβ mouse model and in cells. We believe that hesperetin could be a new therapeutic agent to treat AD-like neurodegenerative disorders.

Author Contributions MI: Concept, design, data collection, analysis, interpretation, and manuscript writing.
TM: Mice grouping, treatment, and performed in vitro experiments.
SUR: Mice grouping, manuscript evaluation, and interpretation.
AK: Mice treatment and behavioral studies.
TA: Data collection and analysis.
MGJ: Behavioral studies, animal handling, and treatment.
MOK: The corresponding author, reviewed and approved the manuscript, and holds all the responsibilities related to this manuscript. All the authors reviewed the revised manuscript.

Funding Information This research work was supported by the Brain Research Program through the National Research Foundation of Korea funded by the Ministry of Science, ICT & Future Planning (2016M3C7A1904391).

Compliance with Ethical Standards

The animal maintenance, treatments, behavioral studies, and surgical procedures were carried out in accordance with the animal ethics committee (IACUC) guidelines issued by the Division of Applied Life Sciences, Department of Biology at Gyeongsang National University, South Korea. The experimental methods were carried out in accordance with the approved 22 guidelines (Approval ID: 125) and all experimental protocols were approved by the animal ethics committee (IACUC) of the Division of Applied Life Sciences, Department of Biology at Gyeongsang National University, South Korea.

Conflict of Interest The authors declare that they have no conflict of interest.

Abbreviations Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase 1; TLR4, Toll-like receptor 4; TNF-α, tissue necrosis factor-α; AD, Alzheimer's disease; CNS, central nervous system; Aβ, amyloid beta; PBS, phosphate buffer saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole

References

1. Nistico R, Pignatelli M, Piccinin S, Mercuri NB, Collingridge G (2012) Targeting synaptic dysfunction in Alzheimer's disease therapy. Mol Neurobiol 46(3):572–587. https://doi.org/10.1007/s12035-012-8324-3
2. Dheer Y, Chitranshi N, Gupta V, Abbasi M, Mirzaei M, You Y, Chung R, Graham SL et al (2018) Bexarotene modulates retinoid-X-receptor expression and is protective against neurotoxic endoplasmic reticulum stress response and apoptotic pathway activation. Mol Neurobiol 55(12):9043–9056. https://doi.org/10.1007/s12035-018-1041-9
3. Tamano H, Suzuki H, Murakami T, Fujii H, Adlard PA, Bush AI, Takeda A (2018) Amyloid beta1-42-induced rapid Zn(2+) influx into dentate granule cells attenuates maintained LTP followed by retrograde amnesia. Mol Neurobiol. https://doi.org/10.1007/s12035-018-1429-6
