SPANDIDOS PUBLICATIONS

Supplementation of aged garlic extract attenuates age-associated memory impairment and cognitive decline: Involvement of molecular pathways in the cortex and hippocampus

TAMANNA MONY[1*], MARCUS JACKSON[1*], AMITAI ZUCKERMAN[1], WENYAN YU[1], THAO THI NGUYEN[2], ASHLEY BALDERRAMA[1], RUNTING LI[1], GRACE Y. SUN[1,3], JIANKUN CUI[1] and ZEZONG GU[1]

[1]University of Missouri School of Medicine, Department of Pathology and Anatomical Sciences, Columbia, MO 65212, USA; [2]Charles W. Gehrke Proteomics Center, University of Missouri, Columbia, MO 65211, USA; [3]University of Missouri, Biochemistry Department, Columbia, MO 65212, USA

Received April 10, 2025; Accepted September 25, 2025

DOI: 10.3892/br.2025.2075

Abstract. The aging of the human population is associated with increases in comorbidities, including functional impairment and cognitive decline. With decreasing numbers of neurons and declining synaptic plasticity, the aging brain exhibits less perfusion, and thus, is more susceptible to attack by reactive oxygen species (ROS). These changes are associated with increased risks of neurodegenerative diseases, which impact the quality of life. Consequently, there is an urgent need to develop nutraceuticals and holistic approaches that may impede the aging process. Aged garlic extract (AGE) is a nutraceutical that has been shown to display neuroprotective properties; in particular, it has the ability to reduce inflammation, apoptosis and ROS. Given these insights, it is reasonable to hypothesize that AGE improves neurological function and cognition by altering neural molecular processes. To test this hypothesis, a battery of behavior tests, including measurements of various cognitive functions, were performed on 82-week-old mice, which were fed a diet supplemented with AGE for 40 weeks. This was followed by label-free global proteomics and machine learning-driven bioinformatics analyses of mouse cortical and hippocampal proteomes for the identification of membrane and cytoskeletal protein targets of the mechanism of action of AGE. Mice in the AGE supplementation group exhibited improvements in learning, memory, exploratory behavior and anxiety ($P<0.05$), but no obvious changes in other functional domains. Analysis of global proteomes revealed the ability of AGE supplementation to induce expression changes, which were most pronounced in the hippocampus. Further comparative bioinformatics analysis also revealed that AGE supplementation increased synaptogenesis signaling in both brain regions and reduced 14-3-3 signaling related to neuronal apoptosis exclusively in the hippocampus. The predicted upstream global proteome modulators influenced by AGE supplementation included microtubule-associated protein tau, amyloid precursor protein, brain-derived neurotrophic factor, TP53, presenilin 1 and superoxide dismutase 1. Taken together, these findings demonstrated the neuroprotective effects of AGE on cognition and protein expression in aging mice, further suggesting its potential use as a nutraceutical to prevent aging-associated neurological comorbidities.

Correspondence to: Dr Zezong Gu, University of Missouri School of Medicine, Department of Pathology and Anatomical Sciences, 1 Hospital Drive, Columbia, MO 65212, USA
E-mail: guze@health.missouri.edu

*Contributed equally

Key words: aged garlic extract, dietary supplement, aging, cognition, memory, anxiety, global proteomics

Introduction

The population of US citizens aged 65 years and older is projected to increase from 58 million in 2022 to 82 million by 2050 (1). The increase in aging-related comorbidities is closely linked to neurological changes characterized by a reduction in the volume of the frontal lobe and hippocampus (2,3), leading to progressive loss of myelin integrity (4), calcium dysregulation (5), mitochondrial dysfunction and accumulation of reactive oxygen species (6). These changes likely contribute to cognitive decline, sensorimotor impairments and increased vulnerability to neurodegenerative disorders (7-9). Alzheimer's disease (AD) and Parkinson's disease are among the most prevalent aging-related neurodegenerative diseases that lack effective preventative treatments (10). Given the multifaceted nature of these diseases, holistic approaches that target multiple pathways hold promise in addressing aging-related cognitive decline.

Garlic has been used as a seasoning or condiment in cooking and folk medicine for thousands of years across various cultures (11). Sulfur-containing compounds, such as allicin and S-allyl cysteine (SAC), are known to contribute to its pungent aroma. Besides its use in cooking, garlic has been shown to exhibit antioxidant properties, and thus, is considered to offer various health benefits. Among various

garlic supplements, aged garlic extract (AGE) has been noted for being an odorless and stable product resulting from prolonged extraction of fresh garlic at room temperature, and has essential bioavailable properties (12-14). Previous research has further indicated that AGE and its active components, such as SAC and N-α-(1-deoxy-D-fructos-1-yl)-L-arginine (FruArg), could offer various systemic health benefits (15). More specifically, studies have highlighted the role of AGE in mitigating aging-related diseases and cardiovascular health risks by lowering cholesterol levels, reducing calcification in coronary arteries, reducing acute phase reactants, and exerting striking protection against systolic and diastolic hypertension (16-20). Animal studies have also revealed that AGE and its active components, such as diallyl trisulfide and SAC, could offer cardioprotective effects, including reducing myocardial damage and supporting glyco- and lipo-metabolism (21,22). Human trials have corroborated these findings, indicating that AGE can improve arterial elasticity, reduce inflammation, and stabilize the ventricular mass in patients with diabetes and cerebrovascular disease (CVD) (23-26). Several single-center randomized clinical trials have also revealed that AGE could attenuate the progression of subclinical atherosclerosis, regenerate peripheral tissue perfusion measured post occlusive reactive hyperemia by laser speckle contrast imaging and increase microcirculation in patients with arteriolosclerosis (27,28). There is strong evidence indicating the ability of AGE to slow the progression of atherosclerosis, thus serving as a nutraceutical in the primary prevention and mitigation of CVD and stroke. Previous studies have suggested that AGE and its bioactive components could enhance resiliency against inflammation whilst mitigating learning and memory deficits by modulating neuronal glucose transport and oxidative stress (15,29,30). These multifaceted health benefits underscore the potential of AGE to serve as a valuable nutraceutical to enhance neuroprotection and improve systemic resilience for brain health.

Previous studies on short-term AGE supplementation have demonstrated improvements in spatial learning and memory in young rats with amyloid-β pathology (9 weeks-old) (30,31). Another study has further demonstrated the ability of the AGE supplement to improve learning and memory deficits in mice with accelerated brain atrophy (12 months old) (32). The proposed molecular mechanisms of short-term AGE supplementation include antiglycation and antioxidation (14), activation of antioxidant enzymes (31), and decreasing oxidative stress and inflammation by modulating the activities of NF-κB and activator protein-1 whilst inhibiting matrix metalloproteinases (33). There is also evidence showing the ability of AGE to inhibit lipid peroxidation, reduce ischemic/reperfusion damage and inhibit oxidative modification of low-density lipoprotein (34). Molecular studies using murine BV-2 microglial cells have further demonstrated the ability of AGE to reduce nitric oxide production, and alter the expression levels of 20 proteins enriched in the cytosol, mitochondria, plasma membrane and cytoskeleton (15,35,36).

Based on the results of previous studies using cultured cells and short-term animal models, it was deemed valuable to investigate the chronic effects of AGE supplementation on different behavioral paradigms, including the assessment of cognitive decline, sensorimotor deficits, memory impairments and anxiety-like behaviors. In the present study, 43-week-old mice were fed a diet supplemented with AGE for 40 weeks, and the chronic effect on their neurological behavior, as well as brain proteomes and molecular pathways, was examined. The results of the present study revealed the long-term benefits of AGE supplementation during the natural aging process.

Materials and methods

Animal husbandry. A total of 48 male C57BL/6J mice (RRID: IMSR_JAX:000664) aged 42 weeks (±3 days) were obtained from The Jackson Laboratory. In the present study, 42-week-old mice were selected because this age is equivalent to middle-age in humans (37,38). On the day of arrival, the mice were randomly placed in a cage, with two mice per cage. Placing two mice per cage has been shown previously to provide an essential environment for social interaction and tracking food consumption (39,40). Mice were housed in an AAALAC accredited animal facility under standard conditions: The ambient temperature 68-79˚F, relative humidity 30-70%, 10-15 air changes per h, and a 12/12-h light/dark cycle.

After 1 week of habituation, the 43-week-old mice (body weight 33.9±0.6 g) were administered either the AGE supplement or the control diet for 40 weeks (~10 months). Control mice were fed the nutritionally complete diet AIN93G (cat. no. F3156-Rodent Diet; AIN-93G Bio-Serv®), and the AGE mice were fed the AIN diet supplemented with AGE (cat. no. F10217 Rodent Diet; AIN-93G-Modified; AGE-CSA-Missouri; Bio-Serv®). AGE (40%; w/w aqueous solution) was provided by Wakunaga Pharmaceutical Co., Ltd. The AGE diet was prepared using the AIN93G base diet from Bio-Serv. AGE solution (2 kg) in 26 kg of the base diet (25.2 kg base diet powder and 2 kg AGE solution) was used to prepare 26 kg of the AGE diet. Because 2 kg of AGE solution contains 0.8 kg dry weight and 1.2 kg of water, the diet was dried at 120˚F in order to bring the moisture down to 5% after pelleting for microbial stability. For the control diet, 8% water was added to the dry mix as a pelleting aid. Mice were given the specific diets along with *ad libitum* access to water. Food intake was determined every week and body weights were measured every 2 weeks. To avoid additional stress, body weight was measured after behavior tests.

After feeding the mice the respective diets for 40 weeks, mice were subjected to behavior assessments. Prior to behavior tests, mice were housed in a reverse 12/12-h light/dark cycle environment for 2 weeks. All animal protocols were approved by the University of Missouri Animal Care and Use Committee (ACUC) (approval no. 25120; Columbia, USA).

