Piperlongumine decreases cognitive impairment and improves hippocampal function in aged mice

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Abstract. Piperlongumine (PL), a biologically active compound from the *Piper* species, has been shown to exert various pharmacological effects in a number of conditions, including tumours, diabetes, pain, psychiatric disorders and neurodegenerative disease. In this study, we evaluated the therapeutic effects of PL on hippocampal function and cognition decline in aged mice. PL (50 mg/kg/day) was intragastrically administrated to 23-month-old female C57BL/6J mice for 8 weeks. Novel object recognition and nest building behaviour tests were used to assess cognitive and social functions. Additionally, immunohistochemistry and western blot analysis were performed to examine the effects of PL on the hippocampus. We found that the oral administration of PL significantly improved novel object recognition and nest building behaviour in aged mice. Although neither the percentage area occupied by astrocytes and microglia nor the level of 4-hydroxynonenal protein, a specific marker of lipid peroxidation, were altered by PL treatment, the phosphorylation levels of *N*-methyl-D-aspartate receptor subtype 2B (NR2B), calmodulin-dependent protein kinase II alpha (CaMKIIα) and extracellular signal-regulated kinase 1/2 (ERK1/2) were markedly increased in the hippocampus of aged mice following the administration of PL. We also found that PL treatment resulted in a CA3-specific increase in the phosphorylation level of cyclic AMP response element binding protein, which is recognized as a potent marker of neuronal plasticity, learning and memory. Moreover, the number of doublecortin-positive cells, a specific marker of neurogenesis, was significantly increased following PL treatment in the dentate gyrus of the hippocampus. On the whole, these data demonstrate that PL treatment may be a potential novel approach in the treatment of age-related cognitive impairment and hippocampal changes.

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Abbreviations: PL, piperlongumine; AD, Alzheimer's disease; EtOH, ethanol; EtOAc, ethyl acetate; MeOH, methanol; CMC, carboxymethyl cellulose; VGLUT1, vesicular glutamate transporter 1; VGLUT2, vesicular glutamate transporter 2; NR2B, N-methyl-D-aspartate receptor subtype 2B; PSD-95, postsynaptic density protein 95; GAD65/67, glutamate decarboxylase 65/67; VGAT, vesicular GABA transporter; CREB, cAMP response element binding protein; CaMKIIα, calcium/calmodulin-dependent protein kinase type II α; ERK1/2, extracellular signal-regulated kinases 1/2; PBS, phosphate-buffered saline; Iba1, ionized calcium-binding adapter molecule 1; GFAP, glial fibrillary acidic protein; 4-HNE, 4-hydroxy-2-nonenal; LTP, long-term potentiation

Key words: aging, cognitive impairment, piperlongumine, hippocampus, neurogenesis

Introduction

The aging population is increasing at a rapid rate worldwide, giving rise to a number of age-related diseases that have a significant social and economic burden on the community. With normal aging, the brain undergoes synaptic dysfunction, extensive neuronal death and declined neurogenesis. Learning and memory impairment and cognitive deficits are well-known characteristics of the aging process (1-3). In addition, aging is associated with various debilitating neurodegenerative conditions, including Alzheimer's disease (AD). Thus, the prevention or delay of the onset of age-related diseases and age-related cognitive decline may improve the quality of life.

The hippocampus, located in the medial temporal lobe of the brain, is crucial for normal learning and memory consolidation. This region is particularly vulnerable to the aging process (2,4). The hippocampus has been shown to undergo several structural and functional changes with age (2). Significant aged-related neuronal atrophy and volume decreases of the hippocampus, as well as hippocampal-dependent learning and memory decline have been demonstrated (5). An upregulation in the levels of ~~of~~ pro-inflammatory genes and inflammatory parameters has also been observed in the hippocampus during aging (6,7). Additionally, changes in synaptic plasticity have been detected in the hippocampi of aged humans and rodents (8,9). Although the mechanisms underlying age-related synaptic plasticity impairment are still under investigation, dysregulations and alterations in the expression levels of several proteins, that play key roles in synaptogenesis and synaptic stabilization, in the hippocampus have been reported (2,10).

Piperlongumine (PL, 5,6-dihydro-1-[(2E)-1-oxo-3-(3,4,5-trimethoxyphenyl)-2-propenyl]-2(1H)-pyridinone) is a natural alkaloid that can be isolated from the long pepper (*Piper longum* L.). PL is found in the fruits and roots of the plant (11). Cumulative evidence has indicated that PL has a number of pharmacological activities, including antidepressant, anxiolytic, anti-fungal, antidiabetic, antinociceptive and antitumour properties (11-16). Moreover, in our previous study, it was demonstrated that administration of PL improves cognitive function in a transgenic mouse model of AD (17). Thus, we hypothesized that PL would enhance cognitive function in aged mice. In the present study, we demonstrate that PL treatment modulates age-related cognitive decline and hippocampal dysfunction in aged mice.

Materials and methods

Preparation of PL. PL was isolated from *Piper longum*. Preparation was performed as described in previous studies (17-19). Dried fruits (500 g) of *Piper longum* were extracted with ethyl acetate (EtOH; 1 liter x 3 times) at room temperature for 1 week. The combined EtOH extracts were concentrated to yield a dry residue (32.5 g), which was subsequently suspended in water (H_2O; 500 ml) and partitioned with EtOAc (3x500 ml). The partial EtOAc extract (6.0 g), which was subjected to a silica gel column chromatography (CC; 5x40 cm), was eluted with a gradient *n*-hexane/acetone system (20:1 to 1:1) to yield 5 fractions (F1-F5). Fractions F3 and F4 were combined and further applied to a reversed phase-C_{18} CC (3x30 cm) with methanol (MeOH)/H_2O (1:1 to 9:1). Subfraction F34.3 (60.8 mg) was purified by high-performance liquid chromatography [mobile phase: MeOH in H_2O containing (0-40 min: 65% MeOH); flow rate: 2 ml/min; UV detection at 205 and 254 nm] to yield a compound (t_R=17.2 min, 14 mg). The chemical structure of the isolated compound was confirmed by comparison with the reported chemical structure of PL using 1D and 2D nuclear magnetic resonance spectroscopy.