4. Dong S, Huang X, Zhen J, Van Halm-Lutterodt N, Wang J, Zhou C, Yuan L (2018) Dietary vitamin E status dictates oxidative stress outcomes by modulating effects of fish oil supplementation in Alzheimer disease model APPswe/PS1dE9 mice. Mol Neurobiol 55(12):9204–9219. https://doi.org/10.1007/s12035-018-1060-6
5. Ayaz M, Junaid M, Ullah F, Subhan F, Sadiq A, Ali G, Ovais M, Shahid M et al (2017) Anti-Alzheimer's studies on beta-sitosterol isolated from Polygonum hydropiper L. Front Pharmacol 8:697. https://doi.org/10.3389/fphar.2017.00697
6. Uddin MS, Al Mamun A, Kabir MT, Jakaria M, Mathew B, Barreto GE, Ashraf GM (2018) Nootropic and anti-Alzheimer's actions of medicinal plants: molecular insight into therapeutic potential to alleviate Alzheimer's neuropathology. Mol Neurobiol. https://doi.org/10.1007/s12035-018-1420-2
7. Ali T, Yoon GH, Shah SA, Lee HY, Kim MO (2015) Osmotin attenuates amyloid beta-induced memory impairment, tau phosphorylation and neurodegeneration in the mouse hippocampus. Sci Rep 5:11708. https://doi.org/10.1038/srep11708
8. Kumar A, Singh A, Ekavali (2015) A review on Alzheimer's disease pathophysiology and its management: an update. Pharmacol Rep 67(2):195–203. https://doi.org/10.1016/j.pharep.2014.09.004
9. Godoy JA, Lindsay CB, Quintanilla RA, Carvajal FJ, Cerpa W, Inestrosa NC (2017) Quercetin exerts differential neuroprotective effects against H2O2 and Abeta aggregates in hippocampal neurons: the role of mitochondria. Mol Neurobiol 54(9):7116–7128. https://doi.org/10.1007/s12035-016-0203-x
10. Barage SH, Sonawane KD (2015) Amyloid cascade hypothesis: Pathogenesis and therapeutic strategies in Alzheimer's disease. Neuropeptides 52:1–18. https://doi.org/10.1016/j.npep.2015.06.008
11. Alkadhi KA, Dao AT (2018) Effect of exercise and Abeta protein infusion on long-term memory-related signaling molecules in hippocampal areas. Mol Neurobiol. https://doi.org/10.1007/s12035-018-1425-x
12. Cheignon C, Tomas M, Bonnefont-Rousselot D, Faller P, Hureau C, Collin F (2018) Oxidative stress and the amyloid beta peptide in Alzheimer's disease. Redox Biol 14:450–464. https://doi.org/10.1016/j.redox.2017.10.014
13. Frost GR, Li YM (2017) The role of astrocytes in amyloid production and Alzheimer's disease. Open Biol 7(12). https://doi.org/10.1098/rsob.170228
14. Dwivedi S, Rajasekar N, Hanif K, Nath C, Shukla R (2016) Sulforaphane ameliorates okadaic acid-induced memory impairment in rats by activating the Nrf2/HO-1 antioxidant pathway. Mol Neurobiol 53(8):5310–5323. https://doi.org/10.1007/s12035-015-9451-4
15. Dinkova-Kostova AT, Abramov AY (2015) The emerging role of Nrf2 in mitochondrial function. Free Radic Biol Med 88(Pt B):179–188. https://doi.org/10.1016/j.freeradbiomed.2015.04.036
16. Ding Y, Chen M, Wang M, Li Y, Wen A (2015) Posttreatment with 11-keto-beta-boswellic acid ameliorates cerebral ischemia-reperfusion injury: Nrf2/HO-1 pathway as a potential mechanism.

Mol Neurobiol 52(3):1430–1439. https://doi.org/10.1007/s12035-014-8929-9
17. Walker DG, Link J, Lue LF, Dalsing-Hernandez JE, Boyes BE (2006) Gene expression changes by amyloid beta peptide-stimulated human postmortem brain microglia identify activation of multiple inflammatory processes. J Leukoc Biol 79(3):596–610. https://doi.org/10.1189/jlb.0705377
18. Rogers J, Strohmeyer R, Kovelowski CJ, Li R (2002) Microglia and inflammatory mechanisms in the clearance of amyloid beta peptide. Glia 40(2):260–269. https://doi.org/10.1002/glia.10153
19. Eikelenboom P, Bate C, Van Gool WA, Hoozemans JJ, Rozemuller JM, Veerhuis R, Williams A (2002) Neuroinflammation in Alzheimer's disease and prion disease. Glia 40(2):232–239. https://doi.org/10.1002/glia.10146
20. Cameron B, Landreth GE (2010) Inflammation, microglia, and Alzheimer's disease. Neurobiol Dis 37(3):503–509. https://doi.org/10.1016/j.nbd.2009.10.006
21. Yamamoto M, Takeda K (2010) Current views of toll-like receptor signaling pathways. Gastroenterol Res Pract 2010:240365. https://doi.org/10.1155/2010/240365
22. Lehnardt S (2010) Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury. Glia 58(3):253–263. https://doi.org/10.1002/glia.20928
23. Hanke ML, Kielian T (2011) Toll-like receptors in health and disease in the brain: mechanisms and therapeutic potential. Clin Sci (Lond) 121(9):367–387. https://doi.org/10.1042/CS20110164
24. Frank S, Copanaki E, Burbach GJ, Muller UC, Deller T (2009) Differential regulation of toll-like receptor mRNAs in amyloid plaque-associated brain tissue of aged APP23 transgenic mice. Neurosci Lett 453(1):41–44. https://doi.org/10.1016/j.neulet.2009.01.075
25. Jin JJ, Kim HD, Maxwell JA, Li L, Fukuchi K (2008) Toll-like receptor 4-dependent upregulation of cytokines in a transgenic mouse model of Alzheimer's disease. J Neuroinflammation 5:23. https://doi.org/10.1186/1742-2094-5-23
26. Olson JK, Miller SD (2004) Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. J Immunol 173(6):3916–3924. https://doi.org/10.4049/jimmunol.173.6.3916
27. Michaud JP, Halle M, Lampron A, Theriault P, Prefontaine P, Filali M, Tribout-Jover P, Lanteigne AM et al (2013) Toll-like receptor 4 stimulation with the detoxified ligand monophosphoryl lipid A improves Alzheimer's disease-related pathology. Proc Natl Acad Sci U S A 110(5):1941–1946. https://doi.org/10.1073/pnas.1215165110
28. Liu S, Liu Y, Hao W, Wolf L, Kiliaan AJ, Penke B, Rube CE, Walter J et al (2012) TLR2 is a primary receptor for Alzheimer's amyloid beta peptide to trigger neuroinflammatory activation. J Immunol 188(3):1098–1107. https://doi.org/10.4049/jimmunol.1101121
29. Zhao B (2005) Natural antioxidants for neurodegenerative diseases. Mol Neurobiol 31(1–3):283–293. https://doi.org/10.1385/MN:31:1-3:283
30. Lan X, Han X, Li Q, Wang J (2017) Epicatechin, a natural flavonoid compound, protects astrocytes against hemoglobin toxicity via Nrf2 and AP-1 signaling pathways. Mol Neurobiol 54(10):7898–7907. https://doi.org/10.1007/s12035-016-0271-y
31. Jung KY, Park J, Han YS, Lee YH, Shin SY, Lim Y (2017) Synthesis and biological evaluation of hesperetin derivatives as agents inducing apoptosis. Bioorg Med Chem 25(1):397–407. https://doi.org/10.1016/j.bmc.2016.11.006
32. Garg A, Garg S, Zaneveld LJ, Singla AK (2001) Chemistry and pharmacology of the Citrus bioflavonoid hesperidin. Phytother Res 15(8):655–669
33. Pollard SE, Whiteman M, Spencer JP (2006) Modulation of peroxynitrite-induced fibroblast injury by hesperetin: a role for intracellular scavenging and modulation of ERK signalling. Biochem Biophys Res Commun 347(4):916–923. https://doi.org/10.1016/j.bbrc.2006.06.153
34. Rainey-Smith S, Schroetke LW, Bahia P, Fahmi A, Skilton R, Spencer JP, Rice-Evans C, Rattray M et al (2008) Neuroprotective effects of hesperetin in mouse primary neurones are independent of CREB activation. Neurosci Lett 438(1):29–33. https://doi.org/10.1016/j.neulet.2008.04.056
35. Choi EJ, Ahn WS (2008) Neuroprotective effects of chronic hesperetin administration in mice. Arch Pharm Res 31(11):1457–1462. https://doi.org/10.1007/s12272-001-2130-1
36. Amin FU, Shah SA, Kim MO (2017) Vanillic acid attenuates Abeta1-42-induced oxidative stress and cognitive impairment in mice. Sci Rep 7:40753. https://doi.org/10.1038/srep40753
37. Carvalho FB, Gutierres JM, Bueno A, Agostinho P, Zago AM, Vieira J, Fruhauf P, Cechella JL et al (2017) Anthocyanins control neuroinflammation and consequent memory dysfunction in mice exposed to lipopolysaccharide. Mol Neurobiol 54(5):3350–3367. https://doi.org/10.1007/s12035-016-9900-8
38. Ali T, Kim MJ, Rehman SU, Ahmad A, Kim MO (2017) Anthocyanin-loaded PEG-gold nanoparticles enhanced the neuroprotection of anthocyanins in an Abeta1-42 mouse model of Alzheimer's disease. Mol Neurobiol 54(8):6490–6506. https://doi.org/10.1007/s12035-016-0136-4
39. Rehman SU, Shah SA, Ali T, Chung JI, Kim MO (2017) Anthocyanins reversed D-galactose-induced oxidative stress and neuroinflammation mediated cognitive impairment in adult rats. Mol Neurobiol 54(1):255–271. https://doi.org/10.1007/s12035-015-9604-5
40. Jo MG, Ikram M, Jo MH, Yoo L, Chung KC, Nah SY, Hwang H, Rhim H et al (2018) Gintonin mitigates MPTP-induced loss of nigrostriatal dopaminergic neurons and accumulation of alpha-synuclein via the Nrf2/HO-1 pathway. Mol Neurobiol 56:39–55. https://doi.org/10.1007/s12035-018-1020-1
41. Liu L, Fujimoto M, Nakano F, Nishikawa H, Okada T, Kawakita F, Imanaka-Yoshida K, Yoshida T et al (2018) Deficiency of tenascin-C alleviates neuronal apoptosis and neuroinflammation after experimental subarachnoid hemorrhage in mice. Mol Neurobiol 55(11):8346–8354. https://doi.org/10.1007/s12035-018-1006-z
42. Abid NB, Yoon G, Kim MO (2017) Molecular cloning and expression of osmotin in a baculovirus-insect system: purified osmotin mitigates amyloid-beta deposition in neuronal cells. Sci Rep 7(1):8147. https://doi.org/10.1038/s41598-017-08396-x
43. Liu Y, Walter S, Stagi M, Cherny D, Letiembre M, Schulz-Schaeffer W, Heine H, Penke B et al (2005) LPS receptor (CD14): a receptor for phagocytosis of Alzheimer's amyloid peptide. Brain 128(Pt 8):1778–1789. https://doi.org/10.1093/brain/awh531
44. Ali T, Kim T, Rehman SU, Khan MS, Amin FU, Khan M, Ikram M, Kim MO (2017) Natural dietary supplementation of anthocyanins via PI3K/Akt/Nrf2/HO-1 pathways mitigate oxidative stress, neurodegeneration, and memory impairment in a mouse model of Alzheimer's disease. Mol Neurobiol 55:6076–6093. https://doi.org/10.1007/s12035-017-0798-6
45. Muhammad T, Ali T, Ikram M, Khan A, Alam SI, Kim MO (2018) Melatonin rescue oxidative stress-mediated neuroinflammation/neurodegeneration and memory impairment in scopolamine-induced amnesia mice model. J NeuroImmune Pharmacol. https://doi.org/10.1007/s11481-018-9824-3
46. Khan MS, Ali T, Kim MW, Jo MH, Chung JI, Kim MO (2018) Anthocyanins improve hippocampus-dependent memory function and prevent neurodegeneration via JNK/Akt/GSK3beta signaling in LPS-treated adult mice. Mol Neurobiol 56:671–687. https://doi.org/10.1007/s12035-018-1101-1
47. Gao H-M, Liu B, Zhang W, Hong J-S (2003) Novel anti-inflammatory therapy for Parkinson's disease. Trends Pharmacol Sci 24(8):395–401. https://doi.org/10.1016/s0165-6147(03)00176-7

48. Tang SC, Lathia JD, Selvaraj PK, Jo DG, Mughal MR, Cheng A, Siler DA, Markesbery WR et al (2008) Toll-like receptor-4 mediates neuronal apoptosis induced by amyloid beta-peptide and the membrane lipid peroxidation product 4-hydroxynonenal. Exp Neurol 213(1):114–121. https://doi.org/10.1016/j.expneurol.2008.05.014
49. Yu MS, Suen KC, Kwok NS, So KF, Hugon J, Chang RC (2006) Beta-amyloid peptides induces neuronal apoptosis via a mechanism independent of unfolded protein responses. Apoptosis 11(5):687–700. https://doi.org/10.1007/s10495-006-5540-1
50. Moser VA, Uchoa MF, Pike CJ (2018) TLR4 inhibitor TAK-242 attenuates the adverse neural effects of diet-induced obesity. J Neuroinflammation 15(1):306. https://doi.org/10.1186/s12974-018-1340-0
51. Rapoport M, Dawson HN, Binder LI, Vitek MP, Ferreira A (2002) Tau is essential to beta -amyloid-induced neurotoxicity. Proc Natl Acad Sci U S A 99(9):6364–6369. https://doi.org/10.1073/pnas.092136199
52. Gotz J, Gotz NN (2009) Animal models for Alzheimer's disease and frontotemporal dementia: a perspective. ASN Neuro 1(4):AN20090042. https://doi.org/10.1042/AN20090042
53. Chun YS, Zhang L, Li H, Park Y, Chung S, Yang HO (2018) 7-Deoxy-trans-dihydronarciclasine reduces beta-amyloid and ameliorates memory impairment in a transgenic model of Alzheimer's disease. Mol Neurobiol 55(12):8953–8964. https://doi.org/10.1007/s12035-018-1023-y
54. Cuadrado-Tejedor M, Garcia-Osta A (2014) Current animal models of Alzheimer's disease: challenges in translational research. Front Neurol 5:182. https://doi.org/10.3389/fneur.2014.00182
55. Matsumura A, Emoto MC, Suzuki S, Iwahara N, Hisahara S, Kawamata J, Suzuki H, Yamauchi A et al (2015) Evaluation of oxidative stress in the brain of a transgenic mouse model of Alzheimer disease by in vivo electron paramagnetic resonance imaging. Free Radic Biol Med 85:165–173. https://doi.org/10.1016/j.freeradbiomed.2015.04.013
56. Barbagallo M, Marotta F, Dominguez LJ (2015) Oxidative stress in patients with Alzheimer's disease: effect of extracts of fermented papaya powder. Mediat Inflamm 2015:624801. https://doi.org/10.1155/2015/624801
57. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB (2014) Reactive oxygen species in inflammation and tissue injury. Antioxid Redox Signal 20(7):1126–1167. https://doi.org/10.1089/ars.2012.5149
58. Gonzalez-Reyes RE, Nava-Mesa MO, Vargas-Sanchez K, Ariza-Salamanca D, Mora-Munoz L (2017) Involvement of astrocytes in Alzheimer's disease from a neuroinflammatory and oxidative stress perspective. Front Mol Neurosci 10:427. https://doi.org/10.3389/fnmol.2017.00427
59. Bao LH, Zhang YN, Zhang JN, Gu L, Yang HM, Huang YY, Xia N, Zhang H (2018) Urate inhibits microglia activation to protect neurons in an LPS-induced model of Parkinson's disease. J Neuroinflammation 15(1):131. https://doi.org/10.1186/s12974-018-1175-8
60. Lee CC, Chang CP, Lin CJ, Lai HL, Kao YH, Cheng SJ, Chen HM, Liao YP et al (2018) Adenosine augmentation evoked by an ENT1 inhibitor improves memory impairment and neuronal plasticity in the APP/PS1 mouse model of Alzheimer's disease. Mol Neurobiol 55(12):8936–8952. https://doi.org/10.1007/s12035-018-1030-z
61. Garwood CJ, Pooler AM, Atherton J, Hanger DP, Noble W (2011) Astrocytes are important mediators of Abeta-induced neurotoxicity and tau phosphorylation in primary culture. Cell Death Dis 2:e167. https://doi.org/10.1038/cddis.2011.50
62. Morishima Y, Gotoh Y, Zieg J, Barrett T, Takano H, Flavell R, Davis RJ, Shirasaki Y et al (2001) Beta-amyloid induces neuronal apoptosis via a mechanism that involves the c-Jun N-terminal kinase pathway and the induction of Fas ligand. J Neurosci 21(19):7551–7560
63. Shankar GM, Walsh DM (2009) Alzheimer's disease: synaptic dysfunction and Abeta. Mol Neurodegener 4:48. https://doi.org/10.1186/1750-1326-4-48