The present study strictly adhered to the guidelines set forth by the University of Missouri and the National Institutes of Health for conducting animal studies and followed an approved method of euthanasia (The American Veterinary Medical Association-approved method). Mice should be euthanized before they experience significant pain or distress. The general appearance of the mice was checked by monitoring signs such as dehydration, severe weight loss, persistent abnormal posture or hypothermia, behavioral changes (such as inability to reach food or water, or lethargy) and clinical signs (such as labored breathing). The mice were euthanized with ~5% isoflurane and it was confirmed that there was no response to a firm toe

pinch, and no signs of breathing and heartbeats, and the mice were continued to be exposed to ~5% isoflurane for ≥1 min to ensure euthanasia. Subsequently, the mice were perfused with saline through a cannula inserted into the left ventricle of the heart to wash out the blood. Removing blood from the brain results in cleaner tissue samples, which can enhance the accuracy and reliability of results. The mice were decapitated, and brain tissues were collected for future studies.

Assessment of multi-domain behavior functions. The aim of the battery of neurobehavioral tests was to assess the impact of dietary AGE supplementation on multiple behavioral domains associated with natural aging in mice (Fig. S1). All behavior tests were carried out in the dark phase (resting phase; with red lights in the testing room). Prior to the behavior tests, mice were placed in the testing room for ≥1 h for acclimation, and tests were conducted from 9:00 am to 3:30 pm. A battery of comprehensive behavior tests was established to evaluate different phenotypes under functional and cognitive behavioral domains, including sensorimotor function, exploratory behavior, anxiety/neophobia, memory and learning. To test sensorimotor functions, a simple neuro-assessment of asymmetric impairment (SNAP) test and an inverted screen test were performed. For anxiety/neophobia responses, the light-dark transition test, emergence test, open-field test, elevated-plus maze and zero maze were used. To assess memory and learning, the novel object recognition (NOR), three-chamber sociability and Barnes maze tests were performed.

SNAP. The SNAP test was used to measure common neurological function domains including sensorimotor responses. The eight test phenotypes consist of interaction, cage grasp, visual placing, pacing/circling, gait/posture, head tilt, visual field and baton, as described previously (41). The SNAP test provides a relatively sensitive, reliable and time-efficient means of assessing neurological deficits in mice. Each individual test has a scoring range of 0-5 based on the established guidelines for atypical neurologic behavior (42).

Inverted screen test. The inverted screen test was used to measure motor function, coordination and muscle strength under the functional behavior domain. It is a quick but insensitive gross screening test that provides a measure of muscular strength. This test was performed as previously described by Deacon with modifications (43). Untrained mice were individually placed on top of a square (7.5x7.5 cm) wire screen which is mounted horizontally on a metal rod. Within 2 sec, the wire screen was rotated to an inverted position (180˚), displacing the mouse to the bottom of the screens. The mouse was kept in this position for 1 min (checking with a stopwatch). This position causes the mouse to fall. Normally, the mouse can hold the screen within a period of time and climb up the incline. The following parameters were measured: i) latency to fall off the screen, ii) could not climb up the screen, and iii) able to climb up the screen within 1-min testing session.

Light-dark transition test. The light-dark box is a behavioral test that measures novelty-seeking and anxiety-like behaviors in mice under the cognitive domain. The test, performed according to the authors' previous studies (44,45), has the same area as the open-field test, except that half of the area is closed, and a small opening (7.5 cm high and 5 cm wide) could be found in the middle. Briefly, animals were placed in the light side of the box and were allowed to explore the box for 5 min. Their movements were tracked and analyzed using the ANY-maze software (RRID: SCR_014289; V7.16, Stoelting Co., Wood Dale, IL; Windows XP OS). A total of 3 measurements were calculated for each mouse: i) latency to first entry into the dark area, ii) number of entries to the dark area, and iii) time spent in the light area.

Open field test. As described previously, the open-field test was performed to measure locomotion, exploratory activity and anxiety-like behaviors (44,46,47). The open-field activity box is 40x40 cm and located on a white platform. Animals were placed into the center of the square arena for 10 min. After each trial, the box was cleaned with 10% ethanol solution and air-dried. The animals' movements were tracked and analyzed using the ANY-maze software. A series of 10x10-cm blocks were identified to track mouse activity, with 4 blocks defined as the center zone and the surrounding 12 blocks as the peripheral zone. Phenotypic parameters such as total distance traveled and time spent in different zones (that is, center vs. periphery) were recorded as indicators of anxiety.

NOR test. The NOR test in the open-field platform evaluates non-spatial learning and recognition memory under the cognition behavioral domain, as previously described (48), with a few modifications. First, the animal was allowed to become familiarized with an object for 5 min. After a 20~30 min interval, the object was placed in a different location in the same arena. Mice were allowed to explore the familiar object in the different location for 5 min. After 24 h, a new (novel) object was placed in the platform together with the familiar object. Normally, the color or shape of the novel object was changed, and the mouse was allowed to explore the object for 5 min. The entire test was recorded and measured using the ANY-maze software. In the aforementioned test, the following parameters were measured: i) Discrimination Index (DI) and ii) percentage of total investigating time. The DI was calculated by taking the difference between time investigating the novel object and the time investigating the familiar object, divided by the total time investigating both objects. The percentage of total investigating time was calculated by dividing the time investigating the novel object by the total time investigating both objects.

Emergence test. The emergence test is widely used to assess neophobia and exploratory behavior in rodents (49,50). The apparatus is an adaptation of the open-field test and intended to evaluate novelty-based anxiety. Naturally, rodents have an innate explorative drive as well as an aversion to brightly lit open spaces. The task involves observation of the time taken for the subject to exit the holding container (shelter) and explore the arena. Mice with high levels of anxiety tend to spend more time in shelter than in the open spaces.

Briefly, a large open field arena surrounded by high walls was used for this test. A cylindrical shaped tube (13.2x4.4x4.4 cm) that serves as the holding container or shelter, was used to restrain the mouse before placing in the arena. The shelter

was equipped with lids and secured in place to prevent it from rolling in the center of the arena. While carrying the mouse in the tube, it was ensured not to close the lid tightly to maintain proper ventilation. After placing the tube in the center, the lid was removed. The number and time the subject spent in the shelter (tube), number of pokes into the shelter (tube) door, and time spent in the lit arena, were recorded and measured using the ANY-maze software.

Elevated plus maze test. The elevated plus maze test was used to evaluate the anxiety and fear domains. The test is based on the concept that rodent's natural behavior is to stay in the enclosed areas and display unconditional fear in open spaces and elevated heights. Anxious animals tend to spend more time in the closed arms than less anxious animals. The elevated plus maze was built according to the description of Lister (51). This maze was made of Plexiglas and consisted of a central square (5x5 cm) from which radiated four arms (5x45 cm). Two of the arms had walls (15 cm high) along the edge (closed arms), whereas the other two arms did not have walls (open arms). A white line was drawn halfway (22.5 cm) along each of the four arms to measure motor activity. The maze was elevated 45 cm above the floor on a plus-shaped plywood stand. The mouse was placed at the junction of the four arms of the maze, facing a closed_arm. They were allowed to explore the apparatus for 5 min while an observer, a video camera, and the automated tracking system recorded their actions. Time spent in the open/closed arms and the duration of time in the center were tracked by ANY-maze software and used those for scoring various cognitive behavior parameters including: i) Number of entries in the open or closed arms, ii) time spent in the open or closed arms, iii) time spent in the center, and iv) percentage of time spent in the open/closed arms.

Zero maze. The zero maze was used to test anxiety- and exploratory-related behaviors in mice. It is an elevated, ring-shaped runway, with the same amount of area devoted to the adjacent open and closed quadrants. Mice were placed in one of the closed quadrants at the start of each trial and were allowed to explore the apparatus for 5 min. The ANY-maze software was used for scoring cognitive behavior parameters including: i) Number of entries in the open or closed segments, ii) time spent in the open or closed segments, iii) time spent in the center, and iv) percentage of time spent in the open/closed segments.

Three-chamber sociability test. A three-chamber social interaction test was performed to assess sociability and social preference as described previously (52). Mice were habituated in the middle compartment for five min before testing. The 'strange' animal (same age and strain) was placed in the wire cage of either the left or right compartment. The strange mouse's location represented stranger zone 1 while the other wire cage remained empty (that is, empty zone). Mice were left to socialize for 10 min. The time spent in the stranger zone 1 (interaction with the new mouse) relative to the time spent in the empty zone was measured.

The social preference test was conducted for another 10 min after termination of the sociability test. A second strange mouse was introduced to the wire cage in place of the empty zone and was considered stranger zone 2. The animal's preference for interacting with a familiar animal (stranger zone 1) or an unfamiliar animal (stranger zone 2) was determined by measuring the same parameters. The indices of sociability and social preference were computed, as previously described (53).

Barnes maze. Spatial learning and memory were tested using the Barnes maze as described (44,54). The maze consists of a circular platform (75 cm diameter) with 20 holes (5 cm diameter) evenly spaced around the platform's perimeter and is elevated 56.5 cm above the floor by a stand. One hole was designated as the escape hole for each mouse, and an escape box was placed underneath the hole. For each mouse, the location of the escape box remained the same throughout all test runs. To create an aversive environment, three 72-watt lights were suspended above the platform, encouraging the mice to flee from the highly lit environment into the darkened environment of the box. Four shapes (+, ■, Δ, •) served as visual cues and each shape respectively was placed on each side of the black background curtains to aid spatial navigation. Behavioral testing consisted of 2 habituation trials on the first day, and 8 evaluation trials (2 trials/day) over the next 4 days. Reversal training consisted of 2 trials per day for 3 days, with an inter-trial interval of 20-30 min. This phase began 24 h after the final acquisition trial. Prior to the start of reversal training, each mouse was assigned a new escape hole, positioned opposite the previously assigned location. Latency (that is, the time mice found the escape box) and total errors (that is, nose-pokes into non-escape holes) were recorded and analyzed by ANY-maze software.

Data and statistical analysis for behavior tests. The data are presented as the mean ± SEM. Results were analyzed by one-way or two-way ANOVA using GraphPad Prism Software (RRID:SCR_002798; V10.0; Dotmatics). Tukey's post hoc test was applied for multiple comparisons. Differences were considered statistically significant at $P<0.05$ for all analyses. An unpaired t-test was applied for individual comparisons between two groups.

Tissue collection for analysis of global proteomes from brain cortex and hippocampus. A total of 10 mice, AGE (n=5) and control diet (n=5), were euthanized at 88 weeks-old (following 11 months of AGE supplementation) for label-free global proteomic quantitation by tandem mass spectrometry (MS^2) analysis. The sample preparation for quantitative proteomic analysis has been described previously (55). Briefly, the left cortex and hippocampi from both hemispheres were dissected and processed. Tissues were lysed with sample buffer containing 2% sodium dodecyl sulphate (SDS), 0.5 M tetraethylammonium bicarbonate (TEAB), pH 8.5, protease inhibitor cocktail (1:100), and phosphatase inhibitor cocktail (1:100). Specimens were homogenized by Glas-Col stringer 099C K43 (Glas-Col LLC, IN) and centrifuged at 17,000 x g for 20 min at 4°C. The protein pellets (insoluble fraction) were collected and further precipitated with 4X (w/v) acetone and incubated at -20°C overnight. Prior to MS^2 analysis, protein pellets were washed two more times with acetone.

Sample processing for MS^2 analysis. Proteins in samples were denatured with 6 M urea, 2 M thiourea and 100 mM ammonium bicarbonate, and then reduced with 10 mM

dithiothreitol (DTT) for 1 h at room temperature (RT) and alkylated at RT in the dark with 30 mM iodoacetamide. Alkylation was halted with 10 mM DTT for 15 min at RT. Protein concentration was determined using Pierce 660 nm Protein Assay (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Proteins from each sample were digested with trypsin (Thermo Fisher Scientific, Inc.) in a sequential digestion. Trypsin was added at a 1:100 enzyme to protein mass ratio. The first digest was incubated at 37˚C for 5 h, and the second digest was incubated overnight. The digested peptides were terminated with trifluoroacetic acid (TFA) 0.5%. For total protein quantification, 5-μg of peptide from each sample was combined for spectral library using the method for data-dependent acquisition with parallel accumulation-serial fragmentation (DDA-PASEF). Subsequently, 500 ng peptides from each sample were loaded to the liquid chromatography-MS and analyzed using the method for data-independent acquisition (DIA-PASEF).

To create a comprehensive spectral library, digested peptides were combined from each sample and fractionated using a high pH reversed-phase peptide fractionation kit according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.). A total of eight fractions were collected, lyophilized and resuspended with 0.1% formic acid in deionized water.

For proteome analyses, an EvoSep One liquid chromatography system was used as described previously (56) and the peptides were analyzed by eluting with a 44 min gradient or EvoSep One 30 samples per day (SPD) program. The system used a 15 cm x 150 μm ID column with 1.5 μm C18 beads (Bruker PepSep) and a 20 μm ID zero dead volume electrospray emitter (Bruker Daltonics). Mobile phases A and B were 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. EvoSep One was coupled online to a modified trapped ion mobility spectrometry quadrupole time-of-flight mass spectrometer (timsTOF Pro 2, Bruker Daltonics) via a nano electrospray ion source (Captivespray, Bruker Daltonics). To generate a spectral library, eight high-pH reversed-phase fractionated samples were analyzed by timsTOF Pro 2 in the DDA-PASEF mode. PASEF and TIMS were set to 'on'. One MS and ten PASEF frames were acquired per cycle of 1.17 sec (~1 MS and 120 MS^2). Target intensity for MS was set at 10,000 counts/sec with a minimum threshold of 2,500 counts/sec. Duty cycle was locked to 100%. Ion mobility coefficient (1/K0) value was set from 0.6 to1.6 V.s/cm2, collision energy was set from 20-59 eV. MS data were collected over m/z range of 100 to 1,700. If the precursor (within mass width error of 0.015 m/z) had >4X signal intensity in subsequent scans, a second MS^2 spectrum was collected. Exclusion was active after 0.4 min. Isolation width was set to 2 for m/z <700 m/z or 3 m/z for m/z >700.

The DIA-PASEF method was optimized with py_diAID, as described previously (57), to cover an m/z range from 300 to 1,350 Da and an ion mobility range of 0.65 to 1.45 V.s/cm2. The method includes two IM windows per DIA-PASEF scan with variable isolation window widths adjusted to the precursor densities. A total of 25 DIA-PASEF scans were deployed at a throughput of 30 SPDs (cycle time: 2.76 sec).

MS^2 statistical analysis. Data were analyzed with Spectronaut version 17 (Biognosys AG) with the default settings, except for the prototypicity filter, which was set to 'only protein group specific'. Protein quantification and statistical analysis were performed using MSstats. The MSstat set up included protein quantification using the top 3 features, a q-value cutoff of 0.01, the use of unique peptides, and the removal of protein with only one feature. No imputation was used, and the data were log_2-transfered based on MS^2 peak area. Normalization was performed using the 'equalize medians' method.

Quantitative MS^2 analysis and bioinformatics. AGE supplementation-induced molecular phenome changes in SDS-insoluble protein were evaluated by computing the expression ratio and P-value for all MS^2-identified proteins from AGE mice relative to age-matched controls. The expression ratio threshold was set to >1.3 and <0.77 for respective increased and decreased proteins (P<0.05). For data visualization, expression ratios and p values were log-transformed to produce fold-change (log_2 expression ratio) and $-log_{10}$ P-value.

The differentially changed proteins in AGE mice cortex and hippocampus were imported into Ingenuity Pathway Analysis (IPA; Redwood City, CA; RRID:SCR_008653) for canonical pathway analysis, machine learning-driven disease pathway analysis, upstream regulator analysis, and neurological behavior prediction. Additionally, comparative analysis was performed by combining expression profiles from AGE mice cortex and hippocampus to obtain 2x2 comparisons for top pathways. The data are licensed under the Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0).

Results

Effects of AGE supplementation on the general health of aging mice and sensorimotor function. No significant differences in average food intake or body weight (Figs. S2 and S3, respectively) were observed when comparing the AGE-supplemented diet-fed mice with the control diet-fed mice. Additionally, no changes in mortality or apparent abnormalities in general health were found in the mice during the 40-week feeding period. Neither group exhibited any significant decrease in body weight throughout the study. The maximum observed body weight loss occurred at the 34th feeding-week, with a weight loss of ~8.1% in the AGE diet-fed group and 6.0% in the control diet-fed group compared with the 1st feeding-week.

The SNAP test was used to assess the impact of AGE supplementation on the sensorimotor function of the aging mice. The inverted screen grip test was performed to assess the effects of AGE on physical strength. No statistically significant differences in coordination, motor strength, proprioception, gait and mental status were observed following 40-week dietary AGE supplementation (Fig. S4).

Effects of AGE supplementation on anxiety and neophobia. Mice fed the AGE diet exhibited less anxiety in the light-dark transition test and reduced neophobia-like behaviors in the emergence test. These mice exhibited a decreased number of entries (Fig. 1A; P<0.01) and time spent in the dark zone (Fig. 1B; P=0.046) relative to the aging controls. Furthermore,

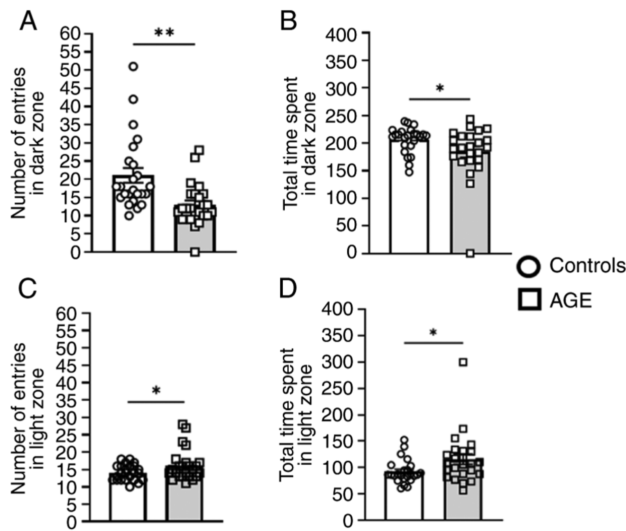

Figure 1. Effects of AGE supplementation on anxiety and neophobia under adverse light condition. The light-dark transition test was used to measure (A) number of entries into dark zone, (B) total time spent in dark zone, (C) number of entries to light zone, and (D) total time spent in light zone. Data are presented as the mean ± SEM; Groups: Control, n=24; AGE diet, n=24. Significantly different from age-matched control group, *P<0.05 and **P<0.01. AGE, aged garlic extract.

the AGE group exhibited an increased number of entries (Fig. 1C; P<0.05) and time spent in the light zone (Fig. 1D; P=0.034).

The AGE-supplemented diet-fed group also exhibited less anxiety and neophobia and increased exploratory behavior in the emergence test (Fig. 2A-G), with statistically significant differences in the following parameters: Less time spent in the shelter zone, more time spent in the arena zone, higher number of line crossings in the arena zone, longer distance travelled in the arena and less time immobile in the arena zone. However, no differences were observed between the AGE-supplemented and control diet groups in the open field test (Fig. S5A-C), as well as the elevated plus maze and zero maze tests (Fig. S6A-F).

Effects of AGE supplementation on exploratory behavior, learning and memory. In the NOR test to evaluate ability of non-spatial learning and recognition memory under the cognition behavioral domain, AGE-supplemented mice displayed a greater DI (Fig. 3A; P=0.027), and increased percentage of investigation time with the novel object location (Fig. 3B; P=0.039), indicating AGE supplementation could improve learning levels, memory and exploratory behaviors in aging mice.

Effects of AGE supplementation on cognition and spatial reference learning in aging mice. It was assessed if AGE supplementation could improve spatial reference learning abilities in aging mice. During the 5-min Barnes maze test, a higher percentage of mice in the AGE diet-fed group found the hiding box for acquired learning compared with that in the control group. For those that found within the allotted time, the latency to enter the hiding box was used to estimate memory and learning abilities. In reversal training, AGE-supplemented diet-fed mice displayed a shorter latency to enter the target zone compared with mice in the control group. The differences were most pronounced in trial 1 on day 2 of reversal training (Fig. 4).

The present study evaluated whether AGE supplementation could improve social recognition, sociability and novelty preference behavior. The three-chamber social interaction test was used for evaluating impact of AGE on the social index of aging mice (time spent with stranger mouse 1 vs. empty cages) and on the novelty preference index [time spent with familiar (stranger mouse 1) vs. stranger mouse 2]. In the present study, AGE supplementation did not improve cognitive domain parameters (Fig. S7A and B) under the current conditions.

Effects of AGE supplementation on proteomes are most pronounced in the hippocampus. The brain proteomic profiles were examined to identify the molecular effects of AGE supplementation in aging mice. Of 5,863 and 5,826 proteins identified using label-free MS^2, 90 and 336 proteins were significantly changed in the AGE-supplemented mouse cortex and hippocampus, respectively (Fig. 5A and B; Table SI). A total of 34 and 197 proteins were decreased in the cortex and hippocampus, respectively, whereas 139 and 56 proteins were increased in the cortex and hippocampus, respectively. Notably, dietary AGE supplementation decreased selenium-binding protein 1 (Selenbp1; fold-change in the cortex, -0.700; P=2.156×10^{-5}; fold-change in the hippocampus, -1.039; P=3.415×10^{-5}) and increased prodynorphin (Pdyn; fold-change in the cortex, 0.734; P=0.007; fold-change in the hippocampus, 1.035; P=0.010) in both brain regions (Fig. 5C).

IPA predicts altered mouse phenomes in AGE-supplemented diet-fed mice. With the bioinformatics tool to examine mouse phenomes, which refers to a full set of observable traits (for example, behavior, physiology and/or disease susceptibility), IPA was able to use curated proteomic datasets to forecast changes in phenotypic outcomes. IPA comparative analysis showed that molecular pathways in both the cortex and hippocampal regions were similarly impacted but with some important distinctions. The commonly affected pathways were 'epithelial adherens junction signaling' (increased; P<0.05), 'synaptogenesis signaling pathway' (increased; P<0.05) and 'Sertoli cell-Sertoli cell junction signaling' (increased; P<0.05) (Fig. 6A; Table SII, sheet: Canonical pathways). Among the top canonical pathways, 14-3-3-mediated signaling related to apoptosis was impacted exclusively in the AGE-supplemented hippocampus (z-score, -1.387; P=1.362×10^{-5}) (Fig. 6A; Table SII, sheet: Canonical pathways). IPA upstream regulator analysis predicted microtubule-associated protein tau (MAPT) to be most impacted by dietary AGE supplementation based on P-value ranking, and this occurred mostly in the hippocampus (cortex P=0.007; hippocampus P=1.212×10^{-14}) (Fig. 6B; Table SII, sheet: Upstream regulators). Amyloid precursor protein (APP) and brain-derived neurotrophic factor (BDNF) were predicted to be increased in the cortex and reduced in the hippocampus (Fig. 6B; Table SII, sheet: Upstream regulators). Presenilin 1 (PSEN1) was exclusively increased in the hippocampus (z-score, 0.658; P=1.992×10^{-10}) (Fig. 6B; Table SII, sheet: Upstream regulators). The p53 cell-cycle regulator was reduced in both the cortex (z-score, -0.442;

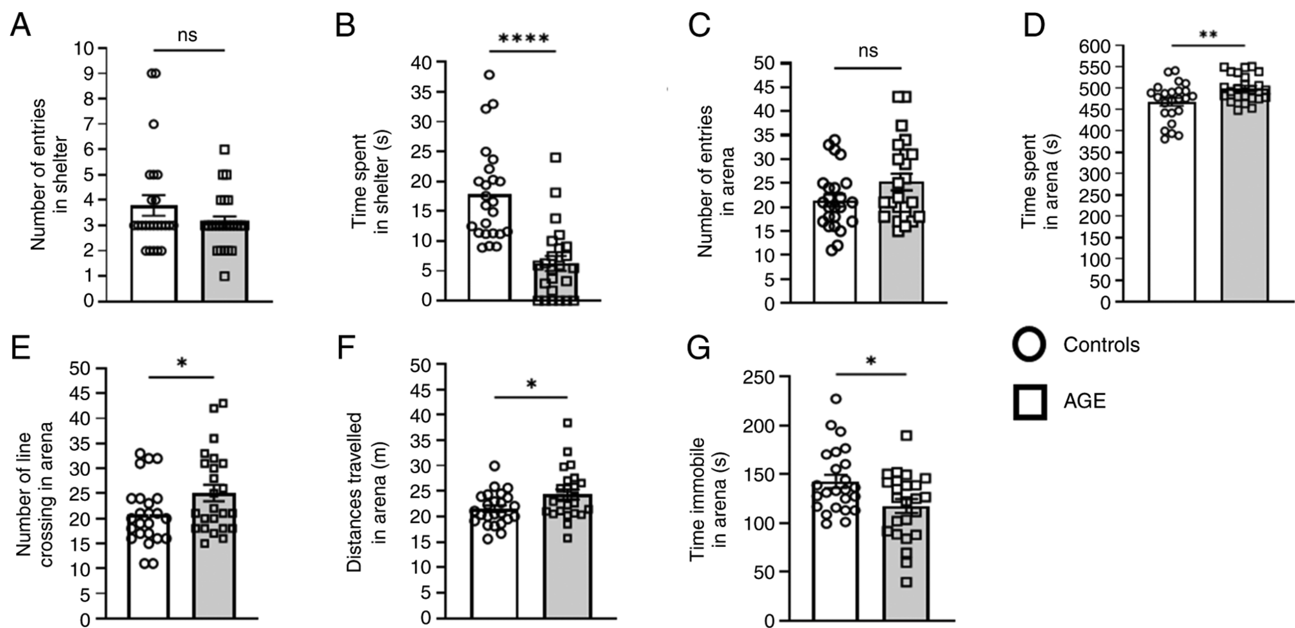

Figure 2. Effects of AGE supplementation on neophobia behaviors. The emergence test was used to measure (A) number of entries into shelter, (B) time spent in shelter, (C) number of entries into arena, (D) time spent in arena, (E) number of line crossings in arena, (F) distance travelled in arena and (G) time immobile in the arena. Data are presented as the mean ± SEM. Groups: Control, n=24; AGE diet, n=24. Significantly different from age-matched control group, *P<0.05, **P<0.01 and ****P<0.0001. AGE, aged garlic extract; ns, not significant.

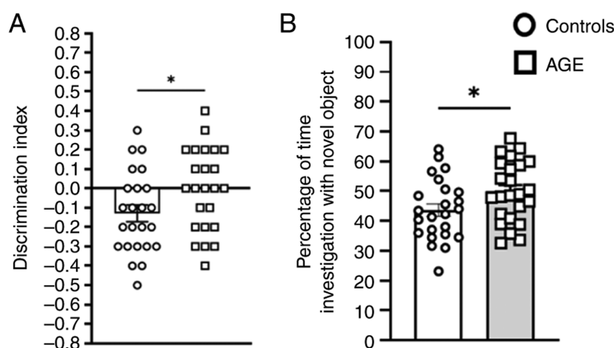

Figure 3. AGE effects on NOR. The NOR test was used to measure (A) Discrimination Index (Scale: -1 to +1), and (B) Percentage of time investigation with novel object. Data are represented as the mean ± SEM. Groups: Control, n=24; AGE diet, n=24. *P<0.05. AGE, aged garlic extract; NOR, novel object recognition.

P=0.002) and hippocampus (z-score, -1.781; P=1.917x10^{-8}) (Fig. 6B; Table SII, sheet: Upstream regulators). IPA behavior predictions suggested that differential expression changes in the cortex and hippocampus proteomes due to AGE supplementation could impact cognition and learning, whereas only changes to the hippocampus proteomes could impact anxiety, memory and spatial memory (Fig. 6C; Table SII, sheet: Behavior prediction).

Discussion

In the natural aging process, there is a well-documented decline in cognition and memory function. Based on existing literature, a strong argument can be made that AGE, along with its bioactive components, may delay cognitive impairment, and thereby improve the behavioral phenotype associated with aging (30,58-61). As reported by Song *et al* (35), garlic (*Allium sativum*), particularly in the form of AGE, contains nutraceutical constituents such as SAC, S-allyl-mercaptocysteine (SAMC) and FruArg. SAC and SAMC, prominent organosulfur compounds, exhibit potent antioxidant, anti-inflammatory and anti-apoptotic activities that are consistent with the observed downregulation of TP53-mediated apoptotic regulators and enhancement of BDNF-associated neurotrophic signaling. FruArg, a carbohydrate derived from garlic via the Maillard reaction, exhibits blood-brain barrier permeability and transcriptional regulatory potential. In lipopolysaccharide-stimulated microglial models, AGE and FruArg reversed 67 and 55% of transcriptomic alterations, respectively, implicating pathways such as Toll-like receptor signaling, IL-6 and nuclear factor erythroid 2-related factor 2 pathways. These findings strengthen the mechanistic framework linking AGE bioactivities to both region-specific proteome shifts and the behavioral improvements reported, highlighting the potential of AGE-derived compounds in attenuating inflammatory activities and enhancing brain resilience.

SAC has been shown to attenuate oxidative stress and prevent neuronal cell death in models of neurodegeneration, while SAMC has demonstrated the ability to modulate gene expression related to mitochondrial function and cellular survival. Building on these findings, the present experiments suggested that the anxiolytic and cognitive benefits observed following AGE supplementation may, at least in part, be mediated by the actions of these sulfur-containing compounds and carbohydrate derivatives on key signaling pathways involved in neuronal health and plasticity.

Although evidence in humans is still being developed, preclinical studies using different aging-related rodent models have shown that AGE supplementation could improve

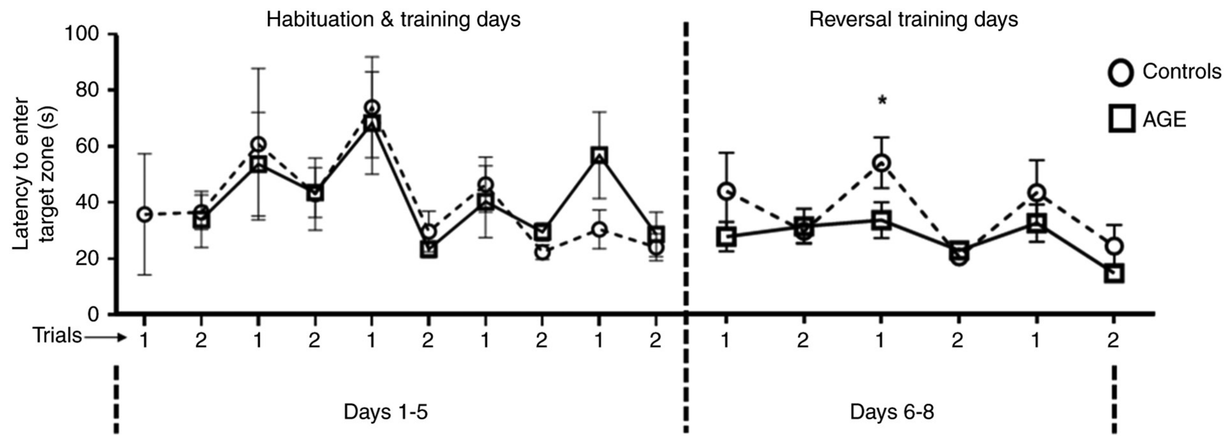

Figure 4. AGE effects on cognition and spatial reference learning. Barnes maze was used to measure latency of first entering the target zone during habituation and training days, and reversal training days. Data are presented as mean ± SEM. Groups: Control, n=24; AGE diet, n=24. On the 2nd reversal training day (testing day 7), significantly different from age-matched control group, *P<0.05, analyzed by the one-tailed, unpaired Student's t-test. AGE, aged garlic extract.

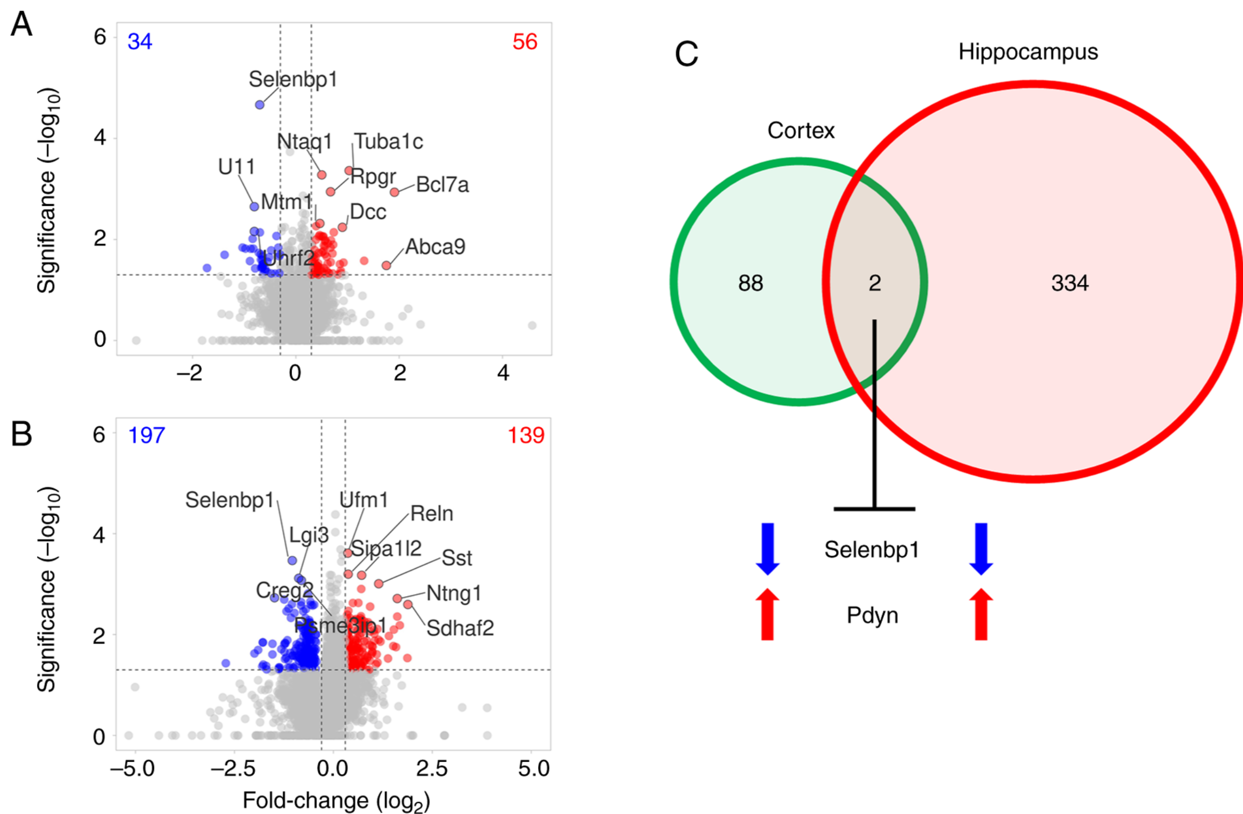

Figure 5. Quantitative proteomics profiling of cortex and hippocampus in AGE-supplemented mice. (A and B) Volcano plots showing global proteomic profiles of AGE mice (A) cortex and (B) hippocampus, respectively; vertical grey lines: -0.34 (decrease threshold) and +0.34 (increase threshold; horizontal grey lines: 1.3 (significance threshold). Differentially expressed proteins are colored red (increased expression), blue (decreased expression), or grey (no change in expression); top 10 hits by Euclidean distance are labeled. (C) Common and distinct differentially expressed proteins in the cortex and hippocampus of AGE-supplemented mice are displayed in the Venn-diagram. Among the identified proteins exhibiting significant changes, Selenbp1 and Pdyn, were shared across both brain regions, the cortex and hippocampus, of AGE-supplemented mice. AGE, aged garlic extract.

cognition through its neuroprotective molecular effects on antioxidant enzymes (58-60), resilience to amyloid-β toxicity (30,58,60,61), and by promoting hippocampal neuroplasticity via modulation of neurotransmitter receptor expression (30). These findings underscore the potential for AGE to improve human health by altering the aging-related brain pathology.

In the present study involving multi-faceted behavioral experiments, aging mice given a diet supplemented with AGE for >40 weeks showed improvements in age-related decline in learning and spatial and working memory assessed in the Barnes maze and NOR tests. These findings are in agreement with results from a previous study using the passive avoidance test, which showed improvements in scopolamine-induced

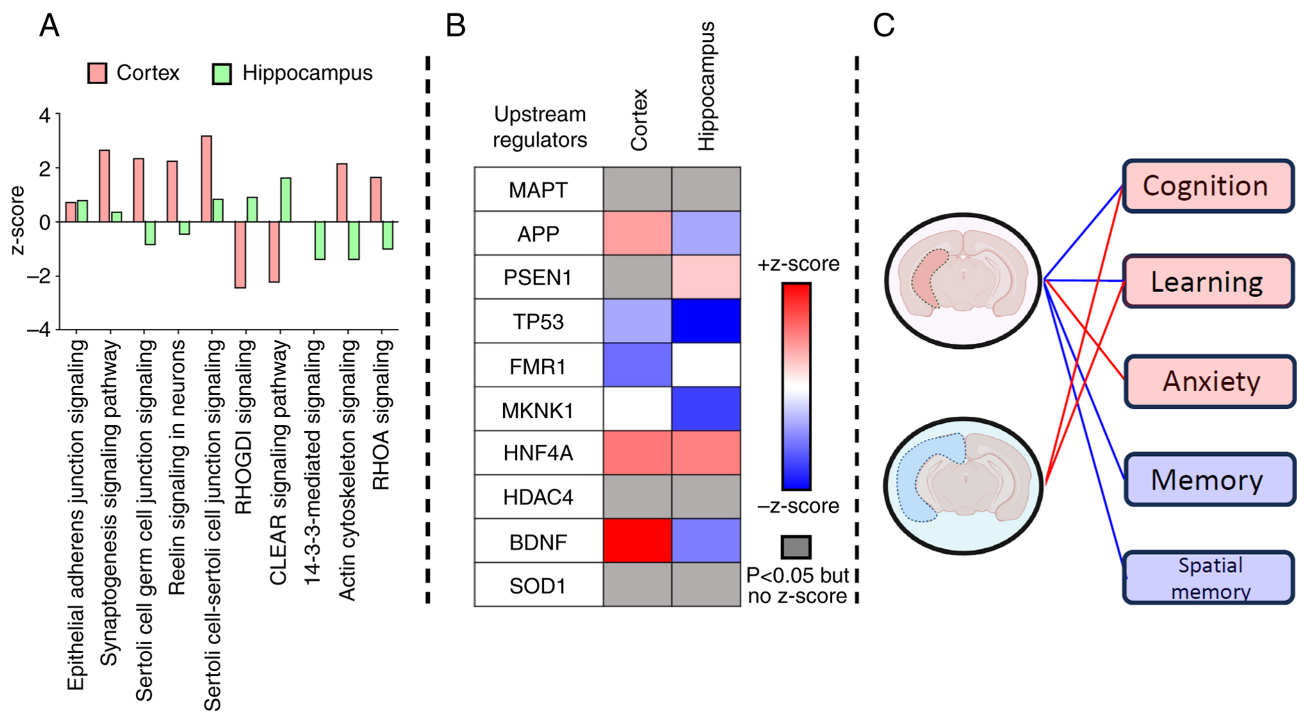

Figure 6. IPA analysis of proteomes in AGE-supplemented mice predicts an altered phenome. (A) Top 10 IPA canonical pathways in the cortex (red bars) and hippocampus (green) proteomes of AGE-supplemented mice (P<0.05). (B) Top 10 most significantly changed upstream regulators in both cortex (left column) and hippocampus (right column) (P<0.05). Upstream regulators were ranked by P-value (rather ranking than z-score) in IPA to assess the statistical enrichment of their downstream targets. MAPT emerged as a top upstream regulator (P<0.01), indicating its targets are significantly overrepresented among the differentially expressed proteins. (C) IPA disease and function analysis to predict neurological behavior based on differential protein expression in the cortex and hippocampus (represented by red lines for increased protein expression and blue lines for decreased protein expression) indicates changes in behavioral phenotypes. Differential expression directionality (red lines: Increasing expression; blue lines: Decreasing expression) in the cortex and hippocampus predicts altered behavioral phenotypes including cognition, learning, anxiety, memory and spatial memory pathways (with red boxes highlighting behaviors driven by increased expression and blue boxes denoting behaviors influenced by decreased expression). IPA, Ingenuity Pathway Analysis; AGE, aged garlic extract; PSEN1, presenilin 1; APP, amyloid precursor protein.

amnesia in mice fed with crude garlic extract (Lasuna) at 10 ml/kg body weight *per os* for 21 days (62). In another study using the senescence-accelerated mouse model of aging, mice fed an AGE diet for 8 months also showed amelioration of memory acquisition deficits and memory retention impairments compared with the age-matched controls without AGE supplementation (63). These data suggest that, in the setting of natural and induced aging models, supplementation of AGE can improve cognition and memory. AGE supplementation has also been shown to attenuate cognitive impairment in the setting of neurodegeneration (30,58,64,65). To the best of our knowledge, the present study is among the first to comprehensively assess multi-domain behavioral functions, demonstrating that a 40-week AGE supplementation regimen could enhance cognition and memory, and mitigate age-associated cognitive decline.

Anxiety and related symptoms such as restlessness, psychomotor agitation and excessive worrying are frequently encountered in later life (66). The current options for the treatment of aging-related anxiety disorders encompass lifestyle modifications, behavioral and cognitive therapies, mindfulness-based interventions, and/or pharmacological treatment, including selective serotonin receptor inhibitors, non-selective serotonin receptor inhibitors and benzodiazepines (66). However, these medications are often associated with severe side effects, further complicating the management of age-related anxiety (66). In the present study, dietary supplementation of AGE could reduce anxiety-like behaviors as manifested by reduced neophobia (fear of new things) and increased exploratory behavior in the emergence test. These results are consistent with a previous study demonstrating the potential of AGE to alleviate anxiety associated with natural aging. A study by Gilhotra and Dhingra (67) investigated the neurochemical mechanisms underlying the anxiolytic effects of garlic extract in mice. Their study focused on GABAergic and nitrergic signaling pathways and employed behavioral paradigms such as the elevated plus maze and the light-dark box test, demonstrating that garlic extract reduced anxiety-like behaviors.

Building on these behavior observations, the present study leveraged machine learning-driven IPA to investigate the molecular mechanisms underlying the anxiolytic effects of AGE. Notably, the anti-anxiety potential of AGE may be linked to its antioxidant properties, modulation of neurotransmitter systems and anti-inflammatory effects. In particular, in Fig. 6 it is illustrated that several upstream regulators, including TP53, MAPK interacting serine/threonine kinase 1 (MKNK1) and BDNF, and their signaling pathways relevant to neurobehavioral functions, were differentially modulated in a region-specific manner following AGE supplementation. In IPA, the z-score is a crucial statistic used to infer the likely activation state (activation or inhibition) of upstream regulators,

which is based on the expression or phosphorylation patterns of its downstream target molecules. As shown in Fig. 6B, TP53 and MKNK1 exhibited strong negative z-scores in the hippocampus, suggesting suppression of stress- or apoptosis-related signaling. Conversely, BDNF was positively regulated in the cortex. These regulatory changes may contribute to the anxiolytic effects of AGE by modulating neuroplasticity, promoting neuroprotection or adjusting stress-response pathways. These molecular changes with behavioral domains are further contextualized in Fig. 6C, highlighting that the pathways linked to anxiety were differentially impacted between the cortex and hippocampus. More detailed mechanistic insights are presented subsequently.

IPA analysis of AGE-induced differential protein expression in the cortex and hippocampus predicted significant changes in the key regulatory proteins MAPT, APP, PSEN1 and BDNF, which likely mediate the distinct proteomic changes associated with AGE supplementation through upstream regulation.

MAPT is normally increased in aging brains, and its phosphorylation is associated with cognitive decline in AD (68,69). MAPT was identified as a top upstream regulator based on P-value ranking within the IPA framework, reflecting significant enrichment of its downstream targets among the differentially expressed proteins ($P<0.01$). While the z-score is applied across regulators, it requires concordant directionality between predicted effects and observed expression changes. In the present dataset, MAPT-associated targets exhibited mixed directional trends, resulting in a non-significant z-score despite strong enrichment. MAPT expression itself did not differ significantly between the cortex and hippocampus (Fig. 6B); rather, the analysis highlighted its inferred regulatory influence. Notably, a greater number of MAPT-related targets were differentially expressed in the hippocampus, pointing to a region-specific sensitivity of MAPT-regulated pathways to dietary AGE supplementation. Future studies are needed to validate the directionality and potential post-translational modifications of MAPT in response to AGE treatment. Although directionality could not be assessed, MAPT emerged as the most significant upstream regulator of molecular changes in the hippocampus ($-\log_{10}$ BH-P-value=13.916), compared with its regulatory impact in the cortex ($-\log_{10}$ BH-P-value=2.143). Notably, >75% of proteins were differentially altered due to AGE supplementation.

APP can be cleaved by β- and γ-secretases, resulting in producing different amyloid-β variants in AD (70). Based on IPA, this protein was predicted to be reduced in the hippocampus (z-score, -0.445; $-\log_{10}$ BH-P-value=10.128) but not in the AGE-supplemented mouse cortex (z-score, 2.714; $-\log_{10}$ BH-P-value=0.945). This prediction suggests a potential ability of AGE to prevent the formation of AD plaques and possibly protect against memory impairment.

PSEN1 or γ-secretase constitutes the catalytic subunit of the γ-secretase complex, and its loss of function due to mutations has been implicated in amyloid-β plaque formation and AD pathogenesis (71). The increase in PSEN1 in the hippocampus (z-score, 0.658; $-\log_{10}$ BH-P-value=9.701) due to AGE supplementation may be a protective mechanism to counteract age-related memory loss.

BDNF is a key regulator for neuronal survival and growth, neurotransmitter modulation and neuronal plasticity, which are essential for learning and memory (72). Upon exogenous administration, BDNF has been shown to participate in learning-related events, memory, depression and cognition (73,74). BDNF mRNA and protein levels are normally reduced in the cortex and hippocampus of older adults relative to younger adults and infants and are positively correlated with brain tissue volume (72,74). In post-mortem AD brains, BDNF is globally reduced in the cortex and hippocampus (72). In the present study, AGE supplementation significantly increased cortical BDNF (z-score, 2.030; $-\log_{10}$ BH-P-value=5.423), suggesting that the AGE effects may preserve the cortical neurons, thus attenuating the age-related decline in cognitive function and short-term memory.

Comparative analysis of the commonly changed pathways in the cortex and hippocampus revealed an increase in synaptogenesis signaling in both brain regions, albeit mostly in the cortex. In aged rodents and primates, loss of synaptic spines in the hippocampus and cortex is known to reduce neural firing rates during working memory tasks and to reduce non-synaptic bouton density associated with learning deficits (75). A study showed that AGE supplementation attenuates oxidative stress and amyloid pathology, restores cortical and hippocampal synaptic proteins, such as synaptophysin and synaptosome associated protein 25, as well as enhanced cognition and memory (76).

In the hippocampus, AGE supplementation was found to reduce 14-3-3 signaling pathways linked to apoptosis and chaperone-mediated autophagy. Aberration of 14-3-3 signaling pathways has been reported in aging, neurodegenerative diseases and cancer (77). Aging has been shown to disturb 14-3-3 chaperone and macroautophagic signaling activity, which resulted in the accumulation of toxic molecular constituents, including MAPT, amyloid-β and α-synuclein (77). Consequently, using AGE supplementation to target 14-3-3 signaling may be a novel mechanism to attenuate neuron cell death during the natural aging process.

In conclusion, the present study revealed the neuroprotective effects of dietary supplementation of AGE in improving age-related cognitive decline and anxiety-like behaviors in aging mice. Modulation of MAPT, APP, PSEN1 and BDNF further suggested the ability of AGE to confer neuroprotective effects against age-related neurodegeneration. Proteomic analysis highlighted the increase in synaptogenesis and reduction in apoptotic signaling, supporting the notion of AGE supplementation as a nutraceutical to mitigate age-related cognitive decline. While these findings provide promising insights into the molecular changes induced by AGE, further validation is essential to strengthen the present conclusions. To corroborate the proteomic data, additional validation experiments using both western blotting and immunohistochemical analyses, as well as other complementary assays, will be performed in future studies. Understanding these mechanisms can offer promising avenues for interventions in age-related neurological disorders. Further research into the translational potential of AGE-based interventions is warranted to address the increasing disease burden of aging-associated cognitive impairments and neuropsychiatric symptoms.

Acknowledgements

The authors would like to thank Dr Dennis K. Miller, Associate Teaching Professor, Department of Psychology Sciences, University of Missouri College of Arts & Science, for his technical guidance in the setup of some neurobehavioral assessments.

Funding

The present study was supported in part by the Wakunaga Pharmaceutical Co., Ltd. (grant no. 00078665) and by the University of Missouri School of Medicine (grant no. C2654010-C8904-0595).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author. The data generated in the present study may be found in the Figshare under accession number 30066604 or at the following URL: https://doi.org/10.6084/m9.figshare.30066604.

Authors' contributions

MT and MJ developed methodology, validated and curated data, conducted formal analysis and investigation, and wrote the original draft. MJ visualized data. AZ conducted formal analysis and investigation. WY validated and curated data. TTN conducted formal analysis and investigation. AB conducted investigation, validated and curated data. RL conducted investigation and validated data. GYS contributed to data interpretation, provided constructive criticism during the revision process, and approved the final version. JC conceptualized the study, developed methodology, validated data, performed formal analysis, conducted investigation and project administration, and curated data. ZG conceptualized and supervised the study, developed methodology, performed software and formal analysis, visualized, validated and curated data, conducted investigation and project administration, provided resources, wrote the original draft, and acquired funding. MT, MJ, AZ, WY, TTN, GYS, JC and ZG wrote, reviewed and edited the manuscript. JC and ZG confirm the authenticity of the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All experimental procedures involving mice were conducted in accordance with the ethical standards of the University of Missouri School of Medicine and the national guidelines for the care and use of laboratory animals. The study protocol was reviewed and approved (approval no. 25120) by the Institutional Animal Care and Use Committee (IACUC) of the University of Missouri School of Medicine (Columbia, USA).

Patient consent for publication

Not applicable.

Competing interests

Financial support was received from Wakunaga Pharmaceutical Co., Ltd., however the funding agent had no influence on the research, results, or interpretation of the study. The authors declare that they have no competing interests.

References

1. Bureau USC: 2023 National Population Projections Tables: Main Series, 2023.
2. No authors listed: 2023 Alzheimer's disease facts and figures. Alzheimers Dement 19: 1598-1695, 2023.
3. Hebert LE, Weuve J, Scherr PA and Evans DA: Alzheimer disease in the United States (2010-2050) estimated using the 2010 census. Neurology 80: 1778-1783, 2013.
4. Bartzokis G, Cummings JL, Sultzer D, Henderson VW, Nuechterlein KH and Mintz J: White matter structural integrity in healthy aging adults and patients with Alzheimer disease: A magnetic resonance imaging study. Arch Neurol 60: 393-398, 2003.
5. Toescu EC, Verkhratsky A and Landfield PW: Ca2+ regulation and gene expression in normal brain aging. Trends Neurosci 27: 614-620, 2004.
6. Melov S: Modeling mitochondrial function in aging neurons. Trends Neurosci 27: 601-606, 2004.
7. Murman DL: The impact of age on cognition. Semin Hear 36: 111-121, 2015.
8. Xia X, Jiang Q, McDermott J and Han JJ: Aging and Alzheimer's disease: Comparison and associations from molecular to system level. Aging Cell 17: e12802, 2018.
9. Yoshimura N, Tsuda H, Aquino D, Takagi A, Ogata Y, Koike Y and Minati L: Age-related decline of sensorimotor integration influences resting-state functional brain connectivity. Brain Sci 10: 966, 2020.
10. Heavener KS and Bradshaw EM: The aging immune system in Alzheimer's and Parkinson's diseases. Semin Immunopathol 44: 649-657, 2022.
11. Yaniv Z and Bachrach U: Handbook of Medicinal Plants. 1st Edition. Haworth Medical Press, Inc. New York, 2005.
12. Amagase H: Clarifying the real bioactive constituents of garlic. J Nutr 136 (3 Suppl): 716S-25S, 2006.
13. Amagase H, Petesch BL, Matsuura H, Kasuga S and Itakura Y: Intake of garlic and its bioactive components. J Nutr 131 (3S): 955S-962S, 2001.
14. Elosta A, Slevin M, Rahman K and Ahmed N: Aged garlic has more potent antiglycation and antioxidant properties compared to fresh garlic extract in vitro. Sci Rep 7: 39613, 2017.
15. Song H, Cui J, Mossine VV, Greenlief CM, Fritsche K, Sun GY and Gu Z: Bioactive components from garlic on brain resiliency against neuroinflammation and neurodegeneration. Exp Ther Med 19: 1554-1559, 2020.
16. Das D, M K, Mitra A, Zaky MY, Pathak S and Banerjee A: A review on the efficacy of plant-derived bio-active compounds curcumin and aged garlic extract in modulating cancer and age-related diseases. Curr Rev Clin Exp Pharmacol 19: 146-162, 2024.
17. Gadidala SK, Johny E, Thomas C, Nadella M, Undela K and Adela R: Effect of garlic extract on markers of lipid metabolism and inflammation in coronary artery disease (CAD) patients: A systematic review and meta-analysis. Phytother Res 37: 2242-2254, 2023.
18. Ried K: Garlic lowers blood pressure in hypertensive subjects, improves arterial stiffness and gut microbiota: A review and meta-analysis. Exp Ther Med 19: 1472-1478, 2020.
19. Varshney R and Budoff MJ: Garlic and heart disease. J Nutr 146: 416S-421S, 2016.
20. Wlosinska M, Nilsson AC, Hlebowicz J, Hauggaard A, Kjellin M, Fakhro M and Lindstedt S: The effect of aged garlic extract on the atherosclerotic process-a randomized double-blind placebo-controlled trial. BMC Complement Med Ther 20: 132, 2020.
21. Asdaq SMB, Challa O, Alamri AS, Alsanie WF, Alhomrani M, Almutiri AH and Alshammari MS: Cytoprotective potential of aged garlic extract (AGE) and its active constituent, S-allyl-l-cysteine, in presence of carvedilol during isoproterenol-induced myocardial disturbance and metabolic derangements in rats. Molecules 26: 3203, 2021.
22. Lin KH, Ng SC, Lu SY, Lin YM, Lin SH, Su TC, Huang CY and Kuo WW: Diallyl trisulfide (DATS) protects cardiac cells against advanced glycation end-product-induced apoptosis by enhancing FoxO3A-dependent upregulation of miRNA-210. J Nutr Biochem 125: 109567, 2024.

23. Gruenwald J, Bongartz U, Bothe G and Uebelhack R: Effects of aged garlic extract on arterial elasticity in a placebo-controlled clinical trial using EndoPAT™ technology. Exp Ther Med 19: 1490-1499, 2020.
24. Hamal S, Cherukuri L, Birudaraju D, Matsumoto S, Kinninger A, Chaganti BT, Flores F, Shaikh K, Roy SK and Budoff MJ: Short-term impact of aged garlic extract on endothelial function in diabetes: A randomized, double-blind, placebo-controlled trial. Exp Ther Med 19: 1485-1489, 2020.
25. Hutchins E, Shaikh K, Kinninger A, Cherukuri L, Birudaraju D, Mao SS, Nakanishi R, Almeida S, Jayawardena E, Shekar C, *et al*: Aged garlic extract reduces left ventricular myocardial mass in patients with diabetes: A prospective randomized controlled double-blind study. Exp Ther Med 19: 1468-1471, 2020.
26. Wlosinska M, Nilsson AC, Hlebowicz J, Fakhro M, Malmsjö M and Lindstedt S: Aged garlic extract reduces IL-6: A double-blind placebo-controlled trial in females with a low risk of cardiovascular disease. Evid Based Complement Alternat Med 2021: 6636875, 2021.
27. Budoff MJ, Ahmadi N, Gul KM, Liu ST, Flores FR, Tiano J, Takasu J, Miller E and Tsimikas S: Aged garlic extract supplemented with B vitamins, folic acid and L-arginine retards the progression of subclinical atherosclerosis: A randomized clinical trial. Prev Med 49: 101-107, 2009.
28. Lindstedt S, Wlosinska M, Nilsson AC, Hlebowicz J, Fakhro M and Sheikh R: Successful improved peripheral tissue perfusion was seen in patients with atherosclerosis after 12 months of treatment with aged garlic extract. Int Wound J 18: 681-691, 2021.
29. Gomez CD, Aguilera P, Ortiz-Plata A, López FN, Chánez-Cárdenas ME, Flores-Alfaro E, Ruiz-Tachiquín ME and Espinoza-Rojo M: Aged garlic extract and S-allylcysteine increase the GLUT3 and GCLC expression levels in cerebral ischemia. Adv Clin Exp Med 28: 1609-1614, 2019.
30. Thorajak P, Pannangrong W, Welbat JU, Chaijaroonkhanarak W, Sripanidkulchai K and Sripanidkulchai B: Effects of aged garlic extract on cholinergic, glutamatergic and GABAergic systems with regard to cognitive impairment in Aβ-induced rats. Nutrients 9: 686, 2017.
31. Wichai T, Pannangrong W, Welbat J, Chaichun A, Sripanidkulchai K and 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 41: 311-318, 2019.
32. Moriguchi T, Saito H and Nishiyama N: Anti-ageing effect of aged garlic extract in the inbred brain atrophy mouse model. Clin Exp Pharmacol Physiol 24: 235-242, 1997.
33. Kim SR, Jung YR, An HJ, Kim DH, Jang EJ, Choi YJ, Moon KM, Park MH, Park CH, Chung KW, *et al*: Anti-wrinkle and anti-inflammatory effects of active garlic components and the inhibition of MMPs via NF-κB signaling. PLoS One 8: e73877, 2013.
34. Borek C: Antioxidant health effects of aged garlic extract. J Nutr 131 (3S): 1010S-1015S, 2001.
35. Song H, Lu Y, Qu Z, Mossine VV, Martin MB, Hou J, Cui J, Peculis BA, Mawhinney TP, Cheng J, *et al*: Effects of aged garlic extract and FruArg on gene expression and signaling pathways in lipopolysaccharide-activated microglial cells. Sci Rep 6: 35323, 2016.
36. Zhou H, Qu Z, Mossine VV, Nknolise DL, Li J, Chen Z, Cheng J, Greenlief CM, Mawhinney TP, Brown PN, *et al*: Proteomic analysis of the effects of aged garlic extract and its FruArg component on lipopolysaccharide-induced neuroinflammatory response in microglial cells. PLoS One 9: e113531, 2014.
37. Flurkey K, Currer JM and Harrison DE: Chapter 20-Mouse models in aging research. In: The Mouse in Biomedical Research (Second Edition). Fox JG, Davisson MT, Quimby FW, Barthold SW, Newcomer CE and Smith AL (eds). Academic Press, Burlington, pp637-672, 2007.
38. Laboratory TJ: When are mice considered 'old'? The Jackson Laboratory, Bar Harbor, ME, 2017. https://www.jax.org/news-and-insights/jax-blog/2017/november/when-are-mice-considered-old.
39. Hohlbaum K, Frahm S, Rex A, Palme R, Thöne-Reineke C and Ullmann K: Social enrichment by separated pair housing of male C57BL/6JRj mice. Sci Rep 10: 11165, 2020.
40. Hennessy MB, Kaiser S and Sachser N: Social buffering of the stress response: Diversity, mechanisms, and functions. Front Neuroendocrinol 30: 470-482, 2009.
41. Chen M, Song H, Cui J, Johnson CE, Hubler GK, DePalma RG, Gu Z and Xia W: Proteomic profiling of mouse brains exposed to blast-induced mild traumatic brain injury reveals changes in axonal proteins and phosphorylated tau. J Alzheimers Dis 66: 751-773, 2018.
42. Shelton SB, Pettigrew DB, Hermann AD, Zhou W, Sullivan PM, Crutcher KA and Strauss KI: A simple, efficient tool for assessment of mice after unilateral cortex injury. J Neurosci Methods 168: 431-442, 2008.
43. Deacon RM: Measuring the strength of mice. J Vis Exp: 2610, 2013.
44. Song H, Konan LM, Cui J, Johnson CE, Langenderfer M, Grant D, Ndam T, Simonyi A, White T, Demirci U, *et al*: Ultrastructural brain abnormalities and associated behavioral changes in mice after low-intensity blast exposure. Behav Brain Res 347: 148-157, 2018.
45. Walker JM, Klakotskaia D, Ajit D, Weisman GA, Wood WG, Sun GY, Serfozo P, Simonyi A and Schachtman TR: Beneficial effects of dietary EGCG and voluntary exercise on behavior in an Alzheimer's disease mouse model. J Alzheimers Dis 44: 561-572, 2015.
46. Siedhoff HR, Chen S, Balderrama A, Sun GY, Koopmans B, DePalma RG, Cui J and Gu Z: Long-term effects of low-intensity blast non-inertial brain injury on anxiety-like behaviors in mice: Home-cage monitoring assessments. Neurotrauma Rep 3: 27-38, 2022.
47. Long JB, Bentley TL, Wessner KA, Cerone C, Sweeney S and Bauman RA: Blast overpressure in rats: Recreating a battlefield injury in the laboratory. J Neurotrauma 26: 827-840, 2009.
48. Huang TN and Hsueh YP: Novel object recognition for studying memory in mice. Bio Protoc 4: e1249, 2014.
49. Leibrock C, Ackermann TF, Hierlmeier M, Lang F, Borgwardt S and Lang UE: Akt2 deficiency is associated with anxiety and depressive behavior in mice. Cell Physiol Biochem 32: 766-777, 2013.
50. Lalonde R and Strazielle C: The relation between open-field and emergence tests in a hyperactive mouse model. Neuropharmacology 57: 722-724, 2009.
51. Lister RG: The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) 92: 180-185, 1987.
52. Kordás K, Kis-Varga Á, Varga A, Eldering H, Bulthuis R, Lendvai B, Lévay G and Román V: Measuring sociability of mice using a novel three-chamber apparatus and algorithm of the LABORAS™ system. J Neurosci Methods 343: 108841, 2020.
53. Kim JW, Seung H, Kwon KJ, Ko MJ, Lee EJ, Oh HA, Choi CS, Kim KC, Gonzales EL, You JS, *et al*: Subchronic treatment of donepezil rescues impaired social, hyperactive, and stereotypic behavior in valproic acid-induced animal model of autism. PLoS One 9: e104927, 2014.
54. Walker JM, Fowler SW, Miller DK, Sun AY, Weisman GA, Wood WG, Sun GY, Simonyi A and Schachtman TR: Spatial learning and memory impairment and increased locomotion in a transgenic amyloid precursor protein mouse model of Alzheimer's disease. Behav Brain Res 222: 169-175, 2011.
55. Jackson M, Chen S, Nguyen TT, Siedhoff HR, Balderrama A, Zuckerman A, Li R, Greenlief CM, Cole G, Frautschy SA, *et al*: The chronic effects of a single low-intensity blast exposure on phosphoproteome networks and cognitive function influenced by mutant tau overexpression. Int J Mol Sci 25: 3338, 2024.
56. Bache N, Geyer PE, Bekker-Jensen DB, Hoerning O, Falkenby L, Treit PV, Doll S, Paron I, Müller JB, Meier F, *et al*: A novel LC system embeds analytes in pre-formed gradients for rapid, ultra-robust proteomics. Mol Cell Proteomics 17: 2284-2296, 2018.
57. Skowronek P, Thielert M, Voytik E, Tanzer MC, Hansen FM, Willems S, Karayel O, Brunner AD, Meier F and Mann M: Rapid and in-depth coverage of the (phospho-)proteome with deep libraries and optimal window design for dia-PASEF. Mol Cell Proteomics 21: 100279, 2022.
58. Nillert N, Pannangrong W, Welbat JU, Chaijaroonkhanarak W, Sripanidkulchai K and Sripanidkulchai B: Neuroprotective Effects of aged garlic extract on cognitive dysfunction and neuroinflammation induced by β-amyloid in rats. Nutrients 9: 24, 2017.
59. Li F and Kim MR: Effect of aged garlic ethyl acetate extract on oxidative stress and cholinergic function of scopolamine-induced cognitive impairment in mice. Prev Nutr Food Sci 24: 165-170, 2019.
60. Pannangrong W, Welbat JU, Chaichun A and 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 69: 269-278, 2020.
61. Jeong JH, Jeong HR, Jo YN, Kim HJ, Shin JH and Heo HJ: Ameliorating effects of aged garlic extracts against Aβ-induced neurotoxicity and cognitive impairment. BMC Complement Altern Med 13: 268, 2013.

62. Mukherjee D and Banerjee S: Learning and memory promoting effects of crude garlic extract. Indian J Exp Biol 51: 1094-1100, 2013.
63. Nishiyama N, Moriguchi T and Saito H: Beneficial effects of aged garlic extract on learning and memory impairment in the senescence-accelerated mouse. Exp Gerontol 32: 149-160, 1997.
64. Chauhan NB and Sandoval J: Amelioration of early cognitive deficits by aged garlic extract in Alzheimer's transgenic mice. Phytother Res 21: 629-640, 2007.
65. Sripanidkulchai B: Benefits of aged garlic extract on Alzheimer's disease: Possible mechanisms of action. Exp Ther Med 19: 1560-1564, 2020.
66. Subramanyam AA, Kedare J, Singh OP and Pinto C: Clinical practice guidelines for geriatric anxiety disorders. Indian J Psychiatry 60 (Suppl 3): S371-S382, 2018.
67. Gilhotra N and Dhingra D: GABAergic and nitriergic influence in antianxiety-like activity of garlic in mice. J Appl Pharm Sci 6: 077-085, 2016.
68. Nelson PT, Alafuzoff I, Bigio EH, Bouras C, Braak H, Cairns NJ, Castellani RJ, Crain BJ, Davies P, Del Tredici K, *et al*: Correlation of Alzheimer disease neuropathologic changes with cognitive status: A review of the literature. J Neuropathol Exp Neurol 71: 362-381, 2012.
69. Ziontz J, Bilgel M, Shafer AT, Moghekar A, Elkins W, Helphrey J, Gomez G, June D, McDonald MA, Dannals RF, *et al*: Tau pathology in cognitively normal older adults. Alzheimers Dement (Amst) 11: 637-645, 2019.
70. Chow VW, Mattson MP, Wong PC and Gleichmann M: An overview of APP processing enzymes and products. Neuromolecular Med 12: 1-12, 2010.
71. Lanoiselée HM, Nicolas G, Wallon D, Rovelet-Lecrux A, Lacour M, Rousseau S, Richard AC, Pasquier F, Rollin-Sillaire A, Martinaud O, *et al*: APP, PSEN1, and PSEN2 mutations in early-onset Alzheimer disease: A genetic screening study of familial and sporadic cases. PLoS Med 14: e1002270, 2017.
72. Tapia-Arancibia L, Aliaga E, Silhol M and Arancibia S: New insights into brain BDNF function in normal aging and Alzheimer disease. Brain Res Rev 59: 201-220, 2008.
73. Croll SD, Ip NY, Lindsay RM and Wiegand SJ: Expression of BDNF and trkB as a function of age and cognitive performance. Brain Res 812: 200-208, 1998.
74. Erickson KI, Miller DL and Roecklein KA: The aging hippocampus: Interactions between exercise, depression, and BDNF. Neuroscientist 18: 82-97, 2012.
75. Morrison JH and Baxter MG: The ageing cortical synapse: hallmarks and implications for cognitive decline. Nat Rev Neurosci 13: 240-250, 2012.
76. Ray B, Chauhan NB and Lahiri DK: Oxidative insults to neurons and synapse are prevented by aged garlic extract and S-allyl-L-cysteine treatment in the neuronal culture and APP-Tg mouse model. J Neurochem 117: 388-402, 2011.
77. Fan X, Cui L, Zeng Y, Song W, Gaur U and Yang M: 14-3-3 proteins are on the crossroads of cancer, aging, and age-related neurodegenerative disease. Int J Mol Sci 20: 3518, 2019.

Copyright © 2025 Mony et al. This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.