Clausena Harmandiana Root Extract Improved Amyloid-β Induced Cognitive Impairments in Rats By Reducing $A\beta_{1-42}$ Protein Levels and Neuroinflammation

Nutchareeporn Nillert
Khon Kaen University
Chantana Boonyarat
Khon Kaen University
Jariya Umka Welbat
Khon Kaen University
Komsun Bunreungthong
Khon Kaen University
Ploenthip Puthongking
Khon Kaen University
Wanassanun Pannangrong (wankun@kku.ac.th)
Khon Kaen University

Research Article

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Abstract

Background: Neuroinflammation caused by amyloid-β (Aβ) is associated with Alzheimer's disease (AD) pathogenesis. In AD, Aβ accumulation can activate the surrounding microglia which followed by the synthesis and release of pro-inflammatory cytokines, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα), results in cognitive impairments. *Clausena harmandiana* (CH) is an herb in the Rutaceae family and has been used in folk medicine for the treatment of illness such as stomachache and headache, and as a health tonic. It is interesting that CH root extract (CHRE) exhibits various anti-inflammatory and other pharmacological activities, but there has not been any study in Alzheimer's disease-like animal models.

Objectives: This study aimed to investigate the effects of CHRE on $A\beta_{1-42}$-induced cognitive impairments, increased $A\beta_{1-42}$ protein levels and neuroinflammation.

Methods: Forty-eight adult male Sprague-Dawley rats (250-300 g) were randomly divided into 6 groups (n=8). The rats were given 0.5% sodium carboxymethylcellulose, Celebrex® (10 mg/kg BW) or CHRE (125, 250, and 500 mg/kg BW) and not given any treatment by oral gavage for 35 days. On day 21, all treated rats were injected with aggregated $A\beta_{1-42}$ at a concentration of 1 μg/μl into both lateral ventricles (1 μl/side), while untreated rats were injected with sterilized normal saline. Ten days later, their recognition memory was assessed using the novel object recognition test. At the end of the experiment, all rats were euthanized by an overdose of thiopental sodium (120 mg/kg BW) and transcardial perfusion with 0.9% normal saline solution, to observe $A\beta_{1-42}$ protein levels and the expression of inflammatory markers (CD11b-positive microglia, IL-1β, and TNFα) in the cerebral cortex and hippocampus.

Results: The results indicated that pretreatment with CHRE at all doses improved impairment of short- and long-term recognition memory. In addition, CHRE significantly decreased $A\beta_{1-42}$ protein levels and the expression of inflammatory markers in both brain regions as well as pretreatment with Celebrex®.

Conclusions: This suggests that CHRE has a potential therapeutic effect against $A\beta_{1-42}$-induced cognitive impairments by reducing $A\beta_{1-42}$ protein levels and neuroinflammation.

Background

Alzheimer's disease (AD) is an irreversible neurodegenerative disorder and the most common type of dementia in the elderly [1]. It is clinically characterized by progressive cognitive dysfunction combined with memory loss [2]. Amyloid-β (Aβ) – a proteolytic fragment generated from sequential cleavage of amyloid precursor protein by β- and γ- secretase enzymes – has been widely recognized as a neuropathological feature of AD [3]. $A\beta_{1-42}$ is considered to be the most significant toxic fragment and the main component of amyloid plaque [4, 5]. When Aβ synthesis exceeds its clearance, it accumulates in various regions of the brain such as the cerebral cortex and hippocampus [6, 7]. The accumulation of aggregated Aβ causes neurotoxicity through various mechanisms, particularly neuroinflammation, which

is an innate immune response in the central nervous system (CNS) against pathological triggers such as neuronal death or protein aggregation [8]. In AD, Aβ-induced neuroinflammation occurs through stimulation of various receptors expression on microglia, particularly clusters of differentiation molecule 11b (CD11b). This is the α-chain of integrin receptor CD11b/CD18, also known as macrophage antigen complex-1 or complement receptor 3 [9]. CD11b is also one of the main phagocytic receptors of microglia, which plays an important role in Aβ clearance [10]. Several studies have demonstrated increased CD11b expression in the brains of AD patients and Aβ-induced rats to be involved in the severity of microglial activation [11–14]. Similarly, microglial activation caused by Aβ is decreased in CD11b knockout mice, indicating that CD11b plays an essential role in Aβ-induced microglial activation [15]. Moreover, CD11b activation also triggers the nuclear factor-κB signaling pathway and subsequent increased production of pro-inflammatory cytokines consisting of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) [16, 17], which are involved in the progression of AD. A previous study also found increased IL-1β and TNFα levels in the brain tissue and cerebrospinal fluid of AD patients [18]. This increase suppresses microglial phagocytosis in Aβ clearance, which in turn intensifies Aβ accumulation and neuroinflammation [19, 20]. Additionally, the overexpression of IL-1β can inhibit long-term potentiation, resulting in impaired learning and memory [21]. Furthermore, chronic TNFα expression leads to increased neuronal death [22] and enhances the synthesis of other pro-inflammatory cytokines [23]. Conversely, the blocking of IL-1β and TNFα decreases Aβ accumulation and neuroinflammation and alleviates cognitive impairments [24, 25]. Recognition memory refers to the capacity to judge whether something recently encountered has been encountered previously [26]. Object recognition memory – the ability to recognize objects – is a critical component of declarative memory and is often impaired in patients affected by neurodegenerative diseases or brain injury [27]. This impairment is also found in Aβ-induced rats [13]. The assessment of object recognition memory in animal models of amnesia is mainly performed using the novel object recognition test [28]. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to prevent and delay the onset of AD [29, 30]. The role of NSAIDs as a potential treatment for AD is controversial [31]. While several studies have reported NSAIDs to be ineffective in AD patients [32], targeting inflammation as a contributor to cognitive loss in the context of accumulation of Aβ remains a possible goal. Multiple reports in both human and animal models have shown that inhibiting inflammation in AD can slow cognitive decline [33, 36]. Many new, highly specific drugs to prevent toxic inflammation and promote the appropriate homeostatic responses to injury have yet to be tested. Celecoxib (Celebrex®) belongs to the class of NSAIDs called selective cyclooxygenase-2 (COX-2) inhibitors, which are highly safe and less toxic than other NSAIDs [34, 35]. Celecoxib has been shown to exhibit anti-inflammatory and neuroprotective effects by decreasing microglial activation and pro-inflammatory cytokine expression in the hippocampus of Aβ-induced rats [36]. This drug also reduces fear learning and memory deficit in olfactory bulbectomized rats [37]. Nevertheless, prolonged exposure to NSAIDs could cause various side effects such as gastrointestinal bleeding and ulcer, arterial wall damage, and nephrotoxicity [38–41]. Researchers have recently shown a growing interest in various natural products with anti-inflammatory (but fewer adverse) effects. *Clausena harmandiana* (CH; song fa dong in Thai) is an herb in the Rutaceae family and has been used in folk medicine for the treatment of illness, stomachache, and headache and as an herbal health tonic [42, 43]. A previous study found nordentatin, a coumarin compound, to be a

major active ingredient that can be isolated from the root bark of CH (CHR) [44]. According to another study, 100 grams of CHR extract (CHRE) contains 0.532 grams of nordentatin [45]. The pharmacological activities of this compound include antioxidant activity via scavenging of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and inhibition of lipid peroxidation as indicated by DPPH and thiobarbituric acid reactive substance tests [45]. In addition, nordentatin can promote neurite outgrowth by increasing of the branching number of cultured P19 neurons [45]. In one study, pretreatment with nordentatin improved cognitive impairments caused by Aβ as assessed using the Y-maze test [46]. Interestingly, there have been no published reports of serious toxicity from CHRE in either in vitro or in vivo models [45, 47]. However, its anti-neuroinflammatory effects and potential ability to alleviate cognitive impairment in AD-like animal models have not been investigated. This study thus aims to investigate the effects of CHRE on $A\beta_{1-42}$-induced cognitive impairments, $A\beta_{1-42}$ protein levels and neuroinflammation.

Methods

Plant material and preparation of CHRE

The root bark of CH (CHR) was collected in Roi Et province, Thailand. The plant was identified voucher specimen (KKU No. 21145; Teerapat Bootchan 67) and deposited at Faculty of Sciences, Khon Kaen Univerisity, Khon Kaen, Thailand. CHRE was prepared by Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand, and permitted to be used in this study. Air-dried CHR was macerated with 70% ethanol and periodically stirred at room temperature for 3 days. The extract was then filtered and concentrated using a rotary evaporator at 40°C and stored in a refrigerator at 2-8°C until use. The extracted yield of CHRE was 19.7% of the wet weight [47].

Animal treatment

Forty-eight healthy adult male Sprague-Dawley rats (250-300 g) were obtained from Nomura Siam International Co., Ltd. (Bangkok, Thailand). The rats were housed in the Northeast Laboratory Animal Center, Khon Kaen University at room temperature (23 ± 2°C) under a 12-h light/dark cycle (lights on from 06:00 to 18:00) with free access to food and water. All studies were carried out following the procedures laid out in the guide for the care and use of laboratory animals under the supervision of the Northeast Laboratory Animal Center, Khon Kaen University, Khon Kaen, Thailand. The experimental design was approved by the Institutional Animal Care and Use Committee of Khon Kaen University (Approval No. IACUC-KKU-25/61; Suppl. Fig.1). After 1 week of acclimatization, the rats were randomly divided into 6 groups (n=8/group). Group 1 was a sham control (SC) group, rats were injected with sterilized normal saline (NS). Group 2 was a vehicle plus amyloid-β (V+Aβ) group, rats were received 0.5% sodium carboxymethylcellulose (NaCMC) and induced memory impairments with $A\beta_{1-42}$ injection. Group 3 was a Celebrex plus amyloid-β (CB+Aβ; a positive control) group, rats were received Celebrex® at 10 mg/kg BW and induced memory impairments with $A\beta_{1-42}$ injection. Groups 4, 5, and 6 were the CHRE125 plus amyloid-β (CHRE125+Aβ), CHRE250 plus amyloid-β (CHRE250+Aβ) and CHRE500 plus amyloid-β (CHRE500+Aβ), rats were received CHRE at 125, 250, and 500 mg/kg BW, respectively and induced

memory impairments with Aβ1-42 injection. NaCMC, Celebrex®, and CHRE were orally administered once daily for 35 consecutive days. On day 21, the rats in groups 2-6 were injected with 1 μl of aggregated $Aβ_{1-42}$ peptide into the lateral ventricle on each side, whereas group 1 was injected with the same dose of sterilized NS. Ten days after $Aβ_{1-42}$ injection, the rats were tested for learning and memory using a novel object recognition (NOR) test (Fig.1). At the end of the experiment, the rats were euthanized by an anesthesia overdose (120 mg/kg BW of thiopental sodium; Jagsonpal Pharmaceuticals Ltd, India) and transcardial perfusion with 0.9% NS solution, and then $Aβ_{1-42}$ protein levels and the expression of CD11b-positive microglia, IL-1β, and TNFα in the cerebral cortex and hippocampus were investigated by immunohistochemistry or western blot analysis.

$Aβ_{1-42}$ injection

Amyloid-β peptide 1-42 ($Aβ_{1-42}$; Enzo Life Sciences, Inc., Farmingdale, NY, USA) was dissolved in 5% acetic acid at a concentration of 1 μg/μl, and the solution was incubated at 37°C for 24 h to induce peptide aggregation [47]. The rats were anesthetized with thiopental sodium (Jagsonpal Pharmaceuticals Ltd, India; 80 mg/kg BW, intraperitoneal), followed by a single injection of aggregated $Aβ_{1-42}$ peptide or sterilized NS into the lateral ventricles bilaterally (1 μl/side) at a rate of 0.2 μl/min [49, 50] using the following coordinates: AP -0.8 mm from bregma, ML ±1.5 mm from bregma, and SI -3.8 mm from dura mater [13, 51]. After injection, the rats were placed on a warm pad (32 - 33°C) until they awoke and were returned to their cages [52].

Novel object recognition test

The novel object recognition (NOR) test was performed in an open field arena (50 cm × 50 cm × 40 cm) in a quiet environment. The test consisted of three phases: habituation, training, and retention. In the habituation phase, each rat was allowed to freely survey the empty arena for 5 min. During the training phase, each rat was allowed 5 min to explore two identical objects that had been placed in the arena at different locations. The retention phase was divided into 2 sub-periods with a 5 min and 24 h delay to assess short- and long-term memory, respectively. Five minutes or 24 h after training, one of the objects was replaced with a novel object, and each rat was again placed in the arena to explore the objects for 5 min. After each rat finished the test, the arena was cleaned with 70% ethanol to remove any odor. Exploration time was recorded using Noldus EthoVision XT version 12 when the rat's mouth, nose, or paw was less than 2 cm from an object. The ability to recognize the novel object was expressed as a discrimination index (DI): the difference between the amount of time spent exploring the novel and familiar object divided by total exploration time (DI = TN-TF/TN+TF). The DI can range from -1 to 1, with positive and negative scores indicating more time spent with the novel and familiar object, respectively, and a zero indicating a null preference [13].

Tissue Processing

After finishing treatment, all rats were deeply anesthetized with an overdose (120 mg/kg BW) of thiopental sodium (Jagsonpal Pharmaceuticals Ltd, India) and transcardially perfused with 0.9% NS

solution. Then, the brains were quickly removed and separated into left and right hemispheres. The left hemisphere was cryoprotected in 30% sucrose solution and submerged in ice-cold 4% paraformaldehyde solution for immunohistochemical investigation using a free-floating technique. The cerebral cortex and hippocampus from the right hemisphere were isolated and stored at -80°C for Western blot analysis.

Immunohistochemistry

The frozen brains were cut into serial coronal sections at 35 μm thickness using a cryostat and then were washed with 0.1M tris-buffered saline (TBS) 3 times for 5 min. Free-floating sections were incubated with 0.3% hydrogen peroxide (H_2O_2) for 15 min to suppress the endogenous peroxidase activity and again washed with TBS. The sections were blocked with 1% bovine serum albumin (BSA; Merck Millipore, Germany) at room temperature for 1 h and incubated with mouse monoclonal anti-integrin αM (CD11b) primary antibody (1:100; Merck Millipore, Germany) at 4°C overnight. After washing with TBS, the sections were incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG secondary antibody (1:500; Invitrogen, Carlsbad, CA, USA) at room temperature for 2 h. The sections were washed with TBS and incubated with 0.001% of diaminobenzidine tetrahydro-chloride dihydrate (DAB; Sigma Aldrich, USA) containing 0.003% H_2O_2 for 20 min. Then, the sections were mounted on gelatin-coated glass slides, dehydrated, and cover-slipped with DPX (Sigma, St. Louis, MO, USA). The immunostained sections were viewed under a light microscope (Nikon ECLIPSE E200 MVR microscope) at X400 magnification and assessed using ImageJ software (Windows version, National Institutes of Health, Bethesda, MD, USA). Six digitized images of the cerebral cortex and hippocampus were obtained from 1 image per area of the frontal cortex (FC), parietal cortex area 2 (PC2) or temporal cortex area 1 (TC1), perirhinal cortex (PRC), and piriform cortex (PiC), as well as 2 images per area of parietal cortex area 1 (PC1), cornu ammonis area 1 (CA1), cornu ammonis area 2/3 (CA2/3), and dentate gyrus (DG). Six sections were then selected from each rat for analysis. Results were represented as a percentage of immunoreactive area difference from control, which was calculated using the following formula: immunoreactive area = [areas of CD11b-positive microglia (positive pixels)/total area of the image (total pixels)] [13, 53].

Western blot analysis

Cerebral cortex or hippocampus tissue was homogenized separately using grinder with the cold lysis buffer (pH 7.6) containing 20 mM tris base (Sigma-Aldrich, Inc., USA), 1 mM ethylene glycol tetraacetic acid (Sigma-Aldrich, Inc., USA), 320 mM sucrose (Loba Chemical Pvt. Ltd., India), 0.1% triton X 100 (Sigma-Aldrich, Inc., USA), 1 mM sodium fluoride (NaF), 10 mM β-glycerophosphate disodium salt hydrate (Sigma-Aldrich, Inc., USA) and Sigma*FAST*™ protease inhibitor cocktail (Sigma Aldrich, USA) to extract total proteins. The homogenized samples were centrifuged at 13,000 rpm at 4°C for 10 min. The protein concentration was determined using a NanoDrop (NanoDrop ND-1000 Spectrophotometer V3.5 User's Manual, NanoDrop Technologies Inc., USA). The samples (100 μg) were heat-denatured at 95°C for 5 min, separated with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad Laboratories GmbH, Munich, Germany), and subsequently transferred onto the nitrocellulose membrane

(Bio-Rad Laboratories GmbH, Munich, Germany). The membrane was blocked with 5% BSA (Merck Millipore, Germany) in 0.1% tris-buffer saline containing tween 20 (TBST) at room temperature for 1 h and then probed with rabbit polyclonal anti-$A\beta_{1-42}$ (1:2,000; Abcam, UK), mouse monoclonal anti-IL-1β (1:300; Santa Cruz Biotechnology, Inc., USA), mouse monoclonal anti-TNF-α (1:300; Santa Cruz Biotechnology, Inc., USA), and mouse monoclonal anti-glyceraldehyde 3 phosphate dehydrogenase (GAPDH; 1:20,000; Abcam, UK) primary antibodies in TBST at 4°C for overnight. After washing with TBST, the membrane was incubated with peroxidase conjugated goat anti-rabbit IgG (1:5,000; Merck Millipore, Germany) or HRP conjugated goat anti-mouse IgG secondary antibodies (1:2,000; Thermo Fisher Scientific Inc., USA) at room temperature for 2 h. The protein bands were visualized with enhanced chemiluminescence (ECL) detection reagents (Thermo Fisher Scientific Inc., USA) and a gel imaging system (Image Quant 400, GH Healthcare, USA) and analyzed using ImageJ software (Windows version, National Institutes of Health, USA). GAPDH was used as a loading control, and the results were represented as the percentage difference from control.

Statistical analysis

All data were presented as mean ± standard error of the mean (SEM) and analyzed using SPSS 23.0 software. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by a Tukey post-hoc test for multiple comparisons. A P value < 0.05 was considered statistically significant.

Results

Effects of CHRE on cognitive impairment in $A\beta_{1-42}$-injected rats

Cognitive impairments, such as impaired object recognition, are typically found in AD patients and animal models. The NOR test was performed on days 31 to 33 to investigate whether CHRE ameliorated such cognitive impairment in $A\beta_{1-42}$ injected rats (Fig. 2). During the training phase, there was no marked difference in the time spent exploring the two identical objects in any group (Fig. 2A). In the retention phase, only the V+Aβ group displayed obvious cognitive impairments, indicated by significant decreases in both short- and long-term DIs compared with the SC group (Fig. 2B-C). These cognitive impairments (both short- and long-term) were ameliorated by administration of CHRE, as demonstrated by the significantly higher DIs in the CHRE125+Aβ, CHRE250+Aβ, and CHRE500+Aβ groups (similar to the CB+Aβ group) compared with the V+Aβ group (Fig. 2B-C). This shows that CHRE improved object recognition impairment in $A\beta_{1-42}$ injected rats. However, there was no significant difference in locomotor activity (velocity of movement or total distance traveled) among groups (Suppl. Fig. 2).

Effects of CHRE on $A\beta_{1-42}$ protein levels in the cerebral cortex and hippocampus of $A\beta_{1-42}$ injected rats

Increases in brain Aβ protein levels are the main contributing factor to AD progression. Western blot was performed to determine whether CHRE decreased $A\beta_{1-42}$ protein levels in the rat brain (Fig. 3). Injection of aggregated Aβ into both lateral ventricles significantly increased $A\beta_{1-42}$ protein levels in the cerebral cortex and hippocampus of the V+Aβ group compared with the SC group (Fig. 3A-B). However,

administration of Celebrex® and CHRE significantly decreased $A\beta_{1-42}$ protein levels in both brain regions of rats in the CB+Aβ, CHRE125+Aβ, CHRE250+Aβ, and CHRE500+Aβ groups compared with the V+Aβ group (Fig. 3A-B), particularly CHRE250 and CHRE500 (Fig. 3A).

Effects of CHRE on the expression of CD11b-positive microglia in the cerebral cortex and hippocampus of $A\beta_{1-42}$ -injected rats

Increased expression of microglial markers, such as CD11b, has been widely reported in AD. IHC was performed to evaluate whether CHRE reduced the expression of CD11b-positive microglia in the rat brain (Fig. 4). Figure 4A-B shows photomicrographs of CD11b-positive microglia in the cerebral cortex and hippocampus of SC and Aβ-induced rats at low magnification. In general, microglia in the cerebral cortex and hippocampus were ramified (small cell bodies, thin and long branches; Fig. 4C-D), and the expression of CD11b-positive microglia was lower than in the SC group (Fig. 4E-F). $A\beta_{1-42}$ injection caused morphological changes to the microglia, which took on an amoeboid form (large cell bodies, thick and short branches) as found in the V+Aβ group (Fig. 4C-D). Moreover, $A\beta_{1-42}$ injection significantly elevated the expression of CD11b-positive microglia in the cerebral cortex and hippocampus of the V+Aβ group compared with the SC group (Fig. 4E-F). However, administration of CHRE significantly reduced the expression of CD11b-positive microglia in both brain regions of rats in the CHRE125+Aβ, CHRE250+Aβ, and CHRE500+Aβ groups (to levels similar to those in the CB+Aβ group) compared with the V+Aβ group (Fig. 4E-F). Some amoeboid microglia were found in all treatment groups (Fig. 4C-D). Interestingly, CHRE250 and CHRE500 decreased the expression of CD11b-positive microglia in the hippocampus to a greater extent than CHRE125 (Fig. 4F).

Effects of CHRE on the expression of IL-1β and TNFα in the cerebral cortex and hippocampus of $A\beta_{1-42}$ -injected rats

The increment of major pro-inflammatory cytokines, including IL-1β and TNFα, plays a crucial role in neuroinflammation and cognitive impairment in AD. Western blotting was performed to assess whether CHRE attenuated the expression of IL-1β (Fig. 5) and TNFα (Fig. 6) in the rat brain. The results showed that $A\beta_{1-42}$ injection dramatically enhanced the expression of IL-1β and TNFα in the cerebral cortex and hippocampus of rats in the V+Aβ group compared with those in the SC group (Fig. 5A-B and 6A-B). However, administration of Celebrex® and CHRE significantly attenuated the expression of IL-1β and TNFα in both brain regions of rats in the CB+Aβ, CHRE125+Aβ, CHRE250+Aβ, and CHRE500+Aβ groups compared with those in the V+Aβ group (Fig. 5A-B and 6A-B). Importantly, CHRE500 reduced the overall expression of both cytokines to a greater extent than CHRE125 (Fig. 5A-B and 6A-B), while only CHRE250 resulted in a greater reduction of IL-1β in the hippocampus (Fig. 5B).

Discussion

This was the first study to assess the pharmacological activities of CHRE in an AD-like animal model. We found that CHRE improved cognitive impairment and decreased $A\beta_{1-42}$ protein levels and inflammatory

markers in $A\beta_{1-42}$-induced rats. The accumulation of Aβ is the key neuropathological feature of AD [54] and cause of cognitive impairments and increased expression of CD11b, IL-1β, and TNFα in the brains of AD patients and Aβ-induced rats [11–13, 18, 27]. Animal models selected for use in AD studies include Aβ-induced AD rats and TgF344-AD transgenic rats. These models mimic pathologies such as cognitive impairment, Aβ accumulation, and neuroinflammation in a way that is remarkably similar to those observed in AD patients [55–57]. Nillert et al. (2017) revealed that intracerebroventricular (ICV) injection of $A\beta_{1-42}$ into both lateral ventricles caused memory impairments, especially with regard to object recognition, via neuroinflammation in rats [13]. We thus employed this model to examine the alteration of object recognition memory using the NOR test in $A\beta_{1-42}$-injected rats after CHRE administration (35 consecutive days). The results of the retention phase showed that $A\beta_{1-42}$ injection impaired both short- and long-term object recognition memory, indicating the successful establishment of an amnesia model. Pretreatment with CHRE at all dosages (125, 250, and 500 mg/kg BW) markedly improved $A\beta_{1-42}$-induced impairment of both short- and long-term memory. This is consistent with a previous report demonstrating that nordentatin at a dose of 50 μmol/kg BW ameliorated cognitive impairments caused by Aβ injection as assessed using the Y-maze test [46].

Aβ aggregation in the brain is closely involved in the progression of AD. Aβ is generated from APP and eliminated by the enzymatic degradation pathways, microglial phagocytosis, cell autophagy, etc. [58, 59]. When this balance is disrupted, increases in Aβ proteins can stimulate their accumulation in the brain [60]. Abnormal Aβ accumulation can initiate an inflammatory response, eventually leading to neurotoxicity [60]. We found that $A\beta_{1-42}$ injection increased Aβ accumulation by increasing the level of $A\beta_{1-42}$ proteins in the cerebral cortex and hippocampus. However, pretreatment with CHRE at all doses reduced these effects. This result is supported by a previous study, which found that 7-hydroxy-5-methoxy-4-methyl-3-(4-methyl piperazin-1-yl)-coumarin (IMM-H004) inhibited Aβ accumulation in the hippocampus [61]. Additionally, another study found that coumarin compounds may interfere the process of Aβ accumulation by interacting with aromatic residues within the hydrophobic core of Aβ [62]. Moreover, these compounds also reduce the release of Aβ from APP via inhibition of β-secretase activity [63]. It is possible that these properties may have been associated with the improvements we observed in $A\beta_{1-42}$ protein levels. Neuroinflammation is a response by the innate immune system to protect the CNS [64]. However, it is a double-edged sword in that it also has the capacity to damage nervous tissue. Although acute neuroinflammation can reduce the accumulation of Aβ plaque in the brain, chronic neuroinflammation may promote the progression of AD [65, 66]. Excessive microglial activation is a predominant feature of neuroinflammation. In AD, Aβ accumulation can activate the surrounding microglia in order to facilitate its clearance [67] by binding it with various microglia receptors including CD11b. Thus, increased CD11b expression is observed in the brains of both AD patients and Aβ-induced rats [11–13]. However, it is still unclear whether microglia lose their efficacy or even become detrimental in the later stages of AD. A major challenge is to identify the specific pathways and molecular mechanisms that could be early indicators of brain damage and protect the brain from degeneration. The evidence suggests that disease-associated microglia (DAM), a recently identified subset of CNS resident macrophages found at sites of neurodegeneration [68], might play such a protective role. Recent studies

have shown that DAM is endowed with a dedicated sensory mechanism, which includes the triggering receptors expressed on the myeloid cell 2 (TREM2) signaling pathway [69, 70], to detect damage within the CNS in the form of neurodegeneration-associated molecular patterns (NAMPs). Transcriptional analysis of DAM and human genome-wide association studies (GWASs) have examined the potential function of DAM pathways in the neurodegenerative brain [68, 71, 72]. Therefore, manipulating DAM may create new therapeutic opportunities for AD. However, the technical limitations involved in analyzing heterogeneous microglia populations make it difficult to accurately determine the immune cell types and states involved in brain disease. Furthermore, chronic microglial activation also stimulates the nuclear factor-κB (NF-κB) signaling pathway and the subsequent release of pro-inflammatory cytokines, especially IL-1β and TNFα [16].

This study showed that $A\beta_{1-42}$ injection enhanced the expression of CD11b-positive microglia in the cerebral cortex and hippocampus. This is consistent with the above-mentioned results showing that increased $A\beta_{1-42}$ protein levels promote microglial phagocytosis through activation of their receptors. Additionally, IL-1β and TNFα expressions also increased in both brain regions of the $A\beta_{1-42}$-injected rats, effects that were reversed via pretreatment with CHRE at all dosages. Similarly, a previous study reported that several coumarins also have anti-inflammatory effects. For example, auraptene was able to inhibit microglial activation and COX-2 expression from astrocytes and neuronal cell death in the hippocampus [55]. In addition, xanthotoxol, IMM-H004, and osthole have been shown to reduce IL-1β, IL-6, TNFα, and nitric oxide (NO) in vivo [73–75]. The ability of coumarins to relieve neuroinflammation may involve interference with the binding of Aβ and microglia receptors [76]. This inhibits the nuclear translocation of NF-κB, the phosphorylation of Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) pathways, leading to suppression of the synthesis and release of pro-inflammatory cytokines [62, 77]. In addition, coumarins exhibit acetylcholinesterase (AChE) inhibitory activity, which in turn increases acetylcholine (ACh) levels in the brain [78–80], ultimately leading to reductions in cognitive impairment caused by $A\beta_{1-42}$ injection. Accordingly, decreases in inflammatory markers in both brain regions were also found in Celebrex®-treated rats (positive control group). This is consistent with previous studies, which reported reversals in COX-2, IL-1α, IL-1β, IL-6 and IL-12 and BDNF in soluble amyloid-β (sAβ)-treated rats that received Celecoxib [36]. Hence, this drug is commonly used as a positive control for studies in rat AD models. The dosing regimen could be critical, as using NSAIDs intermittently versus constant exposure could modulate the immune system differently. Both beneficial and detrimental effects of microglia activation have been described and depend on factors such as age, disease stage, etc. [81, 82]. In the early preclinical stage of AD, mild microglial activation could play a beneficial role. As with NSAIDs, the role of CHRE in anti-inflammatory drugs is being studied. Interestingly, medium or high doses of CHRE were more effective than lower dose in improving $A\beta_{1-42}$ protein levels and neuroinflammation. The reason for this was that all dosages were within the therapeutic range [83], meaning that the CHRE at higher concentrations was able to interact with the aromatic residues of Aβ and interfere with the binding of Aβ and microglial receptors more effectively than at lower concentrations.

All of this shows that the increment of Aβ and pathological changes that occur in the brain due to neuroinflammation are connected with cognitive impairments and the progression of AD. Consequently, reductions of Aβ and neuroinflammation may ameliorate cognitive impairments and delay the progression of AD. We found that administration of CHRE for 35 consecutive days mitigated both short- and long-term recognition memory impairment caused by $A\beta_{1-42}$ injection. It decreased $A\beta_{1-42}$ protein levels and neuroinflammation in the cerebral cortex and hippocampus, both of which are involved in recognition memory [52, 53]. This indicates that the preventive action of CHRE reduced the inflammatory process caused by $A\beta_{1-42}$ and suggests CHRE as a potential pharmacological agent for the treatment of AD.

Conclusions

This study showed that high levels of CHRE improved cognitive impairment in $A\beta_{1-42}$-induced rats through decreased $A\beta_{1-42}$ protein levels and neuroinflammation, particularly the expressions of CD11b-positive microglia, IL-1β, and TNFα in the cerebral cortex and hippocampus.

Abbreviations

ACh: acetylcholine; AChE: acetylcholinesterase; AD: Alzheimer's disease; ANOVA: analysis of variance; Aβ: Amyloid-β; BSA: bovine serum albumin; BW: body weight; CA: California; CA1: cornu ammonis area 1; CA2/3: cornu ammonis area 2/3; CB: Celebrex®; CD11b: clusters of differentiation molecule 11b; CH: *Clausena harmandiana*; CHR: *Clausena harmandiana* root; CHRE: *Clausena harmandiana* root extract; CNS: central nervous system; COX-2: cyclooxygenase-2; DAB: diaminobenzidine tetrahydro-chloride dihydrate; DAM: disease-associated microglia; DG: dentate gyrus; DI: discrimination index; DPPH: 1,1-diphenyl-2-picrylhydrazyl; DPX: dibutylphthalate polystyrene xylene; etc.: et cetera; ECL: enhanced chemiluminescence; FC: frontal cortex; GAPDH: glyceraldehyde 3 phosphate dehydrogenase; GWASs: genome-wide association studies; H_2O_2: hydrogen peroxide; HRP: horseradish peroxidase; ICV: intracerebroventricular; IgG: immunoglobulin G; IL-1β: interleukin-1β; IMM-H004: 7-hydroxy-5-methoxy-4-methyl-3-(4-methyl piperazin-1-yl)-coumarin; Inc.: incorporated; JNK: Jun N-terminal kinase; kg: kilogram; MAPK: mitogen-activated protein kinase; MD: Maryland; mg: milligram; MO: Missouri; NaCMC: sodium carboxymethylcellulose; NaF: sodium fluoride; NAMPs: neurodegeneration-associated molecular patterns; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; NIH: National Institute of Health; NO: nitric oxide; NOR: novel object recognition; NS: normal saline; NSAIDs: non-steroidal anti-inflammatory drugs; NY: New York; PC1: parietal cortex area 1; PC2: parietal cortex area 2; pH: positive potential of the Hydrogen ions; PiC: piriform cortex; PRC perirhinal cortex; sAβ: soluble amyloid-β; SC: sham control; SEM: standard error of the mean; St. Louis: Saint Louis; TBS: tris-buffered saline; TBST: tris-buffer saline containing Tween 20; TC1 temporal cortex area 1; TF: time spent with the familiar; TN: time spent with the novel; TNFα: tumor necrosis factor-α; TREM2: triggering receptors expressed on the myeloid cell 2; USA: United States of America; V: vehicle

Declarations

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Authors' contributions

Conceptualization and methodology of the experiments: WP. Visualization and formal analysis: NN and KB. Funding acquisition: CB. Resources: PP. Software: KB. Supervision: CB. Investigation and drafting of the manuscript: NN. Reviewing and editing of the manuscript: JUW and WP. All authors have read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

The animal experiments were carried out following the guidelines and regulations of Northeast Laboratory Animal Center, Khon Kaen University, Khon Kaen, Thailand, and have received animal ethics approval from the Institutional Animal Care and Use Committee of Khon Kaen University, Khon Kaen, Thailand, (Approval No. IACUC-KKU-25/61).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Department of Anatomy, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand.
[2]Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Khon Kaen University,

Khon Kaen 40002, Thailand.

References

1. Zhang LL, Sui HJ, Liang B, Wang HM, Qu WH, Yu SX, et al. Atorvastatin prevents amyloid-β peptide oligomer-induced synaptotoxicity and memory dysfunction in rats through a p38 MAPK-dependent pathway. Acta Pharmacol. Sin. 2014; *35*(6): 716–26.
2. Du Y, Qu J, Zhang W, Bai M, Zhou Q, Zhang Z, et al. Morin reverses neuropathological and cognitive impairments in APPswe/PS1dE9 mice by targeting multiple pathogenic mechanisms. Neuropharmacology. 2016; 108: 1–13.
3. Querfurth HW, LaFerla FM. Alzheimer's disease. N Engl J Med. 2010; 362(4): 329–44.
4. Sanabria-Castro A, Alvarado-Echeverría I, Monge-Bonilla C. Molecular pathogenesis of Alzheimer's disease: an update. Ann. Neurosci. 2017; 24(1): 46–54.
5. Kametani F, Hasegawa M. Reconsideration of amyloid hypothesis and tau hypothesis in Alzheimer's disease. Front. Neurosci. 2018; 12: 25.
6. Tarasoff-Conway JM, Carare RO, Osorio RS, Glodzik L, Butler T, Fieremans E, et al. Clearance systems in the brain—implications for Alzheimer disease. Nat. Rev. Neurol. 2015; 11(8): 457–70.
7. Wang Y, Zhu T, Wang M, Zhang F, Zhang G, Zhao J, et al. Icariin attenuates M1 activation of microglia and Aβ plaque accumulation in the hippocampus and prefrontal cortex by up-regulating PPARγ in restraint/isolation-stressed APP/PS1 mice. Front. Neurosci. 2019 28; 13: 291.
8. Shastri A, Bonifati DM, Kishore U. Innate immunity and neuroinflammation. Mediat. Inflamm. 2013; 2013: 342931.
9. Khan SQ, Khan I, Gupta V. CD11b activity modulates pathogenesis of lupus nephritis. Front. Med. 2018; 5: 52.
10. Fu H, Liu B, Frost JL, Hong S, Jin M, Ostaszewski B, et al. Complement component C3 and complement receptor type 3 contribute to the phagocytosis and clearance of fibrillar Aβ by microglia. Glia. 2012; 60(6): 993–1003.
11. Hopperton KE, Mohammad D, Trépanier MO, Giuliano V, Bazinet RP. Markers of microglia in post-mortem brain samples from patients with Alzheimer's disease: a systematic review. Mol. psychiatry. 2018; 23(2): 177–98.
12. Czirr E, Castello NA, Mosher KI, Castellano JM, Hinkson IV, Lucin KM, et al. Microglial complement receptor 3 regulates brain Aβ levels through secreted proteolytic activity. J. Exp. Med. 2017; 214(4): 1081–92.
13. Nillert N, Pannangrong W, Welbat JU, Chaijaroonkhanarak W, Sripanidkulchai K, Sripanidkulchai B. Neuroprotective effects of aged garlic extract on cognitive dysfunction and neuroinflammation induced by β-amyloid in rats. Nutrients. 2017; 9(1): 24.
14. Ladeby R, Wirenfeldt M, Dalmau I, Gregersen R, García-Ovejero D, Babcock A, et al. Proliferating resident microglia express the stem cell antigen CD34 in response to acute neural injury. Glia. 2005;

50(2): 121–31.

15. Zhang D, Hu X, Qian L, Chen SH, Zhou H, Wilson B, Miller DS, Hong JS. Microglial MAC1 receptor and PI3K are essential in mediating β-amyloid peptide-induced microglial activation and subsequent neurotoxicity. J. Neuroinflammation. 2011; 8(1): 1–14.
16. Ismail EN, Jantan I, Vidyadaran S, Jamal JA, Azmi N. *Phyllanthus amarus* prevents LPS-mediated BV2 microglial activation via MyD88 and NF-κB signaling pathways. BMC complement. med. ther. 2020; 20(1): 1–13.
17. Liu T, Zhang L, Joo D, Sun SC. NF-κB signaling in inflammation. Signal Transduct. Target. Ther. 2017; 2(1): 1–9.
18. Wang WY, Tan MS, Yu JT, Tan L. Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. Ann. Transl. Med. 2015; 3(10): 136.
19. Tuppo EE, Arias HR. The role of inflammation in Alzheimer's disease. Int. J. Biochem. Cell Biol. 2005; 37(2): 289–305.
20. Morales I, Farias G, Maccioni RB. Neuroimmunomodulation in the pathogenesis of Alzheimer's disease. Neuroimmunomodulation. 2010; 17(3): 202–4.
21. Pickering M, O'Connor JJ. Pro-inflammatory cytokines and their effects in the dentate gyrus. Prog. Brain Res. 2007 1; 163: 339–54.
22. Janelsins MC, Mastrangelo MA, Park KM, Sudol KL, Narrow WC, Oddo S, et al. Chronic neuron-specific tumor necrosis factor-alpha expression enhances the local inflammatory environment ultimately leading to neuronal death in 3xTg-AD mice. Am. J. Pathol. 2008; 173(6): 1768–82.
23. Neta R, Sayers TJ, Oppenheim JJ. Relationship of TNF to interleukins. Immunol. Ser. 1992; 56: 499–566.
24. Kitazawa M, Cheng D, Tsukamoto MR, Koike MA, Wes PD, Vasilevko V, et al. Blocking IL-1 signaling rescues cognition, attenuates tau pathology, and restores neuronal β-catenin pathway function in an Alzheimer's disease model. J. Immunol. 2011; 187(12): 6539–49.
25. Shin JW, Cheong YJ, Koo YM, Kim S, Noh CK, Son YH, Kang C, Sohn NW. α-Asarone ameliorates memory deficit in lipopolysaccharide-treated mice via suppression of pro-inflammatory cytokines and microglial activation. Biomol Ther. 2014; 22(1): 17.
26. Squire LR, Wixted JT, Clark RE. Recognition memory and the medial temporal lobe: a new perspective. Nat. Rev. Neurosci. 2007; 8(11): 872–83.
27. Winters BD, Saksida LM, Bussey TJ. Object recognition memory: neurobiological mechanisms of encoding, consolidation and retrieval. Neurosci. Biobehav. Rev. 2008; 32(5): 1055–70.
28. Antunes M, Biala G. The novel object recognition memory: neurobiology, test procedure, and its modifications. Cogn. Process. 2012; 13(2): 93–110.
29. De Craen AJ, Gussekloo J, Vrijsen B, Westendorp RG. Meta-analysis of nonsteroidal antiinflammatory drug use and risk of dementia. Am. J. Epidemiol. 2005; 161(2): 114–20.

30. Vlad SC, Miller DR, Kowall NW, Felson DT. Protective effects of NSAIDs on the development of Alzheimer disease. Neurology. 2008; 70(19): 1672–7.
31. Weggen S, Rogers M, Eriksen J. NSAIDs: small molecules for prevention of Alzheimer's disease or precursors for future drug development?. Trends Pharmacol. Sci. 2007; 28(10): 536–43.
32. McGeer PL, McGeer EG. NSAIDs and Alzheimer disease: epidemiological, animal model and clinical studies. Neurobiol. Aging. 2007; 28(5): 639–47.
33. Zhang C, Wang Y, Wang D, Zhang J, Zhang F. NSAID exposure and risk of Alzheimer's disease: an updated meta-analysis from cohort studies. Front. Aging Neurosci. 2018; 10: 83.
34. Zhu Y, Yao R, Li Y, Wu C, Heng L, Zhou M, et al. Protective effect of celecoxib on early postoperative cognitive dysfunction in geriatric patients. Front. Neurol. 2018; 9: 633.
35. Meng X, Zhang Q, Zheng G, Pang R, Hua T, Yang S, Li J. Doxorubicin combined with celecoxib inhibits tumor growth of medullary thyroid carcinoma in xenografted mice. Oncol. Lett. 2014; 7(6): 2053–8.
36. Mhillaj E, Morgese MG, Tucci P, Furiano A, Luongo L, Bove M, et al. Celecoxib prevents cognitive impairment and neuroinflammation in soluble amyloid β-treated rats. Neurosci. 2018; 372: 58–73.
37. Borre Y, Lemstra S, Westphal KG, Morgan ME, Olivier B, Oosting RS. Celecoxib delays cognitive decline in an animal model of neurodegeneration. Behav. Brain Res. 2012; 234(2): 285–91.
38. Laine L. Gastrointestinal effects of NSAIDs and coxibs. J. Pain Symptom Manag. 2003; 25(2): 32–40.
39. Niranjan R, Manik R, Srivastava AK, Palit G, Natu SM. Cardiovascular side effect remotely related to NSAIDs: A comparative experimental study on albino rats. J. Anat. Soc. India. 2011; 60(2): 155–9.
40. Ong CK, Lirk P, Tan CH, Seymour RA. An evidence-based update on nonsteroidal anti-inflammatory drugs. Clin Med Res. 2007; 5(1): 19–34.
41. Chiasson JM, Fominaya CE, Gebregziabher M, Taber DJ. Long-term assessment of NSAID prescriptions and potential nephrotoxicity risk in adult kidney transplant recipients. Transplantation. 2019; 103(12): 2675.
42. Daodee S, Wangboonskul J, Yenjai C, Boonyarat C, Monthakantirat O. Determination of five carbazole alkaloids from the root of *Clausena harmandiana* by High Performance Liquid Chromatography. Isan J. Pharm. Sci. 2012; 8(2): 94–100.
43. Noipha K, Thongthoom T, Songsiang U, Boonyarat C, Yenjai C. Carbazoles and coumarins from *Clausena harmandiana* stimulate glucose uptake in L6 myotubes. Diabetes Res. Clin. Pract. 2010; 90(3): 67–71.
44. Wangboonskul J, Tunsirikongkon A, Sasithornwetchakun W. Simultaneous determination of nine analytes in *Clausena harmandiana* Pierre. by new developed high-performance liquid chromatography method and the influence of locations in Thailand on level of nordentatin and dentatin. Pharmacogn. Mag. 2015; 11(41): 1–7.

45. Jantakoon P, Tadtong S, Puthongking P. Neuritogenic and antioxidant activities of nordentatin from *Clausena harmandiana*. JAASP 2012; 1: 180–6.
46. Boonyarut C, Wangboonskul J, Yenjai C, Monthakantirat O. Antioxidant activity and neuroprotective effect of chemical constituents from the roots of *Clausena harmandiana* Pierre against cognitive impairment in animal models. National Research Council of Thailand: Bangkok, Thailand, 2009; 25–26.
47. Wangboonskul J, Prawan A, Takthaisong P, Sasithornwetchakun W, Boonyarat C, Yenjai C, et al. Analgesic, anti-inflammatory, antipyretic activities and acute toxicity of the ethanolic extract of *clausena harmandiana* pierre in animals. JAASP 2012; 1: 159–69.
48. Wichai T, Pannangrong W, Welbat JU, Chaichun A, Sripanidkulchai K, Sripanidkulchai B. Effects of aged garlic extract on spatial memory and oxidative damage in the brain of amyloid-β induced rats. Songklanakarin J Sci Technol. 2019; 41: 311–8.
49. Chen X, Hu J, Jiang L, Xu S, Zheng B, Wang C, et al. Brilliant Blue G improves cognition in an animal model of Alzheimer's disease and inhibits amyloid-β-induced loss of filopodia and dendrite spines in hippocampal neurons. Neuroscience. 2014; 279: 94–101.
50. Thorajak P, Pannangrong W, Welbat JU, Chaijaroonkhanarak W, Sripanidkulchai K, Sripanidkulchai B. Effects of aged garlic extract on cholinergic, glutamatergic and GABAergic systems with regard to cognitive impairment in Aβ-induced rats. Nutrients. 2017; 9(7): 686.
51. Pannangrong W, Welbat JU, Chaichun A, Sripanidkulchai B. Effect of combined extracts of aged garlic, ginger, and chili peppers on cognitive performance and brain antioxidant markers in Aβ-induced rats. Exp. Anim. 2020; 69: 269–278.
52. Cai M, Shin BY, Kim DH, Kim JM, Park SJ, Park CS, et al. Neuroprotective effects of a traditional herbal prescription on transient cerebral global ischemia in gerbils. J. Ethnopharmacol. 2011; 138(3): 723–30.
53. Medeiros R, Kitazawa M, Passos GF, Baglietto-Vargas D, Cheng D, Cribbs DH, et al. Aspirin-Triggered Lipoxin A4 Stimulates Alternative Activation of Microglia and Reduces Alzheimer Disease–Like Pathology in Mice. Am. J. Pathol. 2013; 182(5): 1780–9.
54. Johnson KA, Fox NC, Sperling RA, Klunk WE. Brain imaging in Alzheimer disease. Cold Spring Harb. Perspect. Med. 2012; 2(4): a006213.
55. Okuyama S, Minami S, Shimada N, Makihata N, Nakajima M, Furukawa Y. Anti-inflammatory and neuroprotective effects of auraptene, a citrus coumarin, following cerebral global ischemia in mice. Eur. J. Pharmacol. 2013; 699(1-3): 118–23.
56. Proskauer Pena SL, Mallouppas K, Oliveira AM, Zitricky F, Nataraj A, Jezek K. Early Spatial Memory Impairment in a Double Transgenic Model of Alzheimer's Disease TgF-344 AD. Brain Sci. 2021; 11(10): 1300.
57. Chaney AM, Lopez-Picon FR, Serrière S, Wang R, Bochicchio D, Webb SD, et al. Prodromal neuroinflammatory, cholinergic and metabolite dysfunction detected by PET and MRS in the TgF344-AD transgenic rat model of AD: a collaborative multi-modal study. Theranostics. 2021; 11(14): 6644.

58. Kruppa AJ, Ott S, Chandraratna DS, Irving JA, Page RM, Speretta E, et al. Suppression of Aβ toxicity by puromycin-sensitive aminopeptidase is independent of its proteolytic activity. Biochim Biophys Acta Mol Basis Dis. 2013; 1832(12): 2115–26.
59. Yoon SS, Jo SA. Mechanisms of amyloid-β peptide clearance: potential therapeutic targets for Alzheimer's disease. Biomol Ther. 2012; 20(3): 245–55.
60. Deng Y, Long L, Wang K, Zhou J, Zeng L, He L, et al. Icariside II, a broad-spectrum anti-cancer agent, reverses beta-amyloid-induced cognitive impairment through reducing inflammation and apoptosis in rats. Front. Pharmacol. 2017; 8: 39.
61. Song XY, Wang YY, Chu SF, Hu JF, Yang PF, Zuo W, et al. A new coumarin derivative, IMM-H004, attenuates okadaic acid-induced spatial memory impairment in rats. Acta Pharmacol. Sin. 2016; 37(4): 444–52.
62. Soto-Ortega DD, Murphy BP, Gonzalez-Velasquez FJ, Wilson KA, Xie F, Wang Q, et al. Inhibition of amyloid-β aggregation by coumarin analogs can be manipulated by functionalization of the aromatic center. Bioorg. Med. Chem. 2011; 19(8): 2596–602.
63. Yang HL, Cai P, Liu QH, Yang XL, Li F, Wang J, et al. Design, synthesis and evaluation of coumarin-pargyline hybrids as novel dual inhibitors of monoamine oxidases and amyloid-β aggregation for the treatment of Alzheimer's disease. Eur. J. Med. Chem. 2017; 138: 715–28.
64. Milatovic D, Zaja-Milatovic S, Breyer RM, Aschner M, Montine TJ. Neuroinflammation and oxidative injury in developmental neurotoxicity. Reprod. Dev. Toxicol. 2017: Academic Press: 1051-61.
65. Friedberg JS. Apolipoprotein E alters the association of neuroinflammation with Alzheimer's disease. PhD thesis, Boston University, USA. 2018.
66. Loizou, F. The role of pattern recognition receptors in amyloid-beta-induced inflammation. PhD Thesis, Cardiff University, UK. 2016.
67. Mandrekar-Colucci S, Landreth GE. Microglia and inflammation in Alzheimer's disease. CNS Neurol. Disord. -Drug Targets. 2010; 9(2): 156–67.
68. Keren-Shaul H, Spinrad A, Weiner A, Matcovitch-Natan O, Dvir-Szternfeld R, Ulland TK, et al. A unique microglia type associated with restricting development of Alzheimer's disease. Cell. 2017; 169(7): 1276–90.
69. Griciuc A, Tanzi RE. The role of innate immune genes in Alzheimer's disease. Curr. Opin. Neurol. 2021; 34(2): 228.
70. Deczkowska A, Keren-Shaul H, Weiner A, Colonna M, Schwartz M, Amit I. Disease-associated microglia: a universal immune sensor of neurodegeneration. Cell. 2018; 173(5): 1073–81.
71. Lambert JC, Ibrahim-Verbaas CA, Harold D, Naj AC, Sims R, Bellenguez C, et al. European Alzheimer's Disease Initiative (EADI); Genetic and Environmental Risk in Alzheimer's Disease; Alzheimer's Disease Genetic Consortium; Cohorts for Heart and Aging Research in Genomic Epidemiology. Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. Nat. Genet. 2013; 45(12): 1452–8.

72. Yeh FL, Hansen DV, Sheng M. TREM2, microglia, and neurodegenerative diseases. Trends. Mol. Med. 2017; 23(6): 512–33.
73. He W, Chen W, Zhou Y, Tian Y, Liao F. Xanthotoxol exerts neuroprotective effects via suppression of the inflammatory response in a rat model of focal cerebral ischemia. Cell. Mol. Neurobiol. 2013; 33(5): 715–22.
74. Yang PF, Song XY, Zeng T, Ai QD, Liu DD, Zuo W, et al. IMM-H004, a coumarin derivative, attenuated brain ischemia/reperfusion injuries and subsequent inflammation in spontaneously hypertensive rats through inhibition of VCAM-1. RSC Adv. 2017; 7(44): 27480–95.
75. Kong L, Yao Y, Xia Y, Liang X, Ni Y, Yang J. Osthole alleviates inflammation by down-regulating NF-κB signaling pathway in traumatic brain injury. Immunopharmacol. Immunotoxicol. 2019; 41(2): 349–60.
76. Liu J, Chen Q, Jian Z, Xiong X, Shao L, Jin T, et al. Daphnetin protects against cerebral ischemia/reperfusion injury in mice via inhibition of TLR4/NF-κB signaling pathway. Biomed Res. Int. 2016; 2016: 2816056.
77. Katsori AM, Hadjipavlou-Litina D. Coumarin derivatives: an updated patent review (2012-2014). Expert Opin. Ther. Pat. 2014; 24(12): 1323–47.
78. Kim DH, Kim YC, Jung JW, Lee S, Yoon BH, Cheong JH, et al. Nodakenin, a coumarin compound, ameliorates scopolamine-induced memory disruption in mice. Life Sci. 2007; 80(21): 1944–50.
79. Shen Q, Peng Q, Shao J, Liu X, Huang Z, Pu X, et al. Synthesis and biological evaluation of functionalized coumarins as acetylcholinesterase inhibitors. European journal of medicinal chemistry. 2005; 40(12): 1307–15.
80. Brühlmann C, Ooms F, Carrupt PA, Testa B, Catto M, Leonetti F, et al. Coumarins derivatives as dual inhibitors of acetylcholinesterase and monoamine oxidase. J. Med. Chem. 2001; 44(19): 3195–8.
81. Wyss-Coray T, Mucke L. Inflammation in neurodegenerative disease—a double-edged sword. Neuron. 2002; 35(3): 419–32.
82. Hoozemans JJ, Rozemuller JM, Van Haastert ES, Veerhuis R, Eikelenboom P. Cyclooxygenase-1 and-2 in the different stages of Alzheimer's disease pathology. Curr. Pharm. Des. 2008; 14(14): 1419–27.
83. Cooney L, Loke YK, Golder S, Kirkham J, Jorgensen A, Sinha I, et al. Overview of systematic reviews of therapeutic ranges: methodologies and recommendations for practice. BMC Med. Res. Methodol. 2017; 17(1): 1–9.

Figures

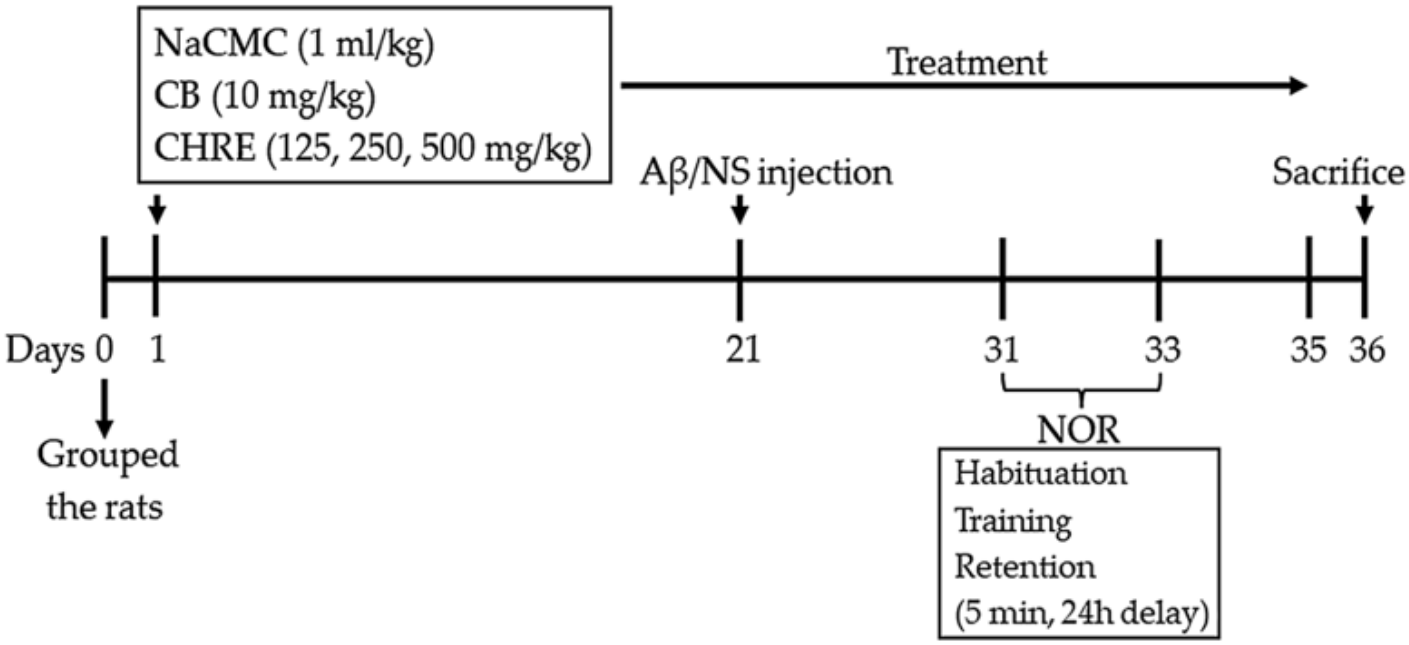

Figure 1

Schematic diagram of drug treatment and behavioral tests. Rats were injected with $A\beta_{1-42}$ into the lateral ventricle on each side after 21 days of drug treatment. NaCMC: sodium carboxymethylcellulose; CB: Celebrex®; CHRE: *Clausena harmandiana* root extract; Aβ: amyloid-β; NS: normal saline; NOR: novel object recognition.

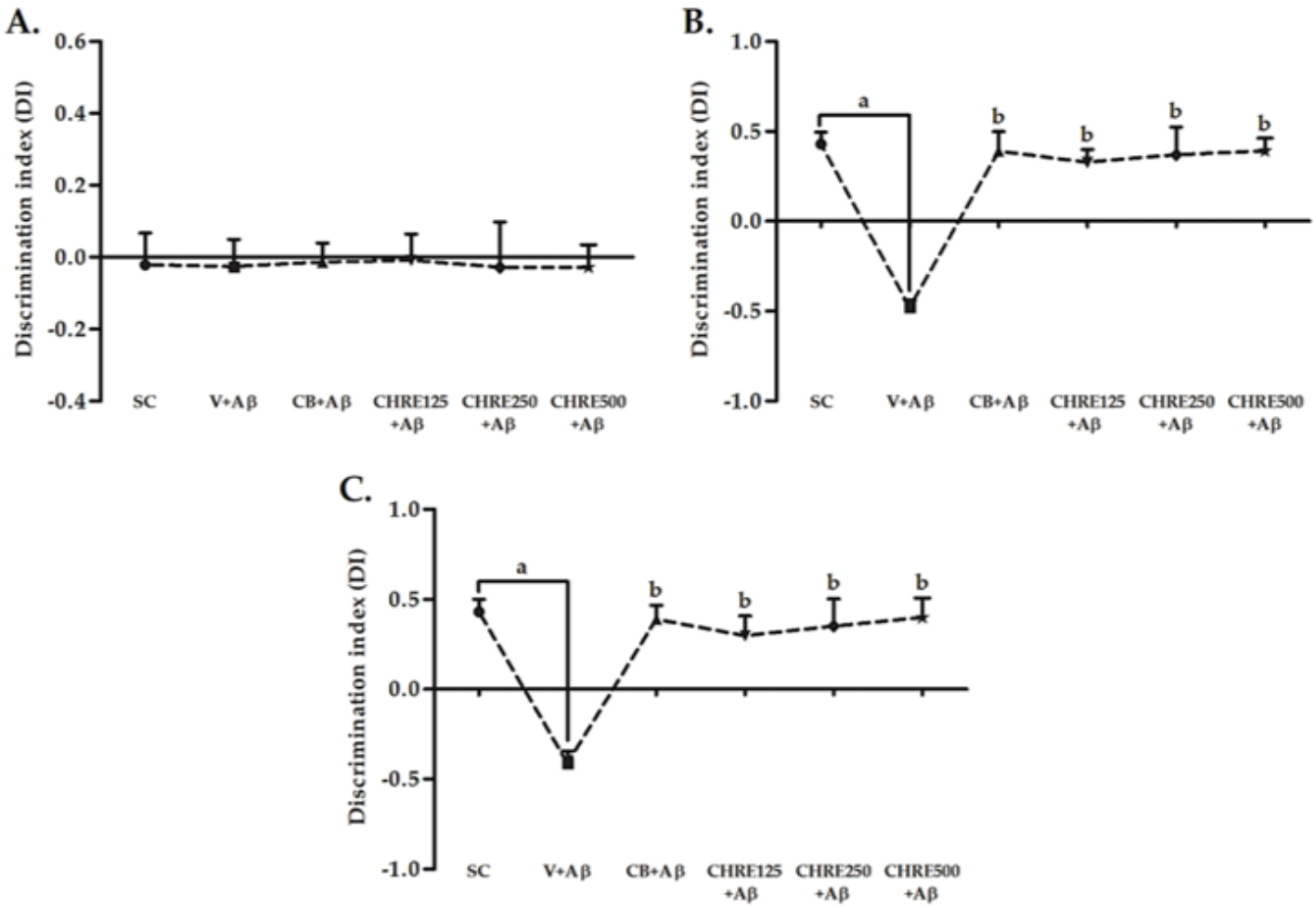

Figure 2

Effects of CHRE on cognitive impairment in $A\beta_{1-42}$-injected rats in the novel object recognition (NOR) test. The discrimination index (DI) in the training phase (**A**), after a 5 min delay (**B**) and a 24 h delay (**C**) of the NOR test. Data are expressed as mean ± SEM; a = significant difference from the SC group at $P < 0.001$, and b = significant difference from the V+Aβ group at $P < 0.001$.

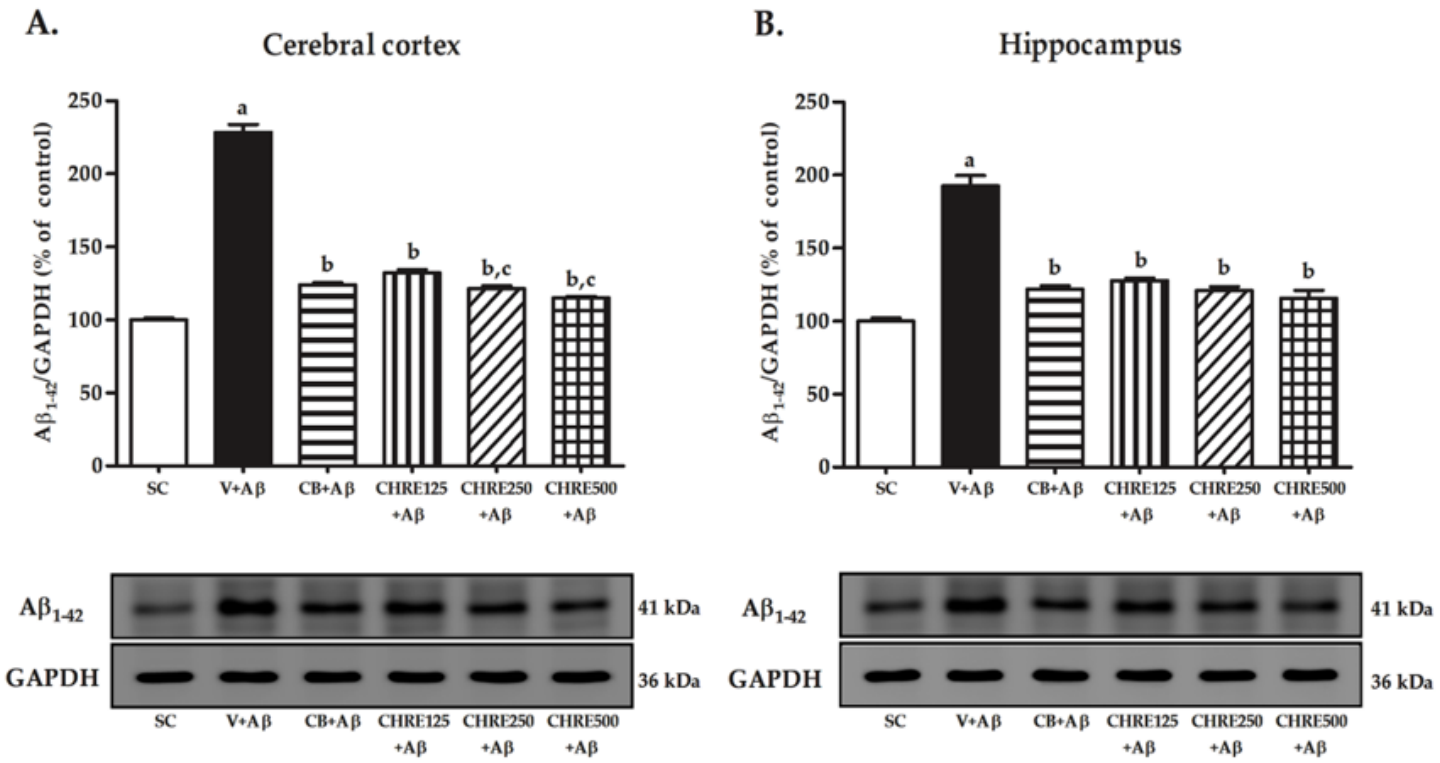

Figure 3

Effects of CHRE on Aβ$_{1-42}$ protein levels in the brain of Aβ$_{1-42}$ injected rats. Representative Western blot imaging and quantitative analysis of Aβ$_{1-42}$ protein levels in the cerebral cortex (**A**) and hippocampus (**B**) using Western blot. GAPDH is used as an internal control. Data are expressed as mean ± SEM; a = significant difference from the SC group at $P < 0.001$, b = significant difference from the V+Aβ group at $P < 0.001$, and c = significant difference from the CHRE125+Aβ group at $P < 0.001$. The original uncropped Western blots for Aβ$_{1-42}$ protein and GAPDH were represented in Suppl. Fig. 3.

Figure 4

Effects of CHRE on the expression of CD11b-positive microglia in the brain of Aβ$_{1-42}$-injected rats. Representative photomicrographs of CD11b-positive microglia in the cerebral cortex (**A**) and hippocampus (**B**) of SC and Aβ-induced rats at low magnification. Representative photomicrographs of CD11b-positive microglia in the cerebral cortex (C) and hippocampus (D) of SC and Aβ$_{1-42}$-injected rats at high magnification. The density of CD11b-positive microglia in the cerebral cortex (**E**) and hippocampus (**F**) measured using immunohistochemistry. Data are expressed as mean ± SEM; a = significant difference from the SC group at $P < 0.001$, b, c, and d = significant difference from the V+Aβ group at $P <$

0.001, $P < 0.01$, and $P < 0.05$, respectively, and e = significant difference from the CHRE125+Aβ group at $P < 0.001$.

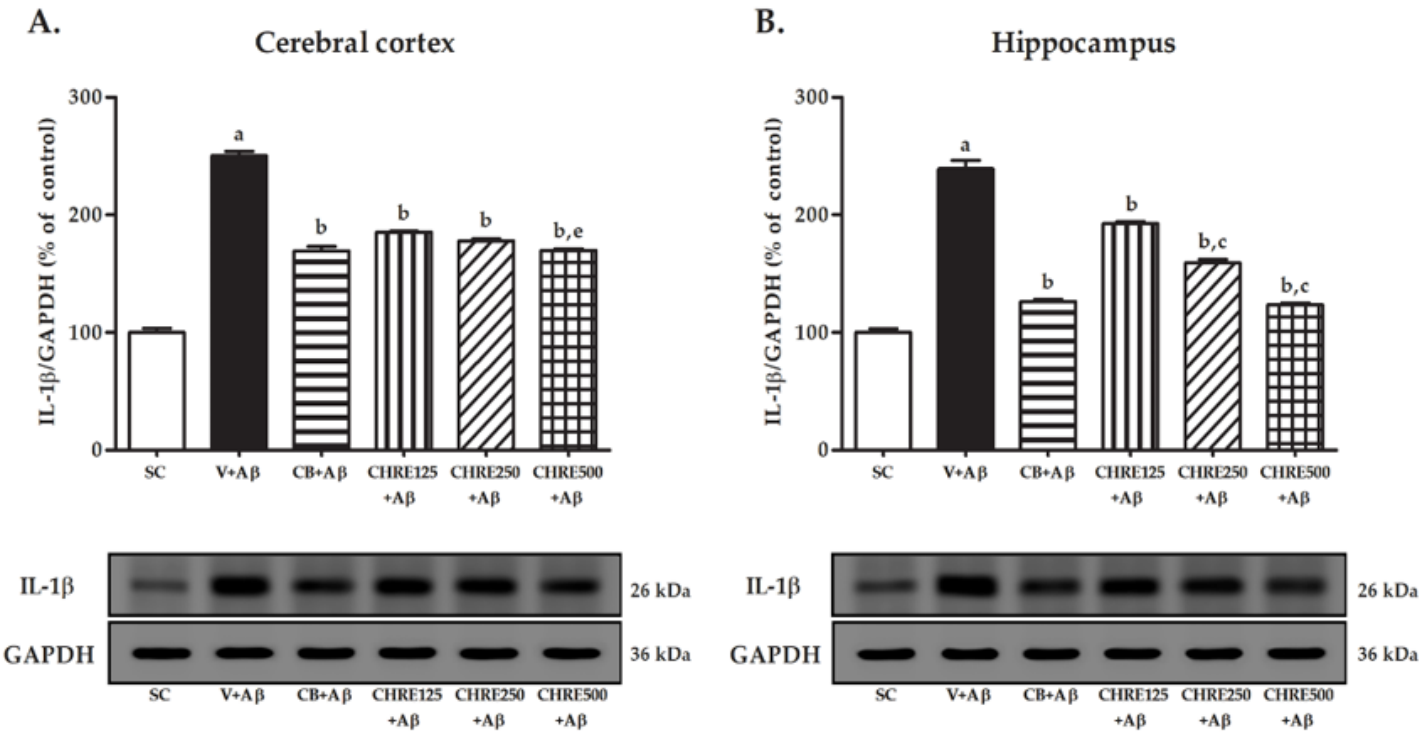

Figure 5

Effects of CHRE on the expression of IL-1β in the brain of $Aβ_{1-42}$-injected rats. Representative western blot imaging and quantitative analysis of IL-1β in the cerebral cortex (A) and hippocampus (B) using Western blot. GAPDH is used as an internal control. Data are expressed as mean ± SEM, a = significant difference from the SC group at $P < 0.001$, b = significant difference from the V+Aβ group at $P < 0.001$, and c and e = significant difference from the CHRE125+Aβ group at $P < 0.001$ and $P < 0.05$, respectively. The original uncropped Western blots for IL-1β and GAPDH were represented in Suppl. Fig. 4.

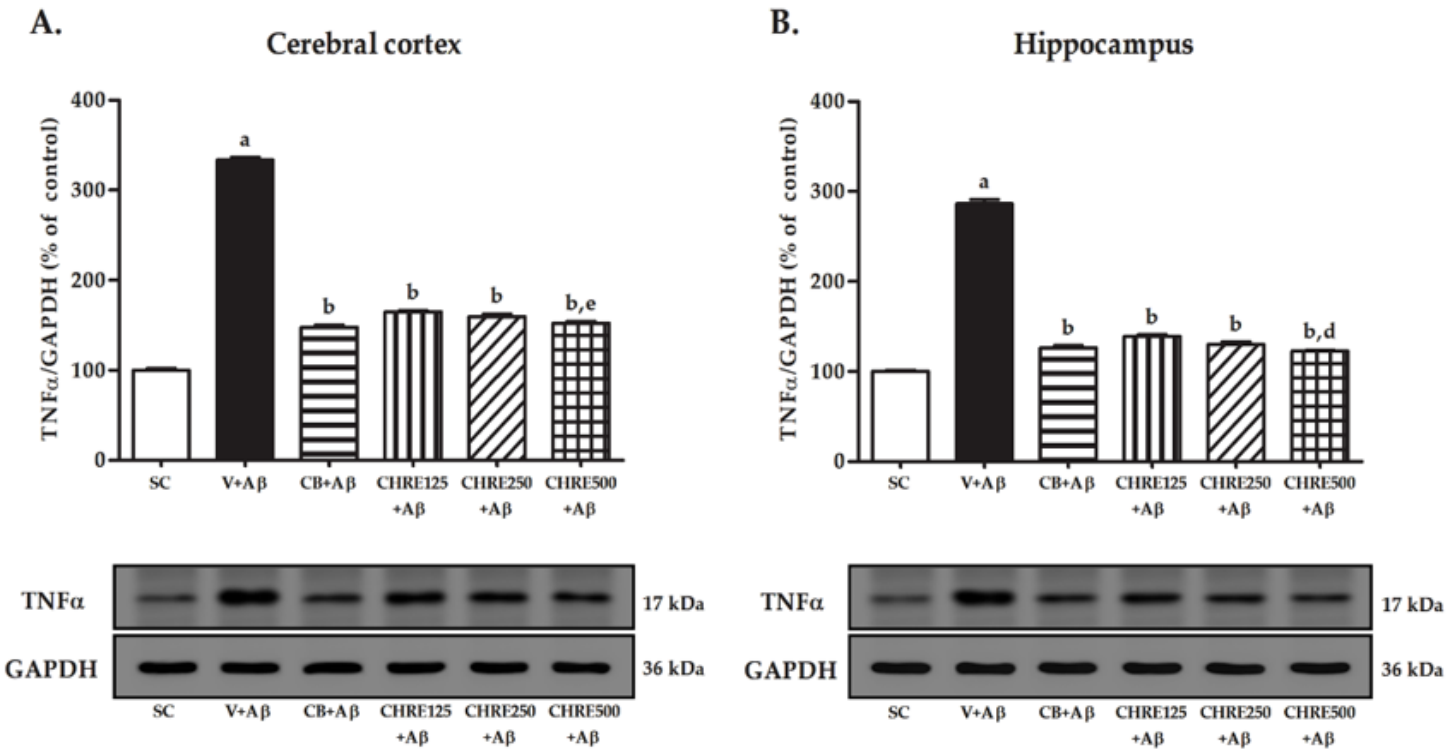

Figure 6

Effects of CHRE on the expression of TNFα in the brain of $Aβ_{1-42}$-injected rats. Representative western blot imaging and quantitative analysis of TNFα in the cerebral cortex (A) and hippocampus (B) using Western blot. GAPDH is used as an internal control. Data are expressed as mean ± SEM, a = significant difference from the SC group at $P < 0.001$, b = significant difference from the V+Aβ group at $P < 0.001$, and d and e = significant difference from the CHRE125+Aβ group at $P < 0.01$ and $P < 0.05$, respectively. The original uncropped Western blots for TNFα and GAPDH were represented in Suppl. Fig. 5.

Supplementary Files

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