Animals. Female C57BL/6J mice, at 3 months (n=7, weighing 19-22 g) and 23 months of age (n=28, 28-34 g), were obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea) and housed in regular polycarbonate plastic cages in an environment with a controlled temperature (21-22˚C) and humidity (50-60%) and a 12-h light/dark cycle (lights on at 7 a.m.). The mice were maintained on an *ad libitum* diet of lab chow (Teklad 2018S, Harlan, WI, USA) with free access to water. The cages were filled to an approximate depth of 1.5 cm with bedding made of chopped wood particles (JSBio, Daejeon, Korea). All materials used were autoclaved and gamma-irradiated. The animal room was maintained in specific-pathogen-free conditions. The C57BL/6J mice at 23 months of age were randomized into the vehicle [0.5% carboxymethyl cellulose (CMC), Aged vehicle, n=14)] and PL (Aged PL, n=14) groups. The PL extract was suspended in 0.5% CMC at a concentration of 5 mg/ml as a stock solution. The 23-month-old female mice were orally administrated 10 μl/g/day of PL stock solution or 0.5% CMC for 8 weeks. The 3-month-old female mice were used as young controls (n=7). Multiple behaviour tests were performed on a single cohort of mice and the following order was obeyed: Open field test → novel object recognition test → nest-building behaviour test (17,20). All the animal experiments were approved by the Institutional Animal Use and Care Committee of the KRIBB (KRIBB-AEC-14074).

Open filed locomotor activity. The mice were individually placed in an open field box (45x45x45 cm^3) for 30 min. The horizontal locomotion of the mouse was measured using a computerized video tracking system, SMART (Panlab, Barcelona, Spain).

Novel object recognition test. The novel object recognition test was performed as described in previous studies (21,22). The mice were individually habituated to a testing chamber (40x20x20 cm^3) with no objects for 5 min and then placed in a testing chamber for 10 min with two identical objects (familiar, acquisition session). The mice were then returned to the home cages. One day later, the mice were placed back into the testing chamber in the presence of one of the original objects and one novel object (novel, recognition session) for 10 min. The original objects were cylindrical wooden blocks 10 cm high x 2 cm in diameter. The novel object was a 10x2.5x2 cm rectangular wooden block. The acquisition and recognition sessions were video-recorded and an observer, who was blinded to the drug treatment, scored the time spent exploring the objects. The chambers and objects were cleaned with ethanol between trials. Exploration was defined as sniffing and touching the object with the nose and/or forepaws. Sitting on the object was not considered exploratory behaviour. A discrimination index was calculated for each animal and expressed using the following formula: [time (number) of contacts with the novel object-time (number) of contacts with the familiar object]/[time (number) of contacts with the novel object + time (number) of contacts with the familiar object] on day 2.

Nest-building behaviour test. The nest building behaviour test was performed as described in a previous study (23). The mice were housed in single cages containing chopped wood particles for 5 days. On the first day of testing, one piece of cotton (5x5 cm; Nestlets, Ancare, Bellmore, NY, USA) was introduced into the home cage to permit nesting. The presence and quality of nesting was rated 1 day later on a 5-point scale ranging from 1 to 5 as follows: 1, nestlet not noticeably

touched (>90% intact); 2, nestlet partially torn up (50-90% remaining intact); 3, mostly shredded, but often no identifiable nest site; 4, an identifiable but flat nest; and 5, a (near) perfect nest. Immediately afterward, the mice were group-housed as before.

Western blot analysis. Western blot analysis was performed as described in a previous study (21). Following 8 weeks of PL treatment, the mice were sacrificed and the hippocampal tissues were rapidly removed and homogenized in a homogenization buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate and 0.1% sodium deoxycholate) containing a cocktail of protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). Protein samples were resolved by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The samples were then transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The blots were incubated with primary antibodies followed by secondary antibodies, and specific signals were visualized using an Enhanced Chemi Luminescence kit (Intron Biotechnology, Gyeonggi-do, Korea). Western blot images were quantified using Quantity One 1-D analysis software version 4.6.1 (Bio-Rad Laboratories, Inc.). The primary antibodies used were vesicular glutamate transporter 1 (VGLUT1; 1:1,000, #135 302, SYSY, Göttingen, Germany), vesicular glutamate transporter 2 (VGLUT2; 1:1,000, #75-067 UC Davis/NIH NeuroMab Facility, Davis, CA, USA), glutamate receptor 1 (GluR1; a gift from Dr J.R. Lee, KRIBB, Daejeon, Korea, 1:1,000), *N*-methyl-D-aspartate receptor subtype 2B [(NR2B, 1:1,000, #4212, Cell Signaling Technology (CST), Danvers, MA, USA)], phosphorylated (p-)NR2B (p-Tyr-1472-NR2B, 1:1,000, #4208, CST), synaptophysin (1:1,000, #S5768, Sigma-Aldrich Co. LLC; Merck KGaA, Darmstadt, Germany), post-synaptic density protein 95 (PSD-95, 1:1,000, #124 014, SYSY), glutamate decarboxylase 65/67 (GAD65/67, 1:1,000, #AB1511, Merck KGaA), gephyrin (1:1,000, #147 011, SYSY), vesicular GABA transporter (VGAT, 1:1,000, #131 002, SYSY), cAMP response element binding protein (CREB, 1:1,000, #06-863, Merck KGaA), p-CREB (p-Ser133-CREB, 1:1,000, #06-519, Merck KGaA), calcium/calmodulin-dependent protein kinase type II α (CaMKIIα, 1:1,000, #sc-13141, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), p-CaMKIIα (p-Thr-286-CaMKIIα, 1:1,000, #sc-12886, Santa Cruz Biotechnology, Inc.), extracellular signal-regulated kinases 1/2 (ERK1/2, 1:1,000, #9102, CST), p-ERK1/2 (p-Thr202/Tyr204-ERK1/2, 1:1,000, #9101, CST) and β-actin (1:1,000, #MAB1501, Merck KGaA). The secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,000, #NCI1460KR, Thermo Fisher Scientific, Inc., Waltham, MA, USA) or goat anti-mouse (1:2,000, #sc-2005, Santa Cruz Biotechnology).

Histological analysis. Immunohistochemistry and immunofluorescence staining were performed as previously described (21,24-26). Following 8 weeks of PL treatment, the mice were deeply anesthetized (250 mg/kg Avertin, intraperitoneally) and transcardially perfused with saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were removed, post-fixed overnight, and then cut into 40-μm-thick coronal sections using a vibratome (Vibratome VT1000A, Leica Microsystems GmbH, Wetzlar, Germany). The free-floating sections were then incubated in PBS containing 3% H_2O_2 (v/v), rinsed 3 times in PBS, and blocked with serum for 1 h at room temperature. The sections were then incubated with the phospho-CREB (Ser133, 1:1,000, #06-519, Merck KGaA), doublecortin (DCX, 1:1,000, #sc-8666, Santa Cruz Biotechnology), 4-hydroxy-2-nonenal (4-HNE, 1:1,000, #HNE11-S, Alpha Diagnostic, San Antonio, TX, USA), ionized calcium-binding adapter molecule 1 (Iba1, 1:1,000, #019-19741, Wako Chemicals USA, Inc., Richmond, VA, USA) and glial fibrillary acidic protein (GFAP, 1:1,000, #Z-0334, Dako, Glostrup, Denmark) primary antibodies overnight at 4˚C. The sections were then washed and incubated with biotinylated secondary anti-rabbit IgG (1:200, #BA-1000, Vector Laboratories, Inc., Burlingame, CA, USA), followed by the avidin-biotinylated peroxidase complex (Vector Laboratories, Inc.) and 3,3'-diaminobenzidine (Sigma-Aldrich Co. LLC; Merck KGaA). Immunofluorescence staining was then performed with an Alexa Fluor 594 goat anti-rabbit IgG antibody (secondary antibody, 1:200, #A11012, Thermo Fisher Scientific, Inc.). Sections containing the hippocampus were selected and the number of doublecortin-positive cells in the dentate gyrus (DG) were counted under a microscope (Olympus Corp., Tokyo, Japan). The intensity of 4-HNE- and p-CREB-stained cells and the percentage area occupied by GFAP- and Iba-1-positive cells in hippocampal CA1, CA3 and DG were assessed using the MetaMorph image analyser (Molecular Devices, LLC, Sunnyvale, CA, USA).

Statistical analysis. GraphPad PRISM (GraphPad Software, Inc., La Jolla, CA, USA) software was used to perform the statistical analyses. Two-sample comparisons were performed using a Student's t-test, while multiple comparisons were made using a one-way ANOVA followed by the Tukey-Kramer's post hoc test. Associations between distance and discrimination index were examined by Pearson's correlation coefficient. All data are presented as the means ± SEM and statistical differences are accepted at the 5% level ($P<0.05$), unless otherwise indicated.

Results

PL improves the performance of aged mice in novel object recognition and nest building tasks. The aged female C57BL/6J mice (23 months old) were randomly separated into the vehicle- and PL-treated groups. PL was administered at a dose of 50 mg/kg/day for 8 weeks, from the ages of 23 to 25 months. The experimental design is presented in Fig. 1A. The aged mice (24 months of age) exhibited a significantly lower locomotor activity in the open field test than the young control mice (Fig. 1B, $P<0.05$). PL treatment did not markedly affect the exploratory behaviour of the aged mice compared to the aged vehicle group (Fig. 1B, $P>0.05$). To determine whether PL can improve cognitive function in aged mice, we performed the novel object recognition test. In the recognition session, with two different objects (one novel and the other familiar), the young control mice explored the novel object for a relatively long time period and a made contact with it a relatively high number of times, yielding a discrimination index

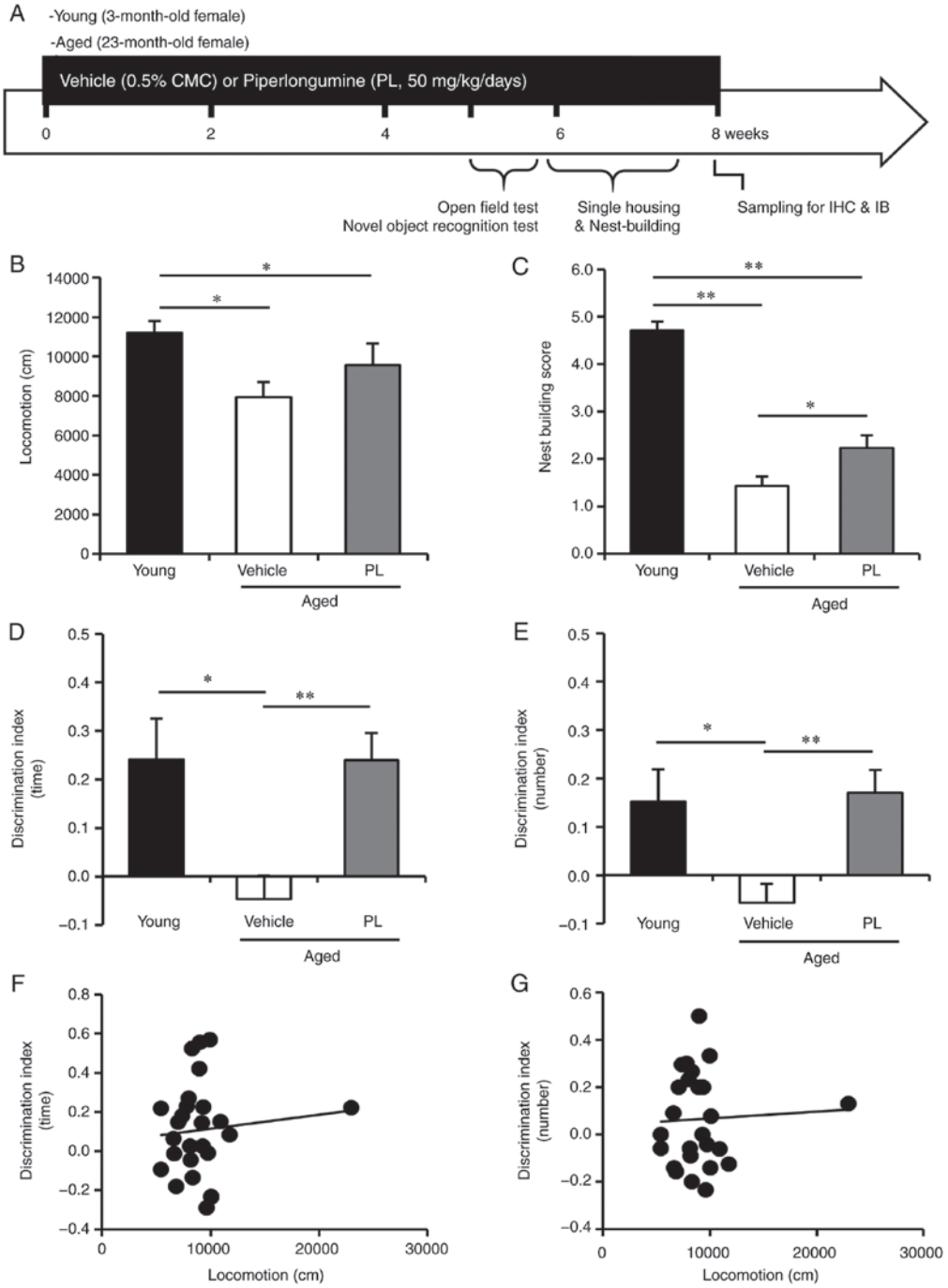

Figure 1. Effect of piperlongumine (PL) on novel object recognition and nest building in aged female mice. (A) Experimental design for PL treatment, behaviour testing and sampling. Open field test, novel object recognition test and nest building behaviour test were performed at 39, 42 and 54 days of PL treatment. (B) Total locomotor activity for a 30-min period in young control mice and aged mice following treatment with the vehicle or PL (young control; n=7, aged vehicle; n=14, aged PL; n=14). (C) The presence and quality of nesting over a 24 h period, rated on a 5-point scale, in young control mice and aged mice following treatment with the vehicle, or PL (young control; n=7, aged vehicle; n=8, aged PL; n=9). (D and E) The discrimination index [(D) the time spent exploring and (E) the number of contacts] of the young mice, and aged mice following treatment with the vehicle or PL in the novel object recognition test (young control; n=6, aged vehicle; n=14, aged PL; n=14). (F and G) The correlation between locomotor activity in open field test and discrimination index in novel object recognition test in aged mice was absent [F, between locomotion (B) and DI (time, D), r=0.09955, P=0.6285; G, between locomotion (B) and DI (number, E), r=0.04872, P=0.8132, n=26]. *P<0.05 and **P<0.01, significant differences from an indicated group, determined by one-way ANOVA, followed by Tukey-Kramer's post-hoc test.

(DI) of approximately 0.24±0.08 and 0.15±0.07, indicating that they had a memory of the familiar object (Fig. 1D and E). By contrast, the aged mice treated with the vehicle exhibited a DI that was significantly lower than that of the younger controls (-0.05±0.05 and -0.06±0.04, Fig. 1D and E), which is consistent with impaired cognition. PL treatment markedly increased the DI in aged mice to approximately 0.24±0.06 and 0.17±0.05 (Fig. 1D and E), reflecting a therapeutic effect of PL on age-related cognitive impairment. PL treatment did not alter the total exploration time (aged vehicle, 10.14±1.11 sec; aged PL, 8.97±0.48 sec, P=0.466) and total number of contacts (aged vehicle, 16.92±2.22; aged PL, 19.71±1.25, P=0.680) to

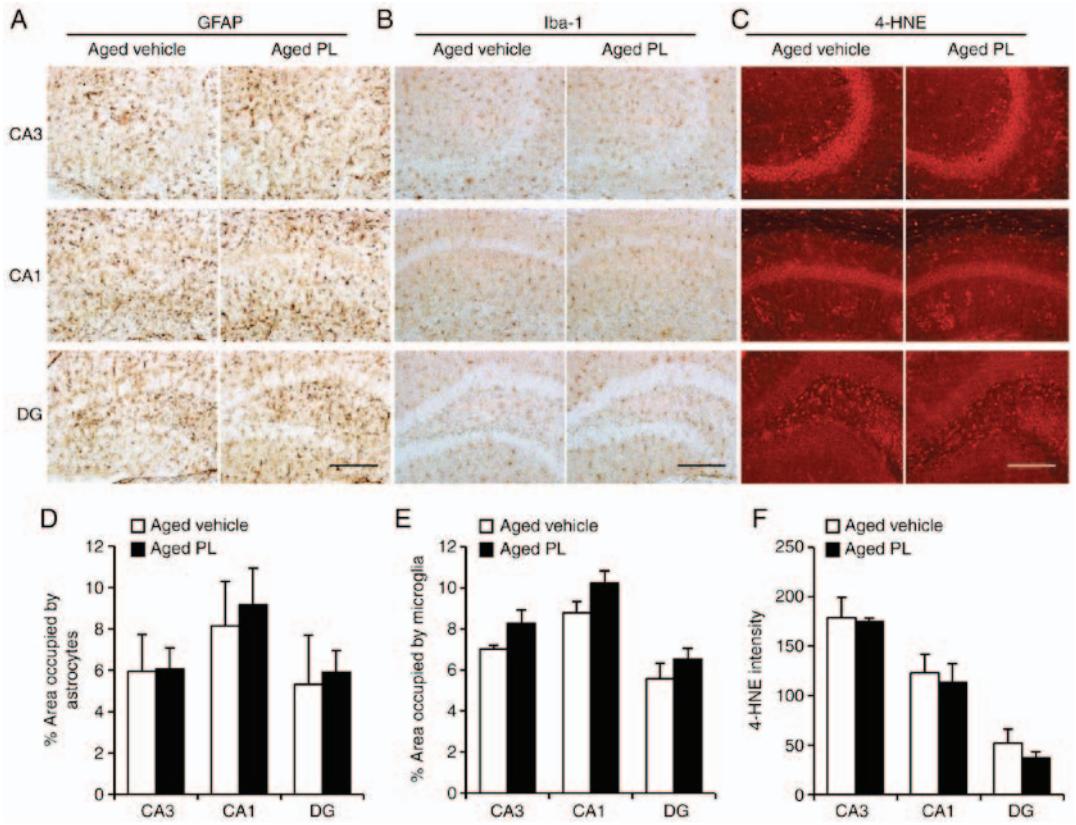

Figure 2. Effect of piperlongumine (PL) on neuroinflammation and oxidative stress in the hippocampus. Activation of microglia and astrocytes was analysed by immunohistochemical staining against glial fibrillary acidic protein (GFAP), ionized calcium binding adaptor molecule 1 (Iba1) and 4-hydroxynonenal (4-HNE), respectively. Images showing (A) GFAP, (B) Iba1, and (C) 4-HNE labelling in the hippocampus of aged mice treated with the vehicle or PL. (D) Percentage area of hippocampus [CA3, CA1 and dentate gyrus (DG)] occupied by astrocytes (thus GFAP labelled; aged vehicle; n=6, aged PL; n=9) in aged mice treated with the vehicle or PL. (E) Percentage area of hippocampus (CA3, CA1, and DG) occupied by microglia (thus Iba-1 labelled; aged vehicle; n=6, aged PL; n=9) in aged mice treated with the vehicle or PL. (F) 4-HNE-intensity in the hippocampus (CA3, CA1, DG) in aged mice treated with the vehicle or PL aged vehicle; n=4, aged PL; n=4). Scale bar, 200 μm. Data are presented as the means ± SEM.

both objects (familiar + novel) on day 2, indicating no influence on the total exploration activity of PL in the novel object recognition test. Additionally, we could not find any association between the distance in the open field test and the DI in the novel object recognition test in the aged mice (Fig. 1F and G, P>0.05).

Previous studies have reported that nest building, which is an indicator of well-being and social context in mice, is decreased in aging in rodent models of AD (27,28). Reduced nesting has also been observed in mice with hippocampal lesions (29). In this study, the nesting score in the nest building test was significantly lower in the aged mice than in the young control mice (Fig. 1C, P<0.05). PL significantly increased the nesting score in the aged mice (Fig. 1C, P<0.05). These results indicate that treatment with PL may improve cognitive and social decline without affecting locomotion in aged mice.

PL did not alter the glia activation and lipid peroxidation in the hippocampus of aged mice. An upregulation of inflammatory responses and oxidative stress have been observed in the hippocampus in aging (30-33). An increase in inflammation in aging implicates the activation of microglia and astrocytes in the brain over this period (34). In aged brains, there is an increase in the number, size and activation of microglia (34). In this study, to investigate the effects of PL on microglia and astrocytes in aging, we measured the percentage area occupied by astrocytes (Fig. 2A and D) and microglia (Fig. 2B and E) in hippocampus through immunohistochemical assay. Additionally, immunofluorescence analysis for oxidative stress (4-HNE, an indicator of lipid peroxidation) in the hippocampus was performed (Fig. 2C and F). PL administration at a dose of 50 mg/kg/day for 8 weeks had no significant effect on glial activation and oxidative stress in the hippocampus at this point in aging.

PL increases the phosphorylation of NR2B, ERK1/2 and CaMKIIα in the hippocampus of aged mice. As the results from the behavioural tests pointed to a reduction in age-related cognitive impairment with PL treatment, we examined the level of synaptic markers in the hippocampus of the aged mice treated with the vehicle or PL. As indicated by the results of western blot analysis, the expression levels of gephyrin, VGAT, GAD65/67, PSD95, VGLUT1, VGLUT2 and synaptophysin were similar between the aged vehicle and aged PL groups (Fig. 3A and B). Additionally, PL had no effect on the protein expression of the AMPA (GluR1) or NMDA (NR2B) receptors (Fig. 3C and D). Of note, the levels of phosphorylation of NR2B (Tyr1472), ERK1/2 (Thr202/Tyr204) and (Thr286) were significantly higher in the aged mice treated with PL than in the aged mice treated with the vehicle (Fig. 3C and D). There was a tendency for the phosphorylation of CREB (Ser133) to be slightly higher in the aged PL group than the aged vehicle

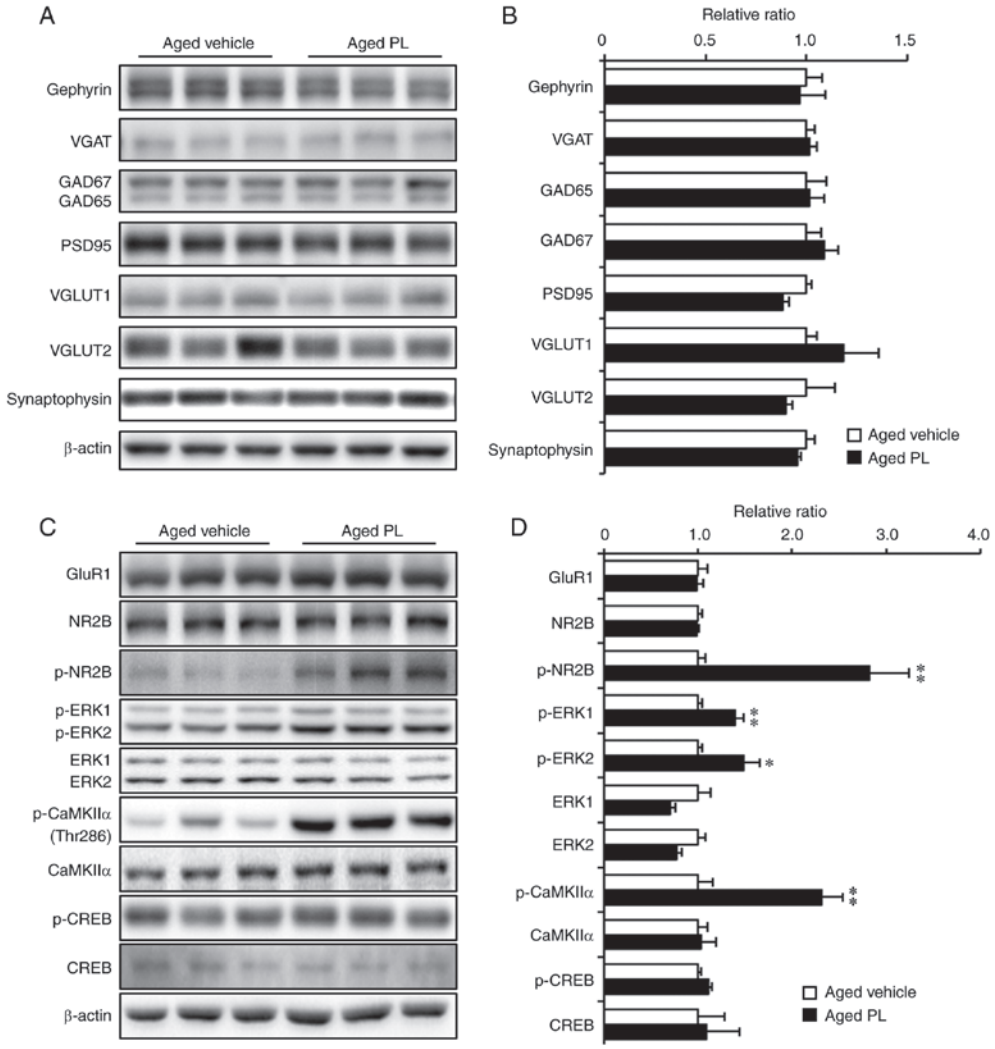

Figure 3. Effect of piperlongumine (PL) on the expression of synaptic proteins and NMDAR signalling proteins. (A and B) Western blot analysis and quantitative analysis of the expression of synaptic proteins [gephyrin, vesicular GABA transporter (VGAT), glutamate decarboxylase 65/67 (GAD65/67), postsynaptic density protein 95 (PSD95), vesicular glutamate transporter 1 (VGLUT1), vesicular glutamate transporter 2 (VGLUT2) and synaptophysin, aged vehicle; n=5, aged PL; n=7] in hippocampal homogenates of aged mice treated with the vehicle or PL. (C and D) Western blot analysis and quantitative analysis of the expression of NMDAR signalling proteins [glutamate receptor 1 (GluR1), *N*-methyl-D-aspartate receptor subtype 2B (NR2B), p-NR2B, extracellular signal-regulated kinase (ERK)1/2, p-ERK1/2, calcium/calmodulin-dependent protein kinase type II α (CaMKIIα), p-CaMKIIα, cAMP response element binding protein (CREB) and p-CREB, aged vehicle; n=5, aged PL; n=7] in hippocampal homogenates of aged mice treated with the vehicle or PL. *P<0.05 and **P<0.01, significant differences from the aged vehicle, as shown by the Student's t-test. Data are presented as the means ± SEM.

group, although this difference was not significant. To further investigate the level of p-CREB in the areas of the hippocampus, we measured the integrated optical density (IOD) of p-CREB by immunohistochemical assay in the CA1, CA3, and DG of the aged vehicle- and aged PL-treated mice (Fig. 4). The IOD in the CA3 was markedly higher in the aged mice treated with PL than in the aged mice treated with the vehicle (Fig. 4A and C, P<0.01); however, the level of p-CREB in the CA1 and DG did not differ significantly between the groups (Fig. 4A, B and D). Taken together, these results suggest that the molecular signalling pathways involving NR2B, CaMKIIα, ERK1/2 and CREB are regulated by PL treatment in the hippocampus of the aged mice.

PL increases neurogenesis in the DG of aged mice. Neurogenesis markedly declines with aging and, thus, the maintenance of an adequate level of hippocampal neurogenesis is another important factor to consider in maintaining cognitive function (35). In this study, to investigate whether PL treatment affects hippocampal neurogenesis, we examined neuronal proliferation by immunohistochemistry using the neuroblast marker, DCX, in the DG of aged mice treated with the vehicle or PL (Fig. 5). The number of DCX-positive cells was markedly lower in the aged mice treated with the vehicle than in the young control group (Fig. 5). However, the number of DCX-positive cells was significantly higher in the PL treated aged mice than in the vehicle treated aged mice (Fig. 5, P<0.05). These results suggest that PL increases adult neurogenesis in the DG of aged mice.

Discussion

Aging is a natural biological process that is associated with physical and cognitive decline. Notably, in both normal aging and under pathological conditions, cognitive decline can diminish the quality of life. In the present study, we found

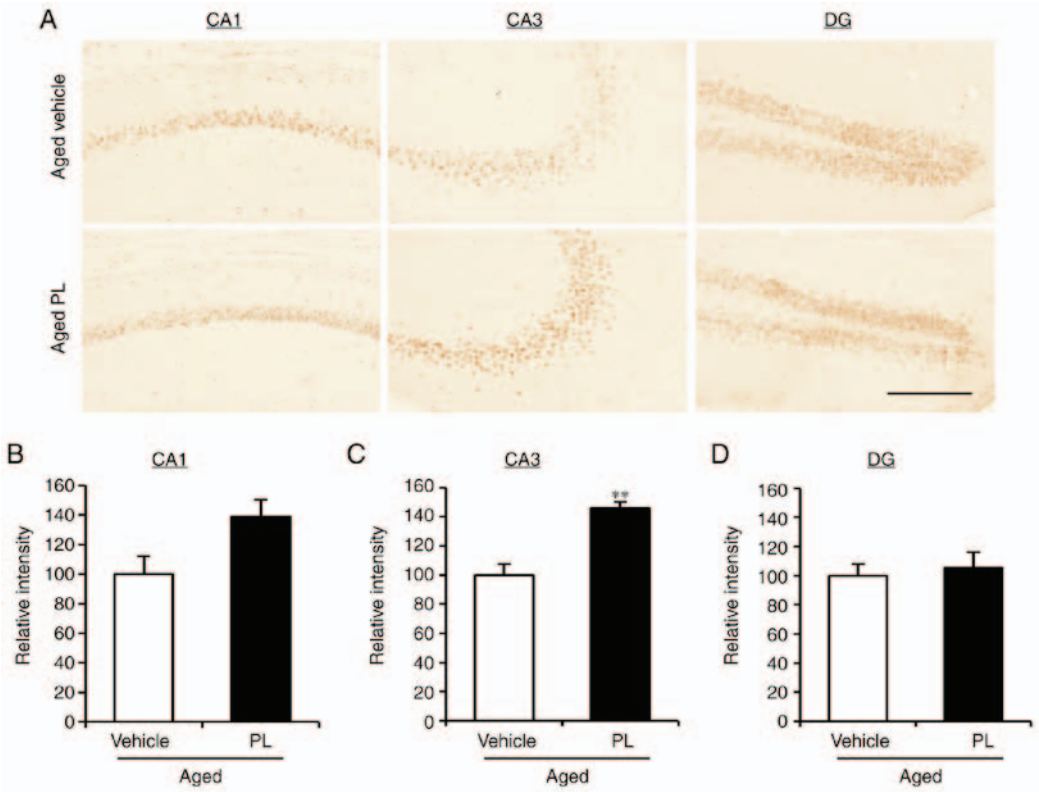

Figure 4. Effect of piperlongumine (PL) on the phosphorylation of CREB in the hippocampus of aged mice. (A) Images showing anti-p-CREB antibody-stained CA1, CA3 and dentate gyrus (DG) of the hippocampus in aged mice treated with the vehicle or PL. Results of the quantitative analysis of the relative intensity of p-CREB in the (B) CA1, (C) CA3, and (D) DG of aged mice treated with the vehicle or PL (aged vehicle; n=5, aged PL; n=4). Scale bar, 200 μm. **P<0.01, significant differences from the aged vehicle, as shown by the Student's t-test. Data are presented as the means ± SEM.

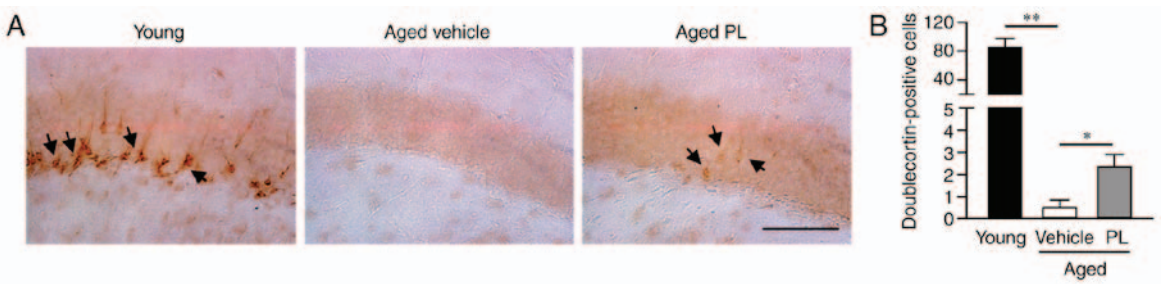

Figure 5. Effect of piperlongumine (PL) on hippocampal neurogenesis. (A) Representative photomicrographs of the DG of the hippocampus of young control, aged vehicle and aged PL groups. Arrows indicate doublecortin (DCX)-positive cells. (B) The number of DCX-positive cells in the DG area of aged mice treated with the vehicle or PL and young mice (young control; n=5, aged vehicle; n=6, aged PL; n=9). Scale bar, 100 μm. *P<0.05 and **P<0.01, significant differences from an indicated group, determined by one-way ANOVA, followed by Tukey-Kramer's post-hoc test. Data are presented as the means ± SEM.

that treatment with piperlongumine (PL), isolated from the long pepper, significantly improved cognitive function in novel object recognition and performance in nest building in 25-month-old female mice. These effects appear to be partly due to the modulation of neuronal activity and neurogenesis in the hippocampus. We found that treatment with PL increased the phosphorylation levels of the NR2B subunit of the NMDA receptor in the hippocampus of aged mice. Furthermore, we observed that PL significantly increased the phosphorylation of ERK1/2 at Thr202/Tyr204, CaMKIIα at Thr286, and CREB at Ser133, and increased the number of doublecortin-positive cells.

PL is a primary constituent of *Piper longum*, which has been reported to kill multiple types of cancer cells through the targeting of the stress response to reactive oxygen species (ROS) (14,36). Diagnosis with certain tumours, such as age-related degenerative diseases, increases with age and the molecular alterations that occur in aging can favour carcinogenesis (37). Senescent cells can drive hyperplastic pathology and promote age-related neurodegeneration (38,39). Recently, PL has been reported to be a potential novel lead for the development of senolytic agents (40) and the selective depletion of senescence cells as an anti-aging strategy may prevent cancer and aging-related degenerative diseases. Although in this study, we did not investigate the anti-tumour activities of PL in aged mice, PL treatment may be beneficial through the apoptosis of age-related senescence cells. Cellular senescence is associated with oxidative stress and inflammation (39). An increase in the expression of GFAP has been the most common change to be

observed in astrocytes with aging (41). The results of this study demonstrated that PL did not affect the size of area occupied by glia, such as microglia and astrocytes, in the hippocampus of the aged mice (Fig. 2). We also observed that lipid peroxidation in the hippocampus was not altered in the aged mice (Fig. 2). However, previously, we have demonstrated that PL effectively decreases astrogliosis and microglia activation in the parietal cortex in animal models of AD (17). The results indicated that the inflammation and microglia activation that was triggered by pathological conditions were effectively suppressed by PL treatment.

The precise mechanism of action through which PL improves cognitive function remains unclear. The results of this study demonstrated that PL modulates the NR2B subunit of the NMDA receptor and CaMKII in the hippocampus (Fig. 3). The phosphorylation of NR2B at Tyr-1472 in hippocampus was increased by treatment with PL (Fig. 3C and D). The level of Tyr-1472 phosphorylation is increased after the induction of long-term potentiation (LTP) in the hippocampus, indicating that the phosphorylation of Tyr-1472 is involved in synaptic plasticity (42). Additionally, CaMKII is the main protein of post-synaptic density and is an essential protein for the induction of NMDAR-dependent LTP (43). CaMKIIα promotes synaptic formation, strengthening, and integration into existing neural circuits (44). Autophosphorylation at Thr286 of CaMKIIα is also required for NMDAR-dependent LTP and hippocampus-dependent learning (45). However, CaMKIIα activation is impaired in an age-dependent manner in the hippocampus and amygdala (46). The loss of CaMKIIα activity results in severe electrophysiological abnormalities that are associated with impaired synaptic plasticity and memory formation, while the overexpression of CaMKIIα improves cognitive performance, as assessed by Morris water maze testing (45,47). NR2B-containing NMDARs is coupled to ERK activation (48). The present study demonstrates that the oral administration of PL also significantly increased ERK1/2 and CREB phosphorylation in the hippocampus (Figs. 3 and 4). One of the key signalling proteins activated downstream of CaMKII and ERK is CREB (49,50). It has been well-documented that CREB plays a role in LTP and memory formation (51). A reduction and deficit in CREB signalling has been observed in aged animals (52). The phosphorylation of Ser133 seems to be a critical step in CREB activation (51,53). Total CREB levels do not appear to change; however, the level of p-CREB is decreased in aged rats (53,54). Additionally, the level of p-CREB expression has been found to be associated with performance in emotional memory tests, where a higher level of p-CREB is indicative of a better emotional memory performance (56,57). In the current study, PL significantly increased the phosphorylation of CREB in the CA3 region of the hippocampus (Fig. 4). Therefore, considering the functional role of these molecules in the regulation of cognitive function, the modulation of CaMKII/ERK/CREB signalling transduction could account for the therapeutic effect of PL.

The age-related decline in adult neurogenesis is a well-documented process (58). In mice, aging is associated with a decreased number of neural stem cells in the hippocampus (59). New-born neurons in aged mice are highly associated with neurogenesis-dependent cognition (60). Moreover, hippocampal neurogenesis in response to exercise and enriched environment contributes to hippocampal plasticity (58,61). Previously, we reported that PL markedly increases sirtuin 1 deacetylase activity in *in vitro* assays (17). Sirtuin 1 is one of seven mammalian sirtuins and has been shown to modulate aging and memory (62,63). Although the regulation of neurogenesis by sirtuin 1 has not been investigated in this study, it has been reported that the activation of sirtuin 1 restores cognitive performance and neurogenesis in mice exhibiting reduced adult neurogenesis and lowered hippocampal cognitive abilities (64). In the present study, there were few DCX-positive neuroblasts in the DG of 25-month-old female mice (Fig. 5). Moreover, the aged mice treated with PL exhibited significantly higher number of DCX-positive cells in the DG than in the aged mice treated with the vehicle (Fig. 5). These results suggest that PL may have an effect on neurogenesis by preventing or reversing age-related decline. However, the precise mechanisms responsible for the effect of PL on neurogenesis in aged mice are not yet clear. Further studies, therefore, are warranted to investigate the effects of PL on neurogenesis, including in *in vitro* models. Additionally, studies on target mediators of signalling pathways involved in the formation of new neurons can be utilized to determine the effect of PL on neurogenesis in the adult brain.

In conclusion, our *in vivo* analysis of aged female mice demonstrates that PL improves some properties of aging, such as age-associated cognitive impairments, synaptic dysfunction and the decline in neurogenesis. Although additional studies are required to elucidate the underlying molecular mechanisms and validate the anti-aging effects of PL in male mice, the results of the present study suggest that the activation of NR2B, CaMKIIα, ERK1/2 and CREB, and the increase in neurogenesis following PL treatment may contribute to hippocampal neuronal activity in the aged brain.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JG, TSP, HYP, GHH, and YKR carried out the experiment and analysed the data. CHL, SK, WKO, and KSK conceived

and planned the experiments. JG, CHL, and KSK wrote the manuscript. YHK, JHH, DHC, DYH and JRN contributed to sample preparation and analysed the data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Use and Care Committee of the KRIBB (KRIBB-AEC-14074).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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