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Nutritional interventions on age-related neurodegenerative diseases

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Tiantian Zhang, Zhigang Liu and Yashi Mi

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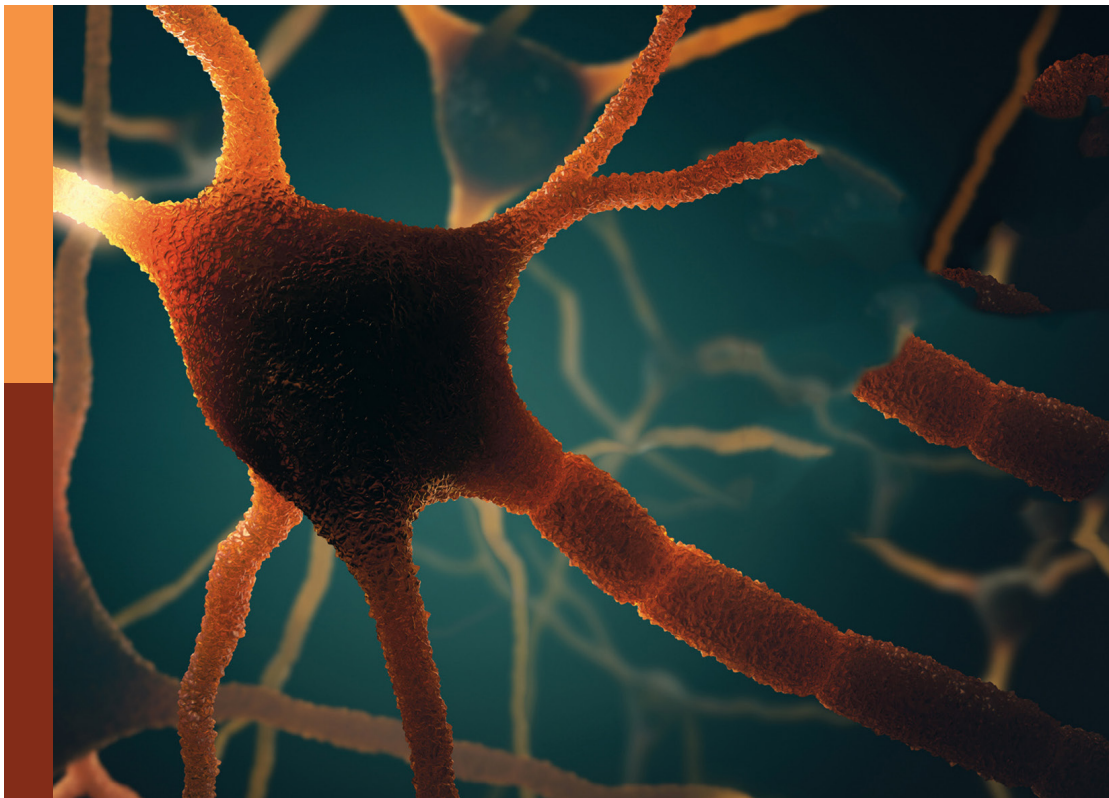

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Nutritional interventions on age-related neurodegenerative diseases

Topic editors

Tiantian Zhang — Ocean University of China, China
Zhigang Liu — Northwest A&F University, China
Yashi Mi — University of Arizona, United States

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Kristy A. Nielson,
Marquette University, United States

*CORRESPONDENCE
Tiantian Zhang
✉ zhangtiantian@ouc.edu.cn

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Editorial: Nutritional interventions on age-related neurodegenerative diseases

Tiantian Zhang[1]*, Zhigang Liu[2] and Yashi Mi[3]

[1]College of Food Science and Engineering, Ocean University of China, Qingdao, China, [2]College of Food Science and Engineering, Northwest A&F University, Yangling, China, [3]Center for Innovation in Brain Science, University of Arizona, Tucson, AZ, United States

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Editorial on the Research Topic
Nutritional interventions on age-related neurodegenerative diseases

With population aging, tactics to promote healthy aging and counteract the progress of age-related neurodegenerative diseases have become more and more important. A balanced and nutritious diet is vital for keeping fit, especially as age increases. The brain has a high requirement for nutrition, and nutritional imbalance can inhibit its structural and functional integrity, seriously affecting cognitive functioning (Dienel, 2019). Recently, it has been reported that nutritional strategies can decrease the risk of age-related neurodegenerative diseases and play a potentially beneficial role in decelerating the progression of brain diseases and retarding the development of some conditions (Cunnane et al., 2020). A number of investigations have indicated that nutritional interventions could enhance the cognitive ability in sufferers with Alzheimer's disease (AD) (Amini et al., 2020). However, the study connecting the effects of nutritional interventions with age-related neurodegenerative diseases is still in the early stages. It is presently unclear whether dietary constituents affect and regulate brain senescence and neuro regression, especially the molecular mechanisms by which nutritional interventions promote brain health. Developing efficacious nutritional interventions to promote wellness aging is becoming a burgeoning and challenging field.

The prime objective of this Research Topic is to explore the latest important findings related to nutritional interventions for age-related neurodegenerative diseases, and further explore the functional meaning of nutrition on brain health and cognitive ability for the elderly people. The current Research Topic covers 12 articles that provide in-depth insight into the effects of nutritional interventions on age-related neurodegenerative diseases.

A prospective longitudinal study by Sun B. et al. aimed to investigate the association of undernourishment with cognitive performances in the Chinese population using the Chinese Longitudinal Healthy Longevity Survey data from 2011 to 2012, 2014, and 2017 to 2018. There was a significant relationship between the Geriatric Nutritional Risk Index and cognitive performances among the elderly in China. Moreover, individuals with more severe malnutrition have poorer cognitive performance, especially in the oldest illiterate women. The results suggested that clinicians should pay more attention to evaluating the nutritional and cognitive status of the elderly in order to promptly intervene and guard against cognitive dysfunction. In addition, an ancillary MAPT-MRI study by Perus et al. determined the effect of multidisciplinary preventive methods on functional brain connectivity in elderly individuals with cognitive impairment, and the results emphasized the significance of cognitive decline status for preventive interventions.

Currently, dietary recommendations for preventing age-related neurodegenerative diseases are yet not largely accepted in guidelines because of contradictions and limited support. A prospective cohort analysis involving 6,647 men and women aged 55–75 years conducted by Nishi et al. aimed to determine the relation between baseline adherence to three prior dietary patterns, namely the Mediterranean diet (MedDiet), the Dietary Approaches to Stop Hypertension (DASH), and the MedDiet-DASH Intervention for Neurodegenerative Delay (MIND), with 2-year change in cognitive function in elderly individuals who were overweight or obese and at a high risk of cardiovascular disease. In elderly Spanish populations who are overweight or obese and have a higher risk of cardiovascular disease, higher baseline adherence to the MedDiet dietary pattern might be related to better cognitive function, compared to lower adherence within 2 years, while higher adherence to the DASH dietary pattern was unconnected with better cognitive function over a 2-year period. Yang et al. found that the ketogenic diet prevented chronic sleep deprivation-induced AD by decreasing ferroptosis and enhancing the neuronal recovery ability via Sirt1/Nrf2 signaling pathway. The ketogenic diet and caloric restriction will increase ketone bodies, especially β-hydroxybutyrate (BHB). Sun W. et al. found that β-hydroxybutyrate treatment avoided myelin loss, reduced the activation of astrocyte and microglia, and activated the neurotrophin brain-derived neurotrophic factor in the corpus callosum and hippocampus.

Vitamins play a significant role in the maintenance of human physiological functions. A quantitative meta-analysis involving 12 studies (n = 1,100) by Hamid et al. tried to determine the connection between vitamin C concentration in plasma and AD while emphasizing the significance and engagement of vitamin C in the etiopathogenesis of AD. Vitamin C deficiency was associated with AD progression, and vitamin C intervention might be a reasonable prevention and treatment method. Notably, clinical research is necessary to illustrate its specific mechanism and role in the pathophysiology and prevention of AD. A case-control study by Cheng et al. involving 360 older people from communities in China evaluated the mediating impact of inflammation on the correlation between vitamin D levels and mild cognitive impairment (MCI). Vitamin D deficiency might enhance the risk of cognitive impairment through a mechanism partially involved in inflammation, thereby vitamin D treatment might improve or delay the cognitive decline caused by inflammation in older people.

Phytochemical ingredients have shown anti-amyloid production, antioxidant, anti-inflammatory and neurotrophic characteristics, which may serve as lead candidates for further prevention of age-related neurodegenerative diseases (Pohl and Kong Thoo Lin, 2018; Yan et al., 2022). Cao et al. detected the levels of β-carboline alkaloids (βCBs), such as harmine, in plasma and tissues of pup rats, aging rats, mice of different physiological states, and healthy volunteers using UPLC-MS/MS and found that the concentration of harmine showed a decreasing trend with advanced age. Zhang et al. found that the food flavoring agent β-caryophyllene could protect neurons and inhibit β-amyloid's neurotoxicity mainly through the JAK2-STAT3-BACE1 pathway. The review "Benefits of dietary polyphenols in Alzheimer's disease" by Wang et al. summarized some of the more promising dietary phytochemicals, especially polyphenols. These substances have been proved to actively regulate some important pathogenesis of AD, such as decreasing β-amyloid plaques and neurofibrillary tangles formation, oxidative stress, neuroinflammation, and synaptic loss. Moreover, this review discussed the latest progress on the potential contribution of gut microbiome in the function of dietary polyphenols.

Seafood is rich in n-3 long-chain polyunsaturated fatty acids, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which play a vital role in brain function (Zhang et al., 2019). Feng et al. found that seafood-derived plasmalogens could protect SH-SY5Y cells against β-amyloid induced toxicity via regulating the transcripts associated with endocytosis, autophagy, apoptosis, neurotransmitter release, and synaptic transmission.

The complex pathogenesis of age-related neurodegenerative diseases results in limited treatment effectiveness, irreversible development of diseases, and significant socio-economic and personal costs (Li et al., 2020). It is necessary to develop a multi-objective diet to prevent the occurrence and development of neurodegenerative diseases. Due to complementary nutritional components, foods from both land and sea contributes to human wellbeing by coordinating resources and nutrient balance. Based on the perspective of the sea-land combination, Wang et al. studied the impacts of Antarctic krill oil (AKO) combined with nobiletin (Nob) and L-theanine (The) on memory loss and cognitive impairment in senescence-accelerated prone 8 mice (SAMP8). Findings showed that AKO exhibited synergistic effects with Nob and The in alleviating recognition memory and spatial memory deficits in SAMP8 mice, respectively. AKO showed synergistic effects with Nob in inhibiting β-amyloid accumulation, neurofibrillary tangles, and apoptosis and neuroinflammation, while the cooperation of AKO and The was involved in synaptic plasticity and anti-neuroinflammation, which indicated that the combination was complex rather than a mechanical addition.

In general, this Research Topic emphasizes the latest advances and innovations in the area of nutritional interventions on age-related neurodegenerative diseases. The Research Topic covers the prevention and treatment of age-related neurodegenerative diseases by the alteration of dietary patterns, macronutrients, micronutrients, and phytochemical compounds. Moreover, the Research Topic covers potential use of combining types of functional ingredients from sea and land toward developing multi-target functional foods. Notably, food is a complex system in which food components can interact, whereby each component can promote or antagonize the effectiveness of other components. In addition, there is inter-individual variability in response to nutritional interventions and their effectiveness for improving cognitive ability. Thus, future research and development directions in this important area should focus on the interaction between food components in alleviating age-related neurodegenerative diseases and personalized nutritional interventions.

Author contributions

TZ wrote the article. ZL and YM reviewed the article and provided critical feedback. All authors

contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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ORIGINAL RESEARCH
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The Relationship of Malnutrition With Cognitive Function in the Older Chinese Population: Evidence From the Chinese Longitudinal Healthy Longevity Survey Study

*Boran Sun, Yihao Zhao, Wenli Lu and Yongjie Chen**

Department of Epidemiology and Statistics, School of Public Health, Tianjin Medical University, Tianjin, China

Background and Objective: Few studies have explored the relationship between malnutrition measured by the Geriatric Nutritional Risk Index (GNRI) and cognitive performance. This study aimed to investigate the association of malnutrition with cognitive function in the Chinese population.

Methods: It was a prospective longitudinal study and used three waves of the Chinese Longitudinal Healthy Longevity Survey (CLHLS) data in 2011–2012, 2014, and 2017–2018. Participants aged 60 years or older without mental illness and cerebrovascular diseases were eligible. The GNRI was used to assess nutritional status as follows: normal nutrition (a GNRI $>$ 98), mild malnutrition (92 $\leq$ a GNRI $\leq$ 98), and moderate-to-severe malnutrition (a GNRI $<$ 92). Cognitive performance was evaluated by the Mini-Mental State Examination (MMSE) scores. The relationship between the GNRI and cognitive function was analyzed using a linear mixed-effects model.

Results: A total of 1,632 subjects were analyzed, including 741 males and 891 females. Of these, 65.0, 19.4, and 15.6% of subjects were at normal nutritional status, mild, and moderate-to-severe malnutrition, respectively. After adjusting for potential confounders, participants under mild and moderate-to-severe malnutrition status have a lower MMSE score [β (95% CI): –0.95 (–1.60, –0.25) and –1.39 (–2.21, –0.57), respectively], compared with those having normal nutrition. Also, there was a linear trend in the association of malnutrition risk with cognitive function in the total population [β (95% CI): –0.74 (–1.13, –0.35)]. However, a significant association of malnutrition with cognitive function was observed only among illiterate females aged above 90 years.

Conclusion: This study suggested that there was a significant relationship between the GNRI and cognitive function in the Chinese elderly. Furthermore, subjects with more serious malnutrition have a worse cognitive function, especially in the oldest illiterate females. Clinicians should put more emphasis on assessing the nutritional and cognitive status of the elderly to timely intervene and prevent cognitive impairment.

Keywords: geriatric nutritional risk index, malnutrition, cognitive function, Chinese elderly, linear mixed-effects model

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Tiantian Zhang,
Ocean University of China, China

Reviewed by:
Xiao Zhen Lv,
Peking University, China
Che Hongxia,
Qingdao University of Science and Technology, China

****Correspondence:***
Yongjie Chen
chenyongjie@tmu.edu.cn

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INTRODUCTION

It is estimated that more than 1.5 billion people will be aged 65 or older by 2050, accounting for 16% of the total population. Consequently, further attention should be paid to the physical and mental health of the elderly (United Nations, 2019). With the population aging, malnutrition caused by an energy imbalance between the intake and requirements has been a serious global public concern. As a previous review reported, 23–60% of the hospitalized elders were malnourished in developed countries (Agarwal et al., 2013), while 48.4% of community-dwelling elderly were identified to have a high malnourished risk in China (Kang et al., 2018). Older people with malnutrition will have increased risks of fall (Lackoff et al., 2020), frailty (Hong et al., 2019), prolonged length of stay in the hospital, morbidity, and mortality (Correia and Waitzberg, 2003; Lim et al., 2012; Leiva Badosa et al., 2017).

Meanwhile, mild cognitive impairment (MCI), as a transitional state between normal aging and dementia, has affected 10–15% of the population aged 65 and above (Anderson, 2019) and gradually became a serious public health problem among old people worldwide. Cognitive impairment is characterized by a decline in memory, attention, language, and other cognitive function beyond age (Eshkoor et al., 2015), which had imposed great burdens on family and society. Compared with those with stable cognitive function, participants with rapid cognitive decline had a 75% higher risk of death (Lv et al., 2019). The prevalence of cognitive impairment was about 16.5 and 16.8% in the European and Chinese elderly, respectively (Hou et al., 2019; Pais et al., 2020). Moreover, 12–15% of old people with MCI will develop irreversible dementia within 1 year (Hill et al., 2017). As there were still no effective disease-modifying pharmacologic treatments for dementia (Tisher and Salardini, 2019), it is crucial to identify protective factors and improve early detection for MCI.

Previous studies have demonstrated that nutritional status played a significant role in the progression of cognitive impairment in older patients with dementia or Alzheimer's disease (AD) (Ousset et al., 2008; Santos et al., 2018; Kimura et al., 2019; Doorduijn et al., 2020). Recent researches have also illustrated that malnutrition was associated with an increased risk of cognitive decline among older adults (Hsu et al., 2019; Assis et al., 2020; Mantzorou et al., 2020; Yu et al., 2021). However, most of the studies have explored the relationship by utilizing Mini Nutritional Assessment (MNA) (Assis et al., 2020; Mantzorou et al., 2020) or Mini Nutritional Assessment Short-Form (MNA-SF) (Hsu et al., 2019; Yu et al., 2021), but not the Geriatric Nutritional Risk Index (GNRI). MNA and MNA-SF were defined as those not using biological indicators, such as albumin. While the GNRI combines two nutritional indicators, namely, albumin and weight loss. Furthermore, usual body weight was replaced by ideal body weight in the NRI formula when usual weight failed to be obtained in the elderly population. The GNRI was initially developed to evaluate the nutrition-related risk in hospitalized older people (Bouillanne et al., 2005). Until present, the GNRI could comprehensively identify the elderly under malnutrition risk and has been proved to predict morbidity and mortality (Hao et al., 2019; Xie et al., 2020). The area under the curve (AUC) of the GNRI predicting a composite of events such as all-cause death was 0.873 in the Chinese population (Zhao Q. et al., 2020). The validity and reliability of the GNRI among Chinese or Asian older adults have been reported in previous studies (Abd Aziz et al., 2019; Nishi et al., 2019; Zhao Y. et al., 2020).

Accordingly, our study aimed to further investigate the relationship of malnutrition, measured by the GNRI, and cognitive function in the Chinese elderly, based on the three waves of the Chinese Longitudinal Healthy Longevity Survey (CLHLS) data in 2011–2012, 2014, and 2017–2018.

MATERIALS AND METHODS

Study Design and Population

Data were based on the CLHLS study. The CLHLS is a nationwide community-based prospective longitudinal study, aiming to investigate health status and factors influencing longevity in the Chinese elderly. Its baseline survey began in 1998, and seven follow-up survey waves were conducted in 2000, 2002, 2005, 2008–2009, 2011–2012, 2014, and 2017–2018, respectively (Yi, 2008; Zeng, 2012). To obtain representative information of Chinese populations at an advanced age, the CLHLS study oversampled the oldest-old and was enriched for centenarians, such as 19.5 thousands centenarians, 26.8 thousands nonagenarians, and 29.7 thousands octogenarians (Zeng, 2013; Zeng et al., 2017). The validity of the CLHLS has been widely reported elsewhere (Gu et al., 2009, 2017).

In addition, a biomedical in-depth CLHLS study was performed in the CLHLS of waves 2008–2009, 2011–2012, and 2014 in sequence. During the in-depth study, physical examinations and almost 30 biomarkers in blood and urine samples were collected by the trained medical staff of the Chinese Center for Disease Control and Prevention local network. More details of the in-depth CLHLS study have been published elsewhere (Zeng et al., 2017; Lv et al., 2019; Liu et al., 2020). The CLHLS study was approved by the Institutional Review Board, Duke University (Pro00062871), and the Biomedical Ethics Committee, Peking University (IRB00001052–13074). All participants gave written informed consent.

In this study, we used three waves of the CLHLS data in 2011–2012, 2014, and 2017–2018. The inclusion criteria were as follows: participants aged 60 years or older; and participants with complete baseline information of the GNRI and Mini-Mental State Examination (MMSE) score. The exclusion criteria were as follows: participants with missing data in covariates; and participants with a history of intracranial tumor, craniocerebral injury, acute infection, mental illness, and cerebrovascular diseases, which were self-reported. Eventually, 1,632 eligible participants were included. The detailed information is presented in **Figure 1**. If the baseline survey was conducted in the wave 2011–2012, participants were followed up consecutively in the 2014 and 2017–2018 waves. Otherwise, if the baseline survey was only in the 2014 wave, follow-up was conducted in the

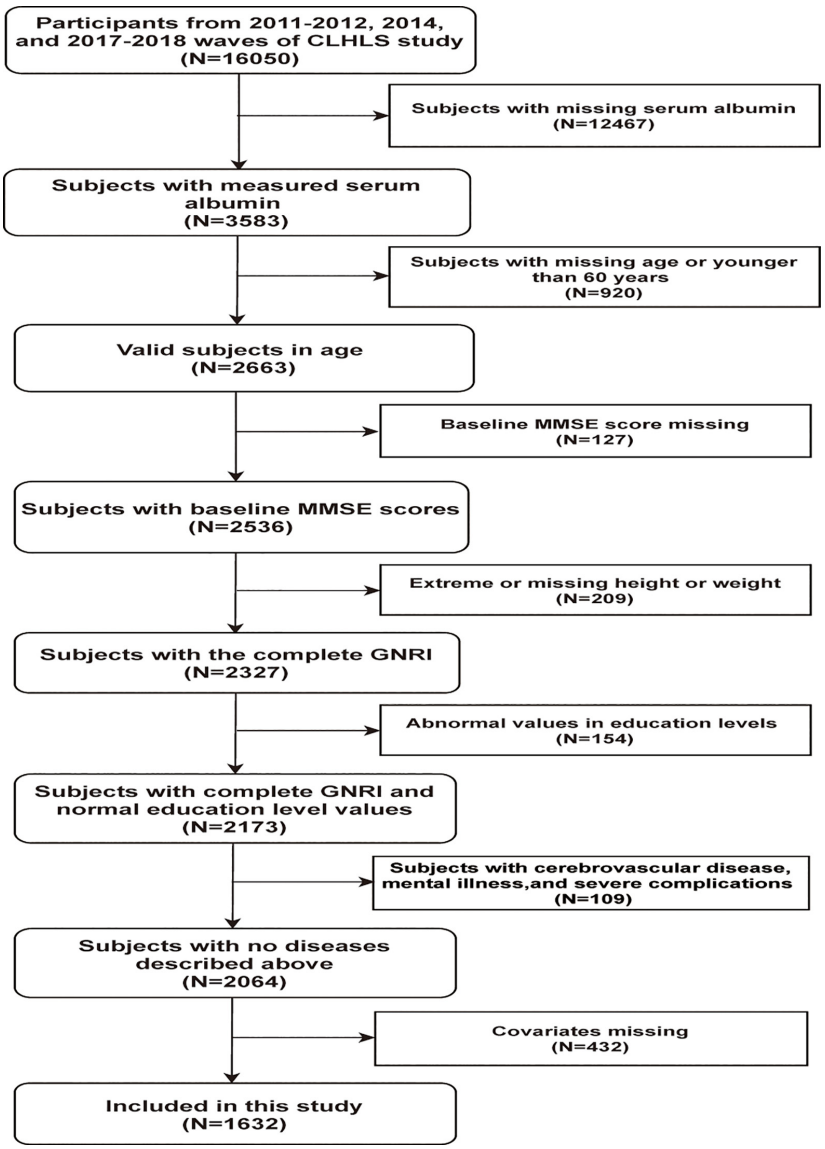

FIGURE 1 | Flowchart of the study population.

wave 2017–2018. Therefore, there is no overlap in our final analyzed population.

Data Collection

The baseline data were collected using a standardized questionnaire in a home-based interview by trained investigators in 2011–2012 and 2014 waves. The information included (1) sociodemographic characteristics such as age, sex (male/female), ethnicity (Han/other), residence (urban/rural), marital status (married/other), education level (no schooling/some schooling), and living alone (yes/no), (2) lifestyle-related variables such as current smoking status (yes/no), drinking status (yes/no), and regular exercising (yes/no), and (3) health status involving a self-reported history of heart disease (yes/no), hypertension (yes/no), diabetes (yes/no), and arthritis (yes/no). Participants were divided into three age groups: 60–79 years, 80–89 years, and $\geq$90 years.

The participants were asked to report their food intake frequency of last month of vegetables, meat, eggs, and fish. The intake frequencies of meat, fish, and eggs were recorded as "almost every day," "occasionally," or "rarely or never." While for vegetables, the intake frequency was "almost every day," "almost every day except in winter," "occasionally," and "rarely or never" (Shi et al., 2015). In the analysis, we classified the frequency of "almost every day except in winter" as "almost every day." The ability of basic activities of daily living (BADL) was measured by a question: Do you need assistance in bathing/dressing/toileting/transferring/eating/continence? According to a previous study using the data of the CLHLS, a score of 0 was given if no help was needed, and a score of 1 was

given if some or complete help was needed. The BADL score ranged from 0 to 6 (Zeng et al., 2017). Based on the BADL score, we divided participants into having BADL disability (BADL score > 0) and not having BADL disability (BADL score = 0).

After the interview, the anthropometric measurements were carried out to obtain height (in meters) and weight (in kilograms). All measurements were carried out three times, and the average values were recorded. Body mass index (BMI) was calculated as weight divided by the square of height.

Serum albumin was measured by trained personnel using an automatic biochemistry analyzer (Hitachi 7180, Japan; Roche Diagnostic, Mannheim, Germany) at the central laboratory at the Capital Medical University in Beijing.

Nutritional Status Assessment

In this study, the GNRI was used to evaluate nutritional status. The GNRI is calculated by albumin and weight loss as follows: GNRI = [1.489 × serum albumin (g/L)] + [41.7 × (actual weight/ideal weight)] (Bouillanne et al., 2005). Weight loss is reflected by ideal body weight and actual weight. Different Lorentz formulas were used to calculate the ideal weight according to sex. For males, ideal weight was calculated by 0.75 × height (cm)–62.5, and ideal weight was obtained using 0.60 × height (cm)–40 for females (Matsuzawa et al., 1990). If the actual weight is greater than the ideal weight, the actual weight/ideal weight is set to 1 (Matsuzawa et al., 1990; Bouillanne et al., 2005). Participants were divided into four groups based on the GNRI as follows: normal nutrition group, a GNRI > 98; mild malnutrition group, a GNRI ≥ 92 but ≤ 98; moderate malnutrition group, a GNRI ≥ 82 but < 92; and severe malnutrition group, a GNRI < 82 (Bouillanne et al., 2005). In this study, since serum albumin failed to be collected in the wave 2008–2009, the GNRI was calculated only using the data of 2011–2012 and 2014 waves in the CLHLS. Moderate and severe malnutrition groups were combined into a moderate-to-severe group. The validity and reliability of the GNRI have been reported in previous studies (Abd Aziz et al., 2019; Nishi et al., 2019; Zhao Q. et al., 2020).

Cognitive Function Evaluation

In each wave of the CLHLS, cognitive function was assessed by the Chinese version of MMSE, which was adapted from the international version that was proposed by Folstein et al. (1975) and validated in previous publications (An and Liu, 2016; Zeng et al., 2017; Zhang et al., 2019). In view of cultural and socioeconomic factors in the Chinese elderly, the Chinese version of the MMSE consists of 13 questions, covering 5 domains of cognitive function, namely, orientation, registration, attention and calculation, recall, and language. With total scores ranging from 0 to 30, a lower score of MMSE indicated a poorer cognitive function. More details about the Chinese version of MMSE are shown in **Supplementary Table 1**.

Statistical Analysis

Continuous variables with normal distribution were presented as means ± SD. Non-normal variables were reported as median (P_{25}, P_{75}), whereas categorical variables were expressed as frequencies (percentages). The distributions of baseline characteristics were compared by one-way ANOVA, Kruskal–Wallis non-parametric test, and χ^2 test across the normal, mild malnutrition, and moderate-to-severe malnutrition groups. For the primary analysis, a linear mixed-effects model was employed to estimate the effects of malnutrition on cognitive function, adjusting for age, sex, ethnicity, residence, marital status, education, living alone, regular exercising, smoking, drinking, history of heart diseases, diabetes, hypertension, and arthritis, BADL disability, and the frequencies of meat, fish, eggs, and vegetables. In the linear mixed-effects model, the MMSE scores from waves 2011–2012, 2014, and 2017–2018 were response variables, and malnutrition assessed by the GNRI was an independent variable. Furthermore, the linear mixed-effects model was stratified by sex, age, and education level. Four sensitivity analyses were conducted. First, the GNRI as a continuous variable was considered as an independent variable in the linear mixed-effects model. Second, serum albumin and BMI were used to construct linear mixed-effects models, respectively. Third, the mediation analysis was used to examine whether the association between malnutrition and cognitive impairment was mediated by hypertension and diabetes. Finally, we imputed the missing covariates using the R software package "*mice*" to evaluate the influence of missing covariates on the results. Two-tailed $P < 0.05$ was considered to be statistically significant, and all analyses were conducted using SAS 9.4 (SAS Institute Inc., Cary, NC, United States).

RESULTS

Characteristics of Participants

Among 1,632 subjects, the average age was 85.57 ± 12.35 years old, and 54.6% of subjects were females. The proportion of participants in the normal nutrition, mild malnutrition, and moderate-to-severe malnutrition groups was 65.0, 19.4, and 15.6%, respectively. As shown in **Table 1**, the risk of malnutrition was higher in an older female with a lower MMSE score, BMI, and serum albumin concentration ($P < 0.001$). Also, subjects who have been married ($P < 0.001$), had regular exercises ($P < 0.001$), and ever attended school ($P < 0.001$) were less likely to be malnourished. Additionally, in contrast with normal nutritional group, people under malnutrition status tended to have BADL disability ($P < 0.001$), rare or never eat meat ($P = 0.001$), fish ($P = 0.007$), eggs ($P = 0.015$), and vegetables ($P < 0.001$). However, ethnicity, residence, living alone, and history of heart diseases, diabetes, and arthritis were not significantly different across the three groups.

Association Between Malnutrition and Cognitive Function

Table 2 shows the association between malnutrition and cognitive function in the total population. Whether adjusting for covariates or not, people in mild and moderate-to-severe malnutrition groups have a poorer cognitive function (adjusted $P = 0.007$ and $P < 0.001$, respectively). Compared with the normal nutrition group, those with mild and moderate-to-severe

TABLE 1 | Characteristics of participants.

Characteristics	Total population (*n* = 1,632)	Nutritional status			*P*-value
		Normal (*n* = 1,060)	Mild malnutrition (*n* = 317)	Moderate-to-severe malnutrition (*n* = 255)	
Age (years, mean ± SD)[a]	85.57 ± 12.35	81.58 ± 11.88	90.96 ± 10.35	95.40 ± 7.67	<0.001
Height (cm, mean ± SD)[a]	153.65 ± 11.04	155.18 ± 11.21	151.05 ± 10.34	150.56 ± 9.88	<0.001
Weight (kg, mean ± SD)[a]	51.20 ± 12.58	55.45 ± 11.90	45.50 ± 9.87	40.63 ± 8.76	<0.001
BMI (kg/m^2, mean ± SD)[a]	21.53 ± 4.03	22.91 ± 3.36	19.87 ± 3.50	17.85 ± 3.03	<0.001
Serum albumin (g/L, mean ± SD)[a]	41.96 ± 4.40	44.13 ± 2.95	39.71 ± 2.82	35.71 ± 3.63	<0.001
Sex[c], n (%)					<0.001
Male	741 (45.4)	553 (52.2)	111 (35.0)	77 (30.2)	
Female	891 (54.6)	507 (47.8)	206 (65.0)	178 (69.8)	
Ethnicity[c], n (%)					0.488
Han	1,406 (86.2)	906 (85.5)	275 (86.8)	225 (88.2)	
Other	226 (13.8)	154 (14.5)	42 (13.2)	30 (11.8)	
Residence[c], n (%)					0.228
Urban	1,582 (96.9)	1,032 (97.4)	307 (96.8)	243 (95.3)	
Rural	50 (3.1)	28 (2.6)	10 (3.2)	12 (4.7)	
Marital status[c], n (%)					<0.001
Married	648 (39.7)	524 (49.4)	91 (28.7)	33 (12.9)	
Other	984 (60.3)	536 (50.6)	226 (71.3)	222 (87.1)	
Education[c], n (%)					<0.001
No schooling	1,043 (63.9)	589 (55.6)	239 (75.4)	215 (84.3)	
Some schooling	589 (36.1)	471 (44.4)	78 (24.6)	40 (15.7)	
Living alone c, n (%)					0.289
Yes	345 (21.1)	212 (20.0)	72 (22.7)	61 (23.9)	
No	1,287 (78.9)	848 (80.0)	245 (77.3)	194 (76.1)	
Smoking[c], n (%)					0.008
Yes	285 (17.5)	207 (19.5)	47 (14.8)	31 (12.2)	
No	1,347 (82.5)	853 (80.5)	270 (85.2)	224 (87.8)	
Drinking[c], n (%)					0.004
Yes	291 (17.8)	212 (20.0)	49 (15.5)	30 (11.8)	
No	1,341 (82.2)	848 (80.0)	268 (84.5)	225 (88.2)	
Regular exercising[c], n (%)					0.001
Yes	255 (15.6)	185 (17.5)	49 (15.5)	21 (8.2)	
No	1,377 (84.4)	875 (82.5)	268 (84.5)	234 (91.8)	
Heart disease[c], n (%)					0.116
Yes	118 (7.2)	87 (8.2)	17 (5.4)	14 (5.5)	
No	1,514 (92.8)	973 (91.8)	300 (94.6)	241 (94.5)	
Hypertension[c], n (%)					<0.001
Yes	1,006 (61.6)	699 (65.9)	171 (53.9)	136 (53.3)	
No	626 (38.4)	361 (34.1)	146 (46.1)	119 (46.7)	
Diabetes[c], n (%)					0.177
Yes	31 (1.9)	25 (2.4)	3 (0.9)	3 (1.2)	
No	1,601 (98.1)	1,035 (97.6)	314 (99.1)	252 (98.8)	
Arthritis c, n (%)					0.938
Yes	136 (8.3)	87 (8.2)	28 (8.8)	21 (8.2)	
No	1,496 (91.7)	973 (91.8)	289 (91.2)	234 (91.8)	
BADL disability					<0.001
Yes	265 (16.2)	87 (8.2)	77 (24.3)	101 (39.6)	
No	1,367 (83.8)	973 (91.8)	240 (75.7)	154 (60.4)	
Meat intake					0.001
Almost every day	1,455 (89.2)	961 (90.7)	278 (87.7)	216 (84.7)	
Occasionally	96 (5.9)	61 (5.8)	21 (6.6)	14 (5.5)	

(Continued)

TABLE 1 | (Continued)

Characteristics	Total population (*n* = 1,632)	Nutritional status			*P*-value
		Normal (*n* = 1,060)	Mild malnutrition (*n* = 317)	Moderate-to-severe malnutrition (*n* = 255)	
Rarely or never	81 (4.9)	38 (3.5)	18 (5.7)	25 (9.8)	
Fish intake					0.007
Almost every day	1,265 (77.5)	830 (78.3)	243 (76.7)	192 (75.3)	
Occasionally	211 (12.9)	140 (13.2)	47 (14.8)	24 (9.4)	
Rarely or never	156 (9.6)	90 (8.5)	27 (8.5)	39 (15.3)	
Eggs intake					0.015
Almost every day	1,410 (86.4)	936 (88.3)	268 (84.5)	206 (80.8)	
Occasionally	103 (6.3)	58 (5.5)	25 (7.9)	20 (7.8)	
Rarely or never	119 (7.3)	66 (6.2)	24 (7.6)	29 (11.4)	
Vegetable intake					<0.001
Almost every day	1,416 (86.8)	947 (89.3)	268 (84.5)	201 (78.8)	
Occasionally	165 (10.1)	90 (8.5)	38 (12.0)	37 (14.5)	
Rarely or never	51 (3.1)	23 (2.2)	11 (3.5)	17 (6.7)	
MMSE score in 2011–2012 wave[b], median (P_{25}, P_{75})	28.0 (21.0, 29.0)	28.0 (25.0, 30.0)	25.0 (15.0, 29.0)	20.0 (11.0, 28.0)	<0.001
MMSE score in 2014 wave[b], median (P_{25}, P_{75})	27.0 (21.0, 29.0)	28.0 (24.0, 30.0)	23.0 (12.0, 27.0)	20.0 (6.0, 26.0)	<0.001
MMSE score in 2017–2018 wave[b], median (P_{25}, P_{75})	28.0 (23.0, 29.0)	28.0 (25.0, 29.0)	24.0 (15.0, 28.0)	24.0 (18.5, 28.5)	<0.001

BMI, body mass index; BADL, basic activities of daily living; MMSE, Mini-Mental State Examination.
[a]*These variables were analyzed using one-way ANOVA.*
[b]*These variables were analyzed using the Kruskal–Wallis non-parametric test.*
[c]*These variables were analyzed using the* χ^2 *test.*

malnutrition have a lower MMSE score of 0.95 and 1.39 points (adjusted β = –0.95, 95% *CI* = –1.60 to –0.25; adjusted β = –1.39, 95% *CI* = –2.21 to –0.57, respectively). Moreover, the MMSE score has shown a significant linear downward trend with increasing malnutrition degrees (adjusted *P* for trend < 0.001).

Table 3 shows the relationship between malnutrition and cognitive function stratified by sex. In males, after adjusting for covariates, a significant association was not found in both mild and moderate-to-severe malnutrition groups (adjusted *P* = 0.196 and *P* = 0.514, respectively). While for female subjects, there was a negative association of mild and moderate-to-severe malnutrition with cognitive function regardless of adjusting covariates or not (adjusted *P* = 0.024, β = –1.12, and 95% *CI* = –2.08 to –0.15; and *P* = 0.001, β = –1.94, and 95% *CI* = –3.09 to –0.79, respectively). A significant trend of a cognitive decline with increasing malnutrition degrees was observed in females (adjusted *P* for trend < 0.001) but not in males (adjusted *P* for trend = 0.289).

The association between malnutrition and cognitive function stratified by age is presented in **Table 4**. After adjusting for covariates, there was no association of mild and moderate-to-severe malnutrition with cognitive function among the elderly aged 60–79 years (adjusted *P* = 0.057 and *P* = 0.821, respectively) and 80–89 years (adjusted *P* = 0.695 and *P* = 0.490, respectively).

TABLE 2 | The association of malnutrition with cognitive function.

Nutrition status	Crude			Adjusted*		
	β	*P*	95% CI	β	*P*	95% CI
Normal (*n* = 1,060)	Ref	Ref	Ref	Ref	Ref	Ref
Mild (*n* = 317)	–4.84	<0.001	–5.57 to –4.10	–0.95	0.007	–1.60 to –0.25
Moderate–to–severe (*n* = 255)	–7.23	<0.001	–8.10 to –6.36	–1.39	<0.001	–2.21 to –0.57
P for trend	–3.88	<0.001	–4.28 to –3.48	–0.74	<0.001	–1.13 to –0.35

**In the adjusted model, age, sex, ethnicity, residence, marital status, education, living alone, regular exercising, smoking status, drinking status, heart disease history, diabetes history, hypertension history, arthritis history, BADL disability, and food intake frequency of vegetables, meat, eggs, and fish were adjusted.*

TABLE 3 | The association of malnutrition with cognitive function stratified by sex.

Subgroups	Crude			Adjusted*		
	β	*P*	95% CI	β	*P*	95% CI
Males (*n* = 741)						
Normal	Ref	Ref	Ref	Ref	Ref	Ref
Mild	–3.23	<0.001	–4.19 to –2.26	–0.61	0.196	–1.53 to 0.31
Moderate-to-severe	–4.30	<0.001	–5.51 to –3.11	–0.39	0.514	–1.55 to 0.78
P for trend	–2.42	<0.001	–2.96 to –1.89	–0.29	0.289	–0.83 to 0.25
Females (*n* = 891)						
Normal	Ref	Ref	Ref	Ref	Ref	Ref
Mild	–4.83	<0.001	–5.88 to –3.78	–1.12	0.024	–2.08 to –0.15
Moderate-to-severe	–7.70	<0.001	–8.90 to –6.50	–1.94	0.001	–3.09 to –0.79
P for trend	–4.03	<0.001	–4.60 to –3.46	–0.99	<0.001	–1.55 to –0.44

**In the adjusted model, age, ethnicity, residence, marital status, education, living alone, regular exercising, smoking status, drinking status, heart disease history, diabetes history, hypertension history, arthritis history, BADL disability, and food intake frequency of vegetables, meat, eggs, and fish were adjusted.*

TABLE 4 | The association of malnutrition with cognitive function stratified by age.

Subgroups	Crude			Adjusted*		
	β	*P*	95% CI	β	*P*	95% CI
60–79 years old (*n* = 515)						
Normal	Ref	Ref	Ref	Ref	Ref	Ref
Mild	–1.01	0.012	–1.79 to –0.22	–0.69	0.057	–1.40 to 0.02
Moderate-to-severe	–0.32	0.722	–2.09 to 1.45	–0.18	0.821	–1.77 to 1.40
P for trend	–0.63	0.037	–1.23 to 0.04	–0.51	0.093	–1.11 to 0.09
80-89 years old (*n* = 420)						
Normal	Ref	Ref	Ref	Ref	Ref	Ref
Mild	–0.75	0.176	–1.84 to 0.34	–0.21	0.695	–1.29 to 0.86
Moderate-to-severe	–1.02	0.162	–2.46 to 0.41	–0.51	0.490	–1.95 to 0.93
P for trend	–0.58	0.077	–1.21 to 0.06	–0.26	0.428	–0.91 to 0.39
≥90 years old (*n* = 697)						
Normal	Ref	Ref	Ref	Ref	Ref	Ref
Mild	–3.30	<0.001	–4.68 to –1.91	–2.04	0.003	–3.37 to –0.71
Moderate-to-severe	–4.39	<0.001	–5.84 to -2.95	–2.54	<0.001	–3.93 to –1.15
P for trend	–2.30	<0.001	–3.01 to –1.58	–1.33	<0.001	–2.03 to –0.63

**In the adjusted model, sex, ethnicity, residence, marital status, education, living alone, regular exercising, smoking status, drinking status, heart disease history, diabetes history, hypertension history, arthritis history, BADL disability, and food intake frequency of vegetables, meat, eggs, and fish were adjusted.*

While among the elderly above 90 years old, a significant association was observed between mild and moderate-to-severe malnutrition and cognitive function (adjusted $P = 0.003$, $\beta = -2.04$, and 95% $CI = -3.37$ to -0.71; and $P < 0.001$, $\beta = -2.54$, and 95% $CI = -3.93$ to -1.15, respectively). Moreover, a linear trend in the association of malnutrition risk with cognitive performance was also found in individuals aged 90 years and above (adjusted P for trend < 0.001).

After adjusting for covariates, a significant association and trend between malnutrition and cognitive impairment were found for the illiterate Chinese elderly (adjusted $P = 0.004$ and 0.007 for mild and moderate-to-severe malnutrition, respectively; adjusted P for trend = 0.002), while the association disappeared among the elderly who ever attended school (**Table 5**).

Sensitivity Analysis

As shown in **Supplementary Table 2**, there is a significant positive relationship of the continuous GNRI and serum albumin with cognitive function, regardless of adjusting or not (crude and adjusted $P < 0.001$). Namely, subjects with a higher GNRI or serum albumin have a better cognitive function, while BMI was not associated with cognitive decline (adjusted $P = 0.499$). In the mediation analysis, as shown in **Supplementary Table 3**, hypertension, rather than diabetes, had a significant partially mediating effect on the relationship between malnutrition and cognitive impairment. The mediating effect was –0.095 (–0.164 to –0.025), and the proportion mediated of hypertension was 9.65%. Moreover, as shown in **Supplementary Table 4**, a significant association remained between mild (adjusted $P = 0.024$) and moderate-to-severe (adjusted $P = 0.004$) malnutrition and cognitive function in the complete dataset.

TABLE 5 | The association of malnutrition with cognitive function stratified by education level.

Subgroups	Crude			Adjusted*		
	β	*P*	95% CI	β	*P*	95% CI
No schooling (*n* = 1,043)						
Normal	Ref	Ref	Ref	Ref	Ref	Ref
Mild	-4.87	<0.001	–5.85 to –3.88	–1.35	0.004	–2.26 to –0.43
Moderate-to-severe	–6.47	<0.001	–7.58 to –5.36	–1.45	0.007	–2.50 to –0.40
P for trend	–3.52	<0.001	–4.04 to –2.99	–0.82	0.002	–1.32 to –0.31
Schooling (*n* = 589)						
Normal	Ref	Ref	Ref	Ref	Ref	Ref
Mild	–1.56	<0.001	–2.45 to –0.67	0.20	0.663	–0.69 to 1.08
Moderate-to-severe	–4.56	<0.001	–5.84 to –3.29	–1.16	0.080	–2.46 to 0.14
P for trend	–2.05	<0.001	–2.59 to –1.51	–0.34	0.250	–0.91 to 0.24

**In the adjusted model, age, sex, ethnicity, residence, marital status, living alone, regular exercising, smoking status, drinking status, heart disease history, diabetes history, hypertension history, arthritis history, BADL disability, and food intake frequency of vegetables, meat, eggs, and fish were adjusted.*

DISCUSSION

A longitudinal study of 1,632 Chinese elderly was used to explore the relationship between malnutrition and cognitive function. We found that malnutrition was associated with cognitive function. Furthermore, subjects with more serious malnutrition have a poorer cognitive performance, especially in illiterate females and the oldest elderly. With increasing degrees of malnutrition, the cognitive function has shown a worsening trend.

Participants were classified into different nutrition status groups according to the GNRI. Notably, 19.4% of participants were under mild malnutrition risk, and 15.6% were moderate-to-severe malnourished. People with malnutrition status were older than those with well-nourished status. This result is in line with studies using alternative methods, such as MNA or MNA-SF, to assess the elderly malnutrition status (Mantzorou et al., 2020; Yu et al., 2021). With the increasing degrees of malnutrition, the MMSE score decreased with a linear trend, which indicated that the cognitive function has become worsening. This is consistent with the finding of a recent study, which revealed that severe nutritional status was related to a faster cognitive decline (Sanders et al., 2016).

Serum albumin, as an indicator to assess nutritional status and inflammation level, has been used to predict adverse clinical outcomes (Don and Kaysen, 2004; Abubakar et al., 2013). Recent studies have shown that a higher albumin level was associated with a lower risk of cognitive impairment (Llewellyn et al., 2010; Yin et al., 2016; Wang et al., 2018). Our results were comparable. Evidence suggested that serum albumin can combine with amyloid-β and can reduce its neurotoxicity by inhibiting its aggregation and fibrosis, thus preventing further progression of cognitive decline (Stanyon and Viles, 2012). Meanwhile, it is reported that BMI has an effect on energy metabolism through regulating body cell mass, which is one of the key factors to reflect nutritional status (Oliveira et al., 2020).

According to previous studies, overweight old people were found to have a lower risk of cognitive impairment or dementia compared with under or normal weight (Hou et al., 2019; Li et al., 2020). However, in our study, we found that BMI was not significantly associated with cognitive function. In fact, BMI may not be a useful tool to assess malnutrition among elders. It did not take into account the distribution of body fat and could not classify lean body mass and fat mass in the elderly (Goyal et al., 2014). A higher risk of cognitive decline in elders with a low BMI may be due to obesity with low muscle mass and increased fat (Riobó Serván et al., 2015). While the GNRI is based on serum albumin and weight loss, which was not selfsame with BMI. Furthermore, a higher weight was given for albumin than for weight according to the formula of the GNRI. Therefore, a significant association of malnutrition with cognitive function was reasonable.

In this study, a strong association between malnutrition and cognitive function was found among individuals aged 90 years and above. We speculated that there was a similar increasing trend of malnutrition risk and cognitive decline with aging, which caused this association. On the one hand, older age has been identified as a risk factor of malnutrition (Guyonnet and Rolland, 2015). Also, previous studies showed that with every 1-year increase in age, the risk of being under malnourished would increase by 8.5% (Wei et al., 2018). On the other hand, it was documented that more than half of the individuals above 60 years old would have a deteriorating cognitive function with aging. Also, the magnitude of the decline in cognitive function was greater in the older elderly (Davis et al., 2018; Zhang et al., 2019).

In addition, a significant association also appeared in female individuals and participants without attending school. A possible explanation might be due to socioeconomic disadvantages, lacking education, and worse financial situation. As reported earlier, females were more likely to have a higher prevalence of malnutrition and cognitive impairment (Au et al., 2017; Ning et al., 2021). Meanwhile, education attainment, as a proxy for cognitive reserve (Staekenborg et al., 2020), was positively associated with the level of the executive function and episodic memory in later life (Liu and Lachman, 2020). Also, participants with a higher educational level would have better nutritional intakes to reduce malnutritional risk (Rippin et al., 2020).

Therefore, although the underlying mechanism of the relationship between malnutrition and cognitive function is not clear until present, the GNRI defined that using serum albumin and weight loss has been validated in exploring the association of malnutrition with cognition function in this study. Researchers should make full use of the GNRI, rather than a single biomarker, in further malnutrition-related studies.

Strengths and Limitations

Our study has some strengths. First, because the CLHLS is a representative study in the Chinese elderly, the conclusion of our current study was more credible and reproducible. Second, compared with the previous study, we used the CLHLS, a longitudinal cohort study, to examine the association between malnutrition and cognitive function, which makes it easier to interpret the causal link between malnutrition and cognitive function. Finally, to our knowledge, the GNRI was rarely used to evaluate the relationship between malnutrition and cognitive function, particularly in the Chinese elderly. Compared to another single indictor, the GNRI has been considered as a reliable comprehensive tool for assessing malnutrition in the elderly. Thus, our study could provide solid evidence on the association of the GNRI with cognitive impairment, which is beneficial for delaying or preventing the occurrence and progression of cognitive impairment. However, this study had some limitations. First, since the CLHLS was designed for the oldest-old Chinese, generalizing the results to other populations should be cautioned. Second, although we collected confounding factors such as smoking, drinking, food intake frequency, hypertension, and history of diabetes, it may lead to a potential recall bias as some of them were self-reported. Third, since biomarkers were assayed for once in the random sample, the changes over time of biomarkers failed to be considered in this study. Finally, this study was enriched for octogenarians and nonagenarians, who might be healthier and less likely to suffer from malnutrition and cognitive impairment. Therefore, this study might underestimate the association of malnutrition with cognitive function.

CONCLUSION

A significant association of malnutrition measured by the GNRI with cognitive performance was observed among the Chinese elderly. Subjects with malnutrition have a poorer cognitive function, especially in illiterate females and the oldest elderly. Furthermore, the cognitive function was worse with malnutrition aggravating. Accordingly, the GNRI should be used broadly to evaluate and improve the nutritional status of the targeted elderly population. Moreover, improving early detection of malnutrition and managing routine nutritional surveillance are crucial for the elderly to prevent cognitive impairment.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories: The CLHLS questionnaires are available at https://sites.duke.edu/centerforaging/programs/chinese-longitudinal-healthy-longevity-survey-clhls/survey-documentation/questionnaires/ and the full datasets used in this analysis are available at https://opendata.pku.edu.cn/dataverse/CHADS.

ETHICS STATEMENT

This study was approved by the Institutional Review Board, Duke University (Pro00062871), and the Biomedical Ethics Committee, Peking University (IRB00001052–13074). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YC: conceptualization, formal analysis, and supervision. WL and YC: methodology, review, and editing. BS and YZ: writing–original draft preparation. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.766159/full#supplementary-material

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Protect Effects of Seafood-Derived Plasmalogens Against Amyloid-Beta (1–42) Induced Toxicity via Modulating the Transcripts Related to Endocytosis, Autophagy, Apoptosis, Neurotransmitter Release and Synaptic Transmission in SH-SY5Y Cells

Junli Feng[1], Gongshuai Song[1], Qing Shen[1], Xi Chen[2], Qingcheng Wang[3]*, Shunyuan Guo[2]* and Manman Zhang[4]**

[1] Zhejiang Province Joint Key Laboratory of Aquatic Products Processing, Collaborative Innovation Center of Seafood Deep Processing, Institute of Seafood, Zhejiang Gongshang University, Hangzhou, China, [2] Zhejiang Provincial People's Hospital, Hangzhou, China, [3] Department of Cardiology, Hangzhou Linping Hospital of Traditional Chinese Medicine, Hangzhou, China, [4] Department of Neurology, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China

To investigate the underlying mechanisms of decreased plasmalogens (Pls) levels in neurodegenerative diseases, here the effects of seafood-derived Pls on undifferentiated and differentiated human SH-SY5Y neuroblastoma cells exposed to amyloid-β_{1-42} was analyzed. Transcriptional profiles indicated that a total of 6,581 differentially expressed genes (DEGs) were significantly identified among different experimental groups, and KEGG analysis indicated that these DEGs were related to AD, endocytosis, synaptic vesicle cycle, autophagy and cellular apoptosis. After Pls treatment, the striking expression changes of *ADORA2A*, *ATP6V1C2*, *CELF6,* and *SLC18A2* mRNA strongly suggest that Pls exerts a beneficial role in alleviating AD pathology partly by modulating the neurotransmitter release and synaptic transmission at the transcriptional level. Besides these, GPCRs are also broadly involved in Pls-signaling in neuronal cells. These results provide evidence for supporting the potential use of Pls as an effective therapeutic approach for AD.

Keywords: Alzheimer's disease, plasmalogens, SH-SY5Y cells, gene expression, transcriptional profiles

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Tiantian Zhang,
Ocean University of China, China

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Hongshun Yang,
National University of Singapore, Singapore
Danuta Jantas,
Maj Institute of Pharmacology, Polish Academy of Sciences (IF PAS), Poland

****Correspondence:***
Qing Shen
leonqshen@163.com;
sq@zjgsu.edu.cn
Qingcheng Wang
wqc198477@sina.com
Shunyuan Guo
gsy9316@126.com
Manman Zhang
zhangmanman@wmu.edu.cn

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INTRODUCTION

Alzheimer's disease (AD), a progressive neurodegenerative disorder, often occurs among aging people (Chung et al., 2019). This disease is characterized by memory loss and cognitive impairment. The pathological features of AD are the deposition of amyloid-β (Aβ) plaques and formation of neurofibrillary tangles, which is primarily constituted by hyperphosphorylated tau proteins in

extra- and intra-nerve cells, leading to synaptic dysfunction and neuronal death. Although different therapeutic approaches have been proposed, only few are tested effectively to block AD progression (Sorrentino et al., 2017). In addition to aging, studies have shown that several risk factors play a crucial role in predisposing for AD. For example, smoking, midlife obesity, hypertension, type 2 diabetes, hypercholesterolemia and history of traumatic brain injury are strongly associated with the onset of AD (Meco et al., 2020).

The molecular pathogenesis of this age-related cognitive decline remains incompletely defined (Wan et al., 2020). Intraneuronal Aβ accumulation is believed to trigger neurodegeneration by disrupting synaptic transmission and endosomal, lysosomal/proteasomal, and mitochondrial functions as well as by facilitating the hyperphosphorylation of microtubular tau protein (Omtri et al., 2018). Genome-wide association studies on AD patient brains have demonstrated significant expression changes in genes that regulate vesicular trafficking, cytoskeleton, energy metabolism, inflammation, ubiquitin-proteosome system, and autophagy (Liang et al., 2007). Besides these, it has been reported that the phosphoinositide 3-kinase (PI3K)/AKT-mTOR signaling pathway, nuclear factor kappa B (NF-κB), and mitogen-activated protein kinase (MAPK) pathways are activated in neurons in several neuropathological conditions (Zheng et al., 2017; Coelho et al., 2019; Saha et al., 2020).

Plasmalogens (Pls) are a special class of dietary glycerophospholipids which can be easily found in animal foodstuffs such as poultry, livestock and seafood. Pls are abundant in the nervous system, especially in the white matter, where Pls are localized in the cytoplasmic side of myelin (Che et al., 2018). Besides their contribution to membrane integrity as structural components, Pls are also involved in multiple cellular processes such as membrane fusion, ion transport, cholesterol efflux, and generation of secondary messengers (Su et al., 2019). Several literatures have reported a direct link between Pls deficiency and AD pathology, whereas the administration of Pls (1 mg/day) was effective to improve cognitive function of mild AD patients (Ginsberg et al., 1995; Wood et al., 2011; Fujino et al., 2017). Che et al. (2018) have reported Pls (20 μg/mL in the medium) could significantly decrease intracellular and extracellular $A\beta_{42}$ levels of CHO-APP/PS1 cells, whereas another study suggested that Pls (5 and 20 μg/mL in the medium) prevented neuronal cell death by activating G-protein coupled receptors (GPCRs) to induce ERK and Akt cellular signaling (Hossain et al., 2013, 2016). However, limited genes have been analyzed in these studies. Therefore, it is necessary to explore the role of Pls in the pathogenesis of AD as a whole.

Aβ peptide is a product of its precursor amyloid polypeptide protein by sequential proteolytic cleavages, and it has various conformations. Among them, $A\beta_{1-42}$ easily forms insoluble aggregates, which are the predominant fibers found in senile neuritic plaques of AD brains (Selkoe, 2001). Previous studies proved that $A\beta_{1-42}$ has neurotoxic potential, interfering with synaptic plasticity and affecting several cellular signaling pathways (Selkoe, 2001). Thus, neurons exposed to appropriate $A\beta_{1-42}$ (less than 10 μM) are routinely used to obtain the *in vitro* AD models (Arbo et al., 2017; Coelho et al., 2019).

In this study, SH-SY5Y neuroblastoma cells were used to obtain human neuron-like cells using the sequential *all-trans* retinoic acid (ATRA) differentiation and brain-derived neurotrophic factor (BDNF) maturation program (Encinas et al., 2000; Goldie et al., 2014). Then, *in vitro* AD model cells were constructed through $A\beta_{1-42}$ induction, and they were treated with seafood-derived Pls (10 μg/mL) for 24 h. Finally, the transcriptional profiles of AD model cells before and after Pls treatment were characterized, and the potential effects of seafood-derived Pls on the AD-related pathological process were uncovered. Results of this study may help us to fully understand the beneficial effects of seafood-derived Pls on pathology of patients with AD.

MATERIALS AND METHODS

Cell Culture and Treatment

The SH-SY5Y human neuroblastoma cell line obtained from BeNa Culture Collection (Beijing, China). The SH-SY5Y cells were grown for three generations before experiments, and they were used in a low passage number (<13). Cells were cultured in flasks in a humidified incubator at 37°C and 5% CO_2 (MCO-15AC, Sanyo Electric Co., Osaka, Japan), and the culture medium was replaced thrice per week. The SH-SY5Y cells were cultured in Dulbecco′s Modified Essential Medium (DMEM, Gibco, San Francisco, CA, United States) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 100 U/mL penicillin/streptomycin (Gibco), and were split into three groups at 80% confluence.

For the control group, the cells were still cultured in DMEM containing 10% FBS and 100 U/mL penicillin/streptomycin to reach 80% confluence, and were harvested at this stage (at day 3). For the other experiment groups, ATRA (Sigma-Aldrich, St. Louis, MO, United States) was added in the medium (DMEM + 10% FBS + 10 μM ATRA + 100 U/mL penicillin/streptomycin), and the same culture medium was changed on day 3. On day 6, the culture medium was changed to serum-free BDNF (Life Technologies, Carlsbad, CA, United States) medium (DMEM + 50 ng/mL BDNF + 100 U/mL penicillin/streptomycin). After 7 days of BDNF exposure, neuron-like phenotype were obtained on day 13. To obtain cells of Pls group (Pls), these neuron-like cells were further treated with seafood-derived Pls (10 μg/mL in medium) for 24 h. For the AD model group (AD), the neuron-like cells were treated with $A\beta_{1-42}$ (1.0–4.0 μM, Sigma-Aldrich) for 24 h before cells collection, whereas cells in the AD model + Pls group (AD_Pls) were further treated with 10 μg/mL seafood-derived Pls in medium for another 24 h and harvested at day 15.

The seafood-derived Pls was extracted and purified from Mussel (*Mytilus edulis*) in the laboratory, according to the method described in a previous study (Wang et al., 2021). The purity of the obtained Pls was 91.56%, with the major components of phosphatidylethanolamine plasmalogens (50.13%) and phosphatidylcholine plasmalogens (41.43%) when

analyzed by HPLC-ELSD (Wang et al., 2021). The remaining 8.44% contained 3.12% PC and 5.08% PE. The proportions of the unsaturated fatty acid in Pls and phospholipids were 63.07 and 58.43%. eicosapentaenoic acid was the major constituent of unsaturated fatty acids, which accounted for 45.82 and 42.35% in Pls and phospholipids, respectively (Song et al., 2020; Wang et al., 2021; Zhang et al., 2021). During the culture and differentiation stages, SH-SY5Y cells were visualized by a Zeiss Axiovert 200 inverted fluorescence microscope (Carl Zeiss, Oberkochen, Germany), and documented with AxioVisionLE (Carl Zeiss).

The 4′,6-Diamidino-2-Phenylindole and Propidium Iodide (PI) Staining

The death of SH-SY5Y cells treated with different concentrations of $A\beta_{1-42}$ was measured by 4′,6-Diamidino-2-Phenylindole (DAPI) (Sigma-Aldrich) and Propidium Iodide (PI) (Sigma-Aldrich) staining. Briefly, differentiated SH-SY5Y cells were cultured on coverslips in a 24-well plate. After the cells adhered, 5 mg/L DAPI was added to the culture supernatant and cultured overnight in dark. Then, $A\beta_{1-42}$ was added to each well to the final concentrations of 1, 2, and 4 μM. After treatment with $A\beta_{1-42}$ for 24 h, 50 mg/L PI was added to each well and stained for 3–5 min. Then, cells were observed under a fluorescence microscope (Carl Zeiss) and analyzed by AxioVisionLE software. Five images were obtained for each cell sample. The total number of nuclei was assessed by DAPI staining, while the nuclei of dead cells were determined by PI staining. Therefore, the cell death rate was determined by comparing the number of PI positive cells to the number of total cells in the images.

RNA Extraction and RNA-Seq Library Preparation

Total RNA was extracted from SH-SY5Y cells of different groups using TRIzol Reagent (Invitrogen, CA, United States). The RNA quantity and quality were analyzed using a NanoDrop ND-1000 instrument (Agilent, CA, United States). The mRNA was purified from 5 μg of total RNA using poly-T oligo-attached magnetic beads after two rounds of purification. The obtained mRNA was fragmented using divalent cations under elevated temperature, and then reverse-transcribed to construct the cDNA library using the mRNASeq sample preparation kit (Illumina, San Diego, United States). The sequencing was performed on an Illumina Novaseq^{TM} 6000 platform (LC Sciences, Houston, Texas, United States). Three biological repeats were assessed for each sample, and all of the data were expressed as the mean ± standard deviation (SD). The raw datasets of sequencing have been submitted to the NCBI Short Read Archive under the accession code PRJNA728528.

RNA-Seq Reads Analysis

The raw transcriptome data were first processed by removing adaptor-containing sequences, poly-N, reads shorter than 150 bp, and low-quality reads. Then the valid reads were aligned to the homo-genome (http://www.ensembl.org/Homo_sapiens/Info/Index) using the HISAT package, which allowed multiple alignments (up to 20 by default) and 2 mismatches while mapping the reads to the reference genes. Mapped reads of each sample were assembled using StringTie, and all of the transcriptome data were merged to generate a comprehensive transcriptome using Perl scripts software. StringTie and edgeR were used to calculate the expression levels of all transcripts by calculating FPKM. These mRNAs were further analyzed using GO (gene ontology) enrichment analysis and KEGG (Kyoto Encyclopedia of Genes and Genomes) signaling pathway enrichment analysis.

Quantitative RT-PCR

To validate the RNA-seq results, the abundance of *ADORA2A*, *APP*, *ATP6V1C2*, *Bcl-2*, *DGKK*, *GSAP*, *GSK3*, *IL33*, *PSEN1*, and *SLC18A2* mRNAs in different experimental groups were analyzed in parallel using an ABI Prism 7500 Sequence Detection System (PE Applied Biosystems, CA, United States). Briefly, total RNA was extracted from samples with three biological repeats, and converted to cDNA using the cDNA reverse transcription kit (Applied Biosystems). Quantitative RT-PCR (qRT-PCR) primers used for determining the transcript abundance of selected mRNAs are listed in **Supplementary Table 1**. *GAPDH* was used as the housekeeping gene. The qPCR amplification was initiated by denaturation at 95°C for 10 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Each reaction was repeated three times. The specificity of the amplification was examined by melting curve analysis. The relative fold changes of the tested genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistics Analysis

All results in this study are represented as the average ± standard deviation (SD) from three independent experiments. Statistical analysis was performed using Microsoft Excel, and one-way analysis of variance (ANOVA) followed by Duncan's new multiple range tests. Differences were assumed to be statistically significant, and indicated with different letters, for $P < 0.05$.

RESULTS

Morphological Characteristics of Cultured SH-SY5Y

In the present study, SH-SY5Y cells were imaged at each stage of treatment using the Zeiss Axiovert 200 inverted microscope. As shown in **Figure 1A**, SH-SY5Y cells treated with ATRA for 5 days had reduced proliferation and presented a more polar morphology, with cell bodies being extended and networks beginning to develop. After further maturation with BDNF, cells migrated to clusters, and the cellular networks became increasingly complex, which were similar to the mature neurons. These mature neuron-like cells were further treated with Pls and $A\beta_{1-42}$ alone or in combination, to obtain cells of Pls group, AD group, and AD_Pls, respectively. However, no obvious morphological differentiation was observed during Pls or $A\beta_{1-42}$ treatments.

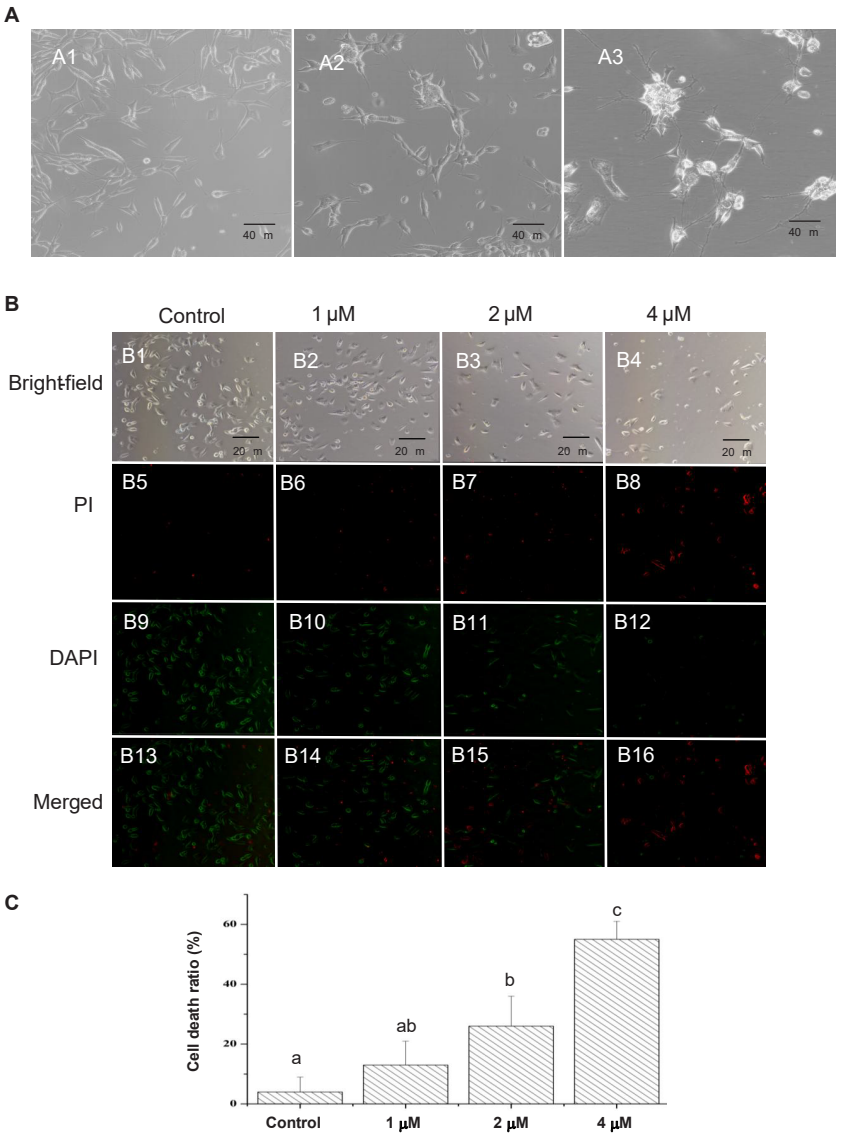

FIGURE 1 | Characteristics of cultured SH-SY5Y cells and effects of $A\beta_{1-42}$ on death of SH-SY5Y cells. **(A)** Morphology of SH-SY5Y cells at different differentiation stages observed using an Axiovert inverted microscope. A1, untreated SH-SY5Y cells (×200 magnification). A2, SH-SY5Y cells treated with ATRA for 5 days (×200 magnification). These cells demonstrated an increased death rate and a more polar morphology, as cell bodies extended longer and networks began to form. A3, after further maturation with BDNF, the cells migrated to clusters and networks became increasingly complex (×200 magnification). **(B)** The SH-SY5Y cells were treated with different concentrations of $A\beta_{1-42}$ for 24 h, the cell death rates were determined by comparing the number of dead cells to the number of total cells in the images (×200 magnification). **(C)** Quantification of cell death ratios after 24 h treatment with the indicated concentrations of $A\beta_{1-42}$. The different letters indicated a significant difference between experiment groups at $p < 0.05$ (Duncan's new multiple range test).

Effects of $A\beta_{1-42}$ on Death of SH-SY5Y Cells

To assess the effects of $A\beta_{1-42}$ on cell death, equal numbers of differentiated SH-SY5Y cells were seeded in each well of a 24-well plate. Nevertheless, the clusters and network structures of the neuron-like SH-SY5Y cells disappeared after trypsin hydrolysis during transferring from the culture flasks to coverslips. **Figure 1B** presented that the cell death ratio notably increased with an increasing in $A\beta_{1-42}$ concentrations, indicating that the damage of $A\beta_{1-42}$ on SH-SY5Y cells was dose-dependent. **Figure 1C** represented that treatment with 2 μM of $A\beta_{1-42}$ leads to 26% cell death, while 55% of the SH-SY5Y cells died when treatment with 4 μM of $A\beta_{1-42}$ for 24 h. To ensure the optimal induction result and an appropriate cell concentration, 2 μM of $A\beta_{1-42}$ was finally used in subsequent experiments.

Transcriptome Sequencing of SH-SY5Y

To investigate the effect of Pls on AD development, the transcriptomes of samples from normal SH-SY5Y cells, AD model and AD_Pls SH-SY5Y cells were sequenced. The RNA

sequence results are summarized in **Table 1**. A total of 466,789,804 raw reads were obtained, of which the valid reads was 428,666,272. Further analysis of these high-quality cleaned reads generated 208,460 assembled transcripts, which corresponded to 58,826 expression genes. In the alignment analysis, the ratio of valid reads which were mapped to homo-genome came up to 96.40–96.72%. The Q30 values were greater than 97.43%, and the GC contents were ranged from 51 to 53%. Besides these, the Pearson correlation coefficients of these transcriptome profiles among libraries and biological repeats further proved the RNA sequence data were reliable (**Supplementary Figure 1**).

Transcriptional Profiles of Different Experimental Groups

As shown in **Figure 2**, 6,581 differentially expressed genes (DEGs) were identified as significant when the experimental groups were compared with each other. Using a fold change cutoff ratio of ≥ 2 or ≤ 0.5, 4,139 DEGs (3721 up-regulated, 418 down-regulated) were further identified when the AD model was compared with control cells. The comparison of AD_Pls and control groups yielded 4,002 DEGs (3578 up-regulated, 424 down-regulated), whereas the comparison of AD and AD_Pls groups yielded 192 DEGs (105 up-regulated, 87 down-regulated). And overall, 59 DEGs were common in all of the three comparisons.

Then, the potential biological functions of these DEGs were analyzed. In the GO analysis performed among control, AD and AD_Pls groups, the most significantly enriched GO terms were "protein binding," "nucleus," cytoplasm," "cytosol," and "nucleoplasm"(**Figure 2B**). Similarly, KEGG enrichment analysis indicated that "PI3K-Akt signaling pathway," "Endocytosis," "MAPK signaling pathway," "Alzheimer disease," "mTOR signaling pathway," "protein processing in endoplasmic reticulum," and "Autophagy" were the most represented pathways (**Figure 2C**). Therefore, these pathways were specifically analyzed.

Differentially Expressed Genes Directly Involved in Alzheimer's Disease

The 86 DEGs directly involved in "Alzheimer disease" pathway were first analyzed. As results presented in **Table 2**, the expression of several hallmarks of AD, such as *APAF1*, *APP*, and *PSEN1* were obviously up-regulated in AD model and AD_Pls cells. Compared with AD model cells, the increased levels of these DEGs were less significant in the AD_Pls group. Moreover, the results indicated that more than half of the AD-related DEGs (48 out of 86) were involved in oxidative phosphorylation (OXPHOS). And expression levels of most OXPHOS transcripts were decreased in AD model and AD_Pls groups. Besides these, several DEGs involved in AD-related calcium signaling pathway (such as *CACNA1D* and *ITPR2*), were also significantly altered (**Supplementary Table 2**).

Differentially Expressed Genes Involved in PI3K-Akt/mTOR and Mitogen-Activated Protein Kinase Signaling Pathways

Then, 178 DEGs involved in PI3K-AKT/mTOR signaling pathway were identified. As the results shown in **Table 3**, most of these DEGs were up-regulated in either AD or AD_Pls groups compared with the control cells. However, the up-regulation extents were less significant after Pls treatment, showing by the 102 of 178 down-regulated DEGs involved in PI3K-AKT/mTOR signaling pathway when the AD_Pls group was compared with AD models. Among them, the down-regulation of *SOS1* was most significantly, whereas *ATP6V1C2* was notably up-regulated after Pls treatment.

The activation of MAPK pathway plays a pivotal role in Aβ-induced neuroinflammation (Zaretsky and Zaretskaia, 2021). Consistent with previous study, 110 DEGs that participated in the MAPK pathway were identified as significant, and 84 of 110 DEGs were up-regulated in the AD group compared with controls. After further treatment with Pls, most of these DEGs were down-regulated, especially *SOS1*, *MAP3K13*, and *NFATC3*.

Differentially Expressed Genes Involved in Endocytosis and Synaptic Vesicle Cycle

Increasing evidences have reported that the onset and progression of AD are associated with endocytosis, as Aβ enters a cell by endocytosis, and then the endocytic vesicle is merged with a lysosome for degradation the peptide (Muraleva et al., 2019). In this study, 115 DEGs related to endocytosis were identified, and most of them were up-regulated in both AD and AD_Pls groups. Further comparison between the AD and AD_Pls groups indicated that the up-regulation of *CBL* and down-regulation of *ARF1* were the most significant after treatment with Pls.

Communication between neurons is mediated by the release of neurotransmitters from the synaptic vesicle, thus impairment of synaptic vesicle dynamics is believed to be one cause of cognitive defects in AD. In this study, 29 DEGs involved in synaptic vesicle cycle were identified. Among them, the calcium sensor *SYT1* was up-regulated by 4.68- and 4.62-folds in AD and AD_Pls groups, respectively (**Table 3**). The expression

TABLE 1 | Summary of the transcriptome sequencing database.

Group	Total reads	Valid reads	Mapped reads	Unique mapped reads	Multiple mapped reads	GC%	≥ Q30%
ADmodel	160,677,104	147,325,300	142,448,010 (96.69%)	114,367,872 (77.62%)	28,080,138 (19.06%)	51.50	97.51
AD model + Pls	147,231,378	137,964,148	133,011,556 (96.40%)	105,793,995 (76.68%)	27,217,561 (19.72%)	51.00	97.43
Control	158,881,322	143,376,824	138,685,824 (96.72%)	107,595,429 (75.02%)	31,090,395 (21.70%)	53.00	97.51
Total	466,789,804	428,666,272	414,145,390	–	–	–	–

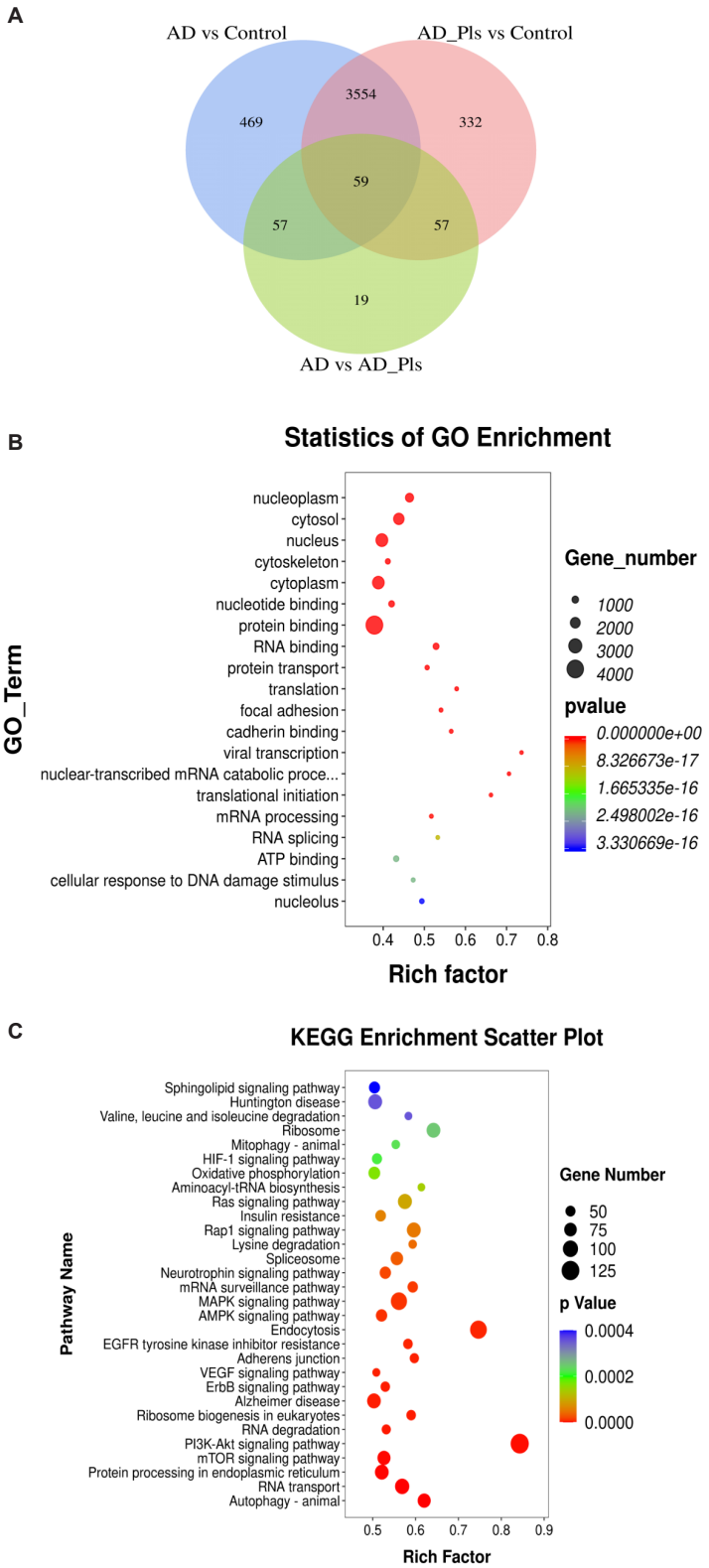

FIGURE 2 | Numbers of DEGs between experimental groups, and their corresponding GO and KEGG enrichment data. **(A)** Venn diagram showing the DEGs significantly identified by comparison of AD model and control groups, AD model + Pls and control groups, and AD model and model + Pls groups (fold change > 2 or fold change < 0.5). **(B)** GO function enrichment analysis of DEGs. **(C)** KEGG pathway enrichment analysis of DEGs.

TABLE 2 | List of the significant ($p < 0.05$) AD-associated DEGs and respective fold changes identified among different groups.

Gene name	Fold change (AD model/ control)	Fold change (AD model + Pls/ control)	Description
Amyloid β formation			
APAF1	3.85	2.72	Apoptotic peptidase activating factor 1
APP	3.92	3.73	Amyloid beta precursor protein
IDE	3.77	2.80	Insulin degrading enzyme
MME	5.22	3.23	Membrane metalloendopeptidase
PSEN1	4.73	4.34	Presenilin 1
AD-related oxidative phosphorylation			
ATP5F1E	0.61	0.70	ATP synthase F1 subunit epsilon, OXPHOS complex V
COX4I2	0.24	0.37	Cytochrome c oxidase subunit 4I2, OXPHOS complex IV
COX7A2L	0.39	0.43	Cytochrome c oxidase subunit 7A2 like, OXPHOS complex IV
CYC1	0.52	0.57	Cytochrome c1, OXPHOS complex III
NDUFA3	0.54	0.72	NADH:ubiquinone oxidoreductase subunit A3, OXPHOS complex I
NDUFS8	0.57	0.74	NADH:ubiquinone oxidoreductase core subunit S8, OXPHOS complex I
SDHD	1.58	1.76	Succinate dehydrogenase complex subunit D, OXPHOS complex II
UQCRB	0.48	0.52	Ubiquinol-cytochrome c reductase binding protein, OXPHOS complex III

TABLE 3 | List of the significant ($p < 0.05$) PI3K-AKT/mTOR and MAPK signaling pathways related DEGs and respective fold changes identified among different groups.

Gene name	Fold change (AD model/ control)	Fold change (AD model + Pls/ control)	Description
PI3K-AKT/mTOR signaling pathway			
ATP6V1C2	0.15	2.10	ATPase H^+ transporting V1 subunit C2
FN1	2.02	1.64	Fibronectin 1
FOXO3	4.91	3.94	Forkhead box O3
ITGA9	3.19	2.27	Integrin subunit alpha 9
KIT	4.55	3.37	KIT proto-oncogene receptor tyrosine kinase
LAMA1	3.49	2.47	Laminin subunit alpha 1
*SOS1**	4.56	1.64	SOS Ras/Rac guanine nucleotide exchange factor 1
MAPK signaling pathway			
MAP3K13	2.57	1.48	Mitogen-activated protein kinase kinase kinase 13
NFATC3	3.47	1.53	Nuclear factor of activated T cells 3

**Indicate genes involved in both PI3K-AKT/mTOR and MAPK signaling pathways.*

levels of *ATP6V1C2* (responsible for the vesicle acidification) and *SLC18A2* (involved in the vesicle retrieved), were notably decreased to 0.03- and 0.0005-fold of control cells in the AD group, whereas their levels recovered to 2.10- and 0.47-fold of controls in AD_Pls group.

Differentially Expressed Genes Related to Autophagy and Apoptosis

The dramatic increase in autophagic vacuoles is another feature of AD. To explore the changes in autophagy in AD and AD_Pls cells, 81 autophagy-related DEGs were assessed. **Table 4** showed that most of these DEGs were up-regulated in AD and AD_Pls groups, but the up-regulation levels of *MTMR3 and STX17* were markedly reduced after Pls treatment. Nevertheless, the transcript levels of genes encoding lysosomal proteolysis (*CTSB* and *CTSD*) did not significantly change among different experimental groups.

Autophagy is closely related to cell apoptosis, thus the apoptosis pathway is also assessed. KEGG analysis indicated that 65 DEGs related to apoptosis were identified as significant (**Table 4**). When the three experimental groups were compared with each other, *PTPN13* was notably up-regulated in both AD and AD_Pls groups. *ITPR2* was up-regulated by 5.06-folds in the AD group, whereas only 2.92-folds in the AD_Pls group. When the AD and AD_Pls groups were compared, the expression levels of *DDIT3* were increased whereas *CASP2* level was decreased after treatment with Pls.

Other Differentially Expressed Genes

Besides the aforementioned DEGs, the transcription level of *BHLHB9* was up-regulated thousands of times in both AD and AD_Pls groups, whereas *KLHL11*, *AKAP2*, DGKK, *ZNF445*, *PDE1A*, and *MYH15* mRNAs were up-regulated dozens of times (**Table 5**). On the contrary, the transcription levels of *GPCR22* and *TRAPPC* were significantly down-regulated in both AD and AD_Pls groups. Finally, when the AD and AD_Pls groups were compared, the expression levels of *MATR3*, *CELF6*, and *ADORA2* in AD_Pls cells were significantly down-regulated after treatment with Pls (**Table 5**).

Quantitative RT-PCR

To validate the transcriptome results, 10 transcripts were selected and further analyzed by qRT-PCR. As expected, the expression patterns of *ADORA2A*, *APP*, *ATP6V1C2*, *Bcl-2*, *DGKK*, *GSAP*, *GSK3*, *IL33*, *PSEN1*, and *SLC18A2* mRNAs were consistent with those obtained by RNA-seq (**Figure 3**). Compared with control cells, the expression levels of *DGKK* were markedly increased in AD and AD_Pls groups. *SLC18A2* and *ADORA2A* were the most significantly down-regulated transcripts in the AD group and AD_Pls groups, respectively. However, discrepancies were also observed between data obtained from the qRT-PCR and RNA-seq. For example, the up-regulation levels of *DGKK* mRNA in AD and AD_Pls groups obtained from qRT-PCR were 25.53 and 16.82, whereas these values were 30.68 and 27.51 when detected by RNA-seq. These discrepancies might result from different sensitivities among different technologies.

TABLE 4 | List of significant ($p < 0.05$) endocytosis, synaptic vesicle cycle, autophagy, and apoptosis-associated DEGs and respective fold changes identified among different groups.

Gene name	Fold change (AD model/ control)	Fold change (AD model + Pls/ control)	Description
Endocytosis			
ARF1	1.22	0.44	ADP ribosylation factor 1
CBL	0.35	1.94	Cbl proto-oncogene
CHMP4A	1.60	2.45	Charged multivesicular body protein 4A
SH3KBP1	1.64	2.28	SH3 domain containing kinase binding protein 1
Synaptic vesicle cycle			
ATP6V1C2	0.15	2.10	ATPase H + transporting V1 subunit C2
SLC18A2	0.00	0.47	Solute carrier family 18 member A2
SYT1	4.68	4.62	Synaptotagmin 1
Autophagy			
ATG2B	4.15	3.76	Autophagy related 2B
ATG4A	2.74	2.37	Autophagy related 4A cysteine peptidase
ITPR1	4.87	4.25	Inositol 1,4,5-trisphosphate receptor type 1
LAMP1	1.74	1.42	Lysosomal associated membrane protein 1
MTMR3	5.47	3.79	Myotubularin related protein 3
*PIK3R1**	2.10	3.24	Phosphoinositide-3-kinase regulatory subunit 1
STX17	3.64	1.35	Syntaxin 17
Apoptosis			
CASP2	2.33	1.13	Caspase 2
DDIT3	1.54	2.75	DNA damage inducible transcript 3
ITPR2	5.06	2.92	Inositol 1,4,5-trisphosphate receptor type 2
PTPN13	5.34	5.35	Protein tyrosine phosphatase, non-receptor type 13

**indicate genes involved in both autophagy and apoptosis.*

TABLE 5 | Other DEGs identified among different groups.

Gene name	Fold change (AD model/ control)	Fold change (AD model + Pls/ control)	Description
BHLHB9	1347.47	7905.61	Basic helix-loop-helix family member b9
PDE1A	27.51	16.27	Phosphodiesterase 1A
ARMCX4	17.29	7.53	Armadillo repeat containing X-linked 4
TIAF1	16.03	2.03	TGFB1-induced anti-apoptotic factor 1
CELF6	0.77	0.03	CUGBP Elav-like family member 6
ADORA2A	0.92	0.05	Adenosine A2a receptor
MATR3	5.09	0.03	Matrin 3

DISCUSSION

A key limitation in the field of neuroscience is the lack of suitable *in vitro* models resembling mature neurons. Although animal models are informative, they cannot provide a full explanation of the molecular mechanisms underlying neuronal function as human transcriptional networks are complex, with species-specific gene expression modulation patterns (Goldie et al., 2014). SH-SY5Y neuroblastoma cells have been used extensively to model networks and pathways related to human cognitive disorders. Although there is debate about the need to differentiate SH-SY5Y, an increasing number of studies have suggested the consideration of experimental methodology and applicability of the cell model in answering complex functional questions related to human neural architecture (Goldie et al., 2014). Previous studies have reported that the ATRA differentiation-BDNF maturation program not only induced significant increases in the expression of neuron-specific marker genes (such as *Sv2*, *NeuN*, and *NPY*), but also yielded SH-SY5Y cells with phenotype approaching mature neurons (Agholme et al., 2010; Goldie et al., 2014). Consistent with these studies, the expression levels of *Sv2C* in AD model and AD_Pls cells were 3.88- and 3.36-folds higher than that of the controls, and the *NPY2R* levels were up-regulated by 8.78- and 6.98-folds in AD and AD_Pls groups when compared with the controls (**Supplementary Table 2**). These data indicated that differentiated SH-SY5Y cells displayed mature neuron-like appearance and function. These neuron-like cells were further treated to obtain AD models, and subsequently used to investigate the potential role of seafood-derived Pls in AD pathogenesis.

Down-regulation of OXPHOS genes in animal models of AD has been reported in previous studies, and it is suggested that these reductions could trigger an overall bioenergetic crisis in the neurons, resulting in cell death (Area-Gomez and Schon, 2017; Mastroeni et al., 2017). Altered Ca^{2+} homeostasis in AD and AD_Pls cells was also observed at the transcriptional level in this study, which has been considered as an upstream event of AD pathogenesis and often occurs before the development of overt symptoms (Tong et al., 2018). Collectively, these data indicate that exposure to $A\beta_{1-42}$ cause energy defects and Ca^{2+} dysregulation in SH-SY5Y cells, whereas PLs might effectively attenuate this crisis due to less significant down-regulation of these genes.

The PI3K-AKT/mTOR signaling pathway has been considered as the root cause of neuropsychiatric disorders such as AD (Sharma and Mehan, 2021). Up-regulation of PI3K-AKT/mTOR signaling pathway is associated with axonal dysregulation, leading to harmful consequences including over-production of reactive oxygen species, mitochondrial instability, lower oxidative phosphorylation and ATP levels, and even neuronal apoptosis (Seitz et al., 2012). Conversely, PI3K-AKT/mTOR signaling pathway inhibitors play a neuroprotective role, because of their effects in inhibiting cell apoptosis (Seitz et al., 2012). Consistent with those previous studies, **Table 3** shows that the up-regulation degrees of DEGs related to PI3K-AKT/mTOR and MAPK signaling pathways were less significant after Pls treatment. These data indicate the PI3K-AKT/mTOR and MAPK signaling pathway is activated after $A\beta_{1-42}$ exposure, whereas Pls treatment inhibits the activation of these signaling pathways.

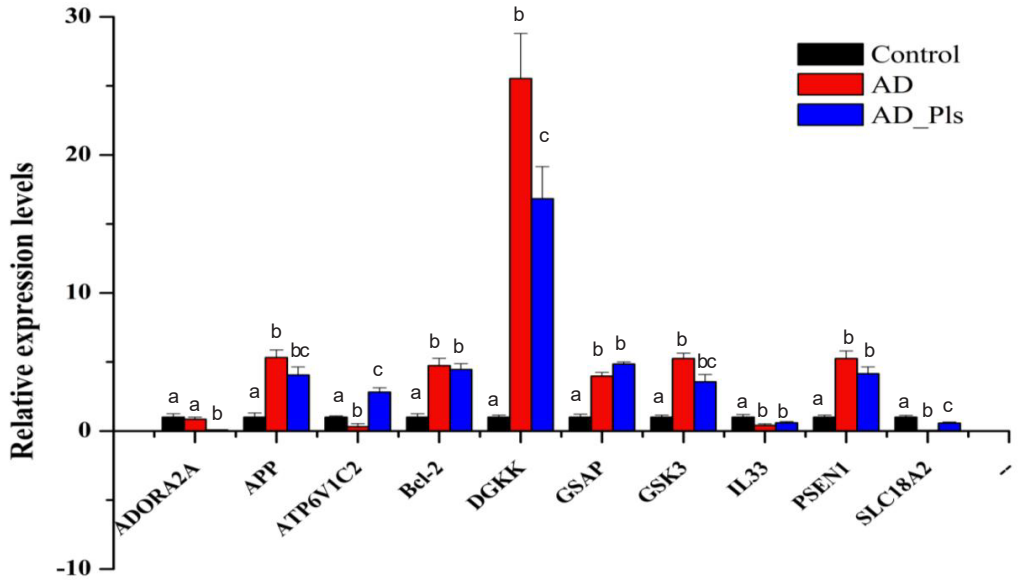

FIGURE 3 | A comparison of the expression levels of several genes in AD model, AD model + Pls, and control groups. These genes were selected as KEGG analysis indicated that they were AD-related (*APP, GSAP, GSK3,* and *PSEN1*), autophagy-related (*Bcl-2*), fatty acid metabolism-related (*DGKK*), or their mRNAs levels were significantly changed among different groups (*ATP6V1C2, SLC18A2,* and *IL33*). The expression level of each gene in the control group was set as 1, and that in AD and AD_Pls groups was quantified relative to it. *GAPDH* was selected as an endogenous control. The results were represented as average ± SD (n = 3), and different letters denoted a significant difference between each group at $p < 0.05$ (Duncan's new multiple range test).

ATP6V1C2 encodes vacuolar-H^+ ATPase (V-ATPase), which is a proton pump that is required for acidification of lysosome/vacuole (Zhao et al., 2018). Thus, V-ATPase would influence cellular processes such as endocytosis, vacuole fusion and protein degradation in eukaryotes. As lysosomes are the final-degradation organelles of Aβ peptide and acidic environment is the determinant of hydrolytic enzyme activation, it is speculated that down-regulation of *ATP6V1C2* mRNA levels in AD model cells would lead to increasing of pH value in lysosomes, resulting in inactivation of peptidases and inefficient Aβ clearance (Zhao et al., 2018; Zhou et al., 2021). However, the acidic lysosomal environment recovered after Pls induction by elevated *ATP6V1C2* transcript levels in AD_Pls cells (**Table 3**). In addition, *ATP6V1C2* also modulates the concentration of neurotransmitters into synaptic vesicles and is crucial in synaptic transmission (Zhao et al., 2018). A very recent study has reported that the pleiotropic roles of low *ATP6V1A* levels in AD pathogenesis are mediated via the synaptic vesicle cycle, phagosome, and oxidative phosphorylation (Zhou et al., 2021), which is consistent with results of this study.

SLC18A2 is another notable DEG involved in synaptic vesicle cycle, which encodes a neurotransmitter transporter responsible for the packaging of small molecule neurotransmitters (acetylcholine, histamine, dopamine, norepinephrine, epinephrine, and serotonin) into synaptic vesicles (Hu et al., 2020). *SLC18A2* is expressed in monoaminergic neurons of the central nervous system, and abnormal expression of *SLC18A2* has been proposed to contribute to vulnerability toward epilepsy-related psychiatric disorders and cognitive impairment in adults (Markos et al., 2016; Treble-Barna et al., 2017). The *SLC18A2* inhibitor (Deutetrabenazine) is proved to be effective in the cure of involuntary movements in patients with tardive dyskinesia, and the *SLC18A2* blocker (Tetrabenazine) is the only US Food and Drug Administration-approved drug for Huntington's disease (Hu et al., 2020). In this study, the transcript levels of *ATP6V1C2* and *SLC18A2* were significantly down-regulated in AD model, yet their levels were quickly recovered after further treatment with Pls (**Table 4**). Thus, it is suspected that Pls may also alleviate $A\beta_{1-42}$-induced neurotoxicity through modulating neurotransmitter system.

Autophagy plays a modulatory role in the internalization and uptake of Aβ, and likely impacts the degradation or formation of Aβ plaques (Bordi et al., 2016). Nevertheless, sometimes conflicting results were obtained according to earlier investigations of autophagy induction in AD models (Bordi et al., 2016). Through a custom-designed microarray analysis, a previous study reported that genes related to autophagosome formation and lysosomal biogenesis were up-regulated, whereas the autolysosomal proteolytic function was not evidently altered at early stages of AD (Lipinski et al., 2010). Results of this study were consistent with these reports, as shown by the overall up-regulation of autophagy Initiation, autophagophore formation and elongation, and autophagosome-lysosome fusion-related genes, whereas the expression levels of lysosomal proteolysis were not evidently changed. Therefore, it is hypothesized that the activation of autophagy in AD model cells may represent an acute attempt by the affected neuronal cells to rid themselves of the harmful effects of $A\beta_{1-42}$ exposure. However, abnormally accelerated endocytosis and accumulation of $A\beta_{1-42}$ eventually become counterproductive

as lysosomal proteolysis function is insufficient, leading to the acceleration of AD onset.

Among other DEGs, the up-regulation of *BHLHB9* was most notable (**Table 5**). BHLHB9 is also known as GPCR-associated sorting protein 3 (GASP-3), it promotes neurosynaptogenesis by influencing the phosphorylation and proteolytic processing of APP and PSENs in transgenic mice (Mishra and Heese, 2011). The marked up-regulation of *BHLHB9* mRNA levels in both the AD and AD_Pls groups may be related to the complex neural network and synaptic structures formed in AD and AD_Pls cells compared with control cells. However, it is noticed the *BHLHB9* mRNA level is 5.87-fold higher after further treatment with Pls. This result is consistent with the identified roles of *BHLHB9* in improving memory and learning abilities in animal models, as well as the expected effect of Pls on alleviating AD pathology (Mishra and Heese, 2011). Other DEGs in **Table 5** are also relevant to neurodegenerative diseases. *PDEs* are targets for therapy of AD, as many PDE inhibitors have shown encouraging cognitive improvement effects (Nabavi et al., 2019). *ARMCX4*, also known as *GASP-4*, is one of the highly dysregulated priority genes in brains of Parkinson's disease (Abu-Helo and Simonin, 2010). *TIAF1* encodes a small TGF-β1-induced factor, and a significant up-regulation of Aβ levels occurred rapidly following TIAF1 self-association in degenerating and dead neurons (Chang, 2009).

Table 5 also showed that expression levels of *ADORA2*, *CELF6,* and *MATR3* were significantly altered after Pls treatment. *MATR3* is one of the newly identified dementia-causing genes (Park et al., 2020). *ADORA2A* encodes the adenosine A2A receptor, which is a GPCR that mediates synaptic transmission and neuronal excitability in the central nervous system (Domenici et al., 2019). Moreover, *ADORA2A* has been reportedly essential for $A\beta_{1-42}$ toxicity as $A\beta_{1-42}$ did not induce learning deficits or synaptotoxicity in *ADORA2A* knockout mice (Chen et al., 2007). In addition, preclinical data have also supported the use of adenosine A2A receptor as therapeutic target in neuropsychiatric disorders (Domenici et al., 2019). *CELF6* encodes a RNA-binding protein, which is highly expressed in several monoaminergic cell populations and in cells of the hypothalamus commonly targeted in psychiatry. A recent study has revealed that many targets mRNA of *CELF6* encode proteins involved in synaptic transmission (Rieger et al., 2020). When the transcriptional alterations of *ATP6V1C2, SLC18A2*, *ADORA2*, and *CELF6* are considered together, it is speculated that seafood-derived Pls alleviate the pathology of AD mainly by modulating synaptic vesicle trafficking, promoting neurotransmitter transport, and synaptic transmission.

The expression of several mRNAs encoding GPCRs (*ADORA2A*) and GASPs (*BHLHB9* and *ARMCX4*) were significantly altered in this study. These findings were consistent with previous research, as it has been reported that Pls activate orphan GPCRs to enhance the phosphorylation of ERK and Akt kinases, resulting in the inhibition of caspase-3 activity and thus inhibiting neuronal cell death (Hossain et al., 2016). Besides this, GPCRs also regulate tau phosphorylation and Ca^{2+} dysregulation through various cellular kinases in AD neurons (Chidambaram and Chinnathambi, 2020). Based on these reports and findings of this study, it is suspected that GPCRs are extensively involved in AD pathogenesis, and they may contribute to the protective role of seafood-derived Pls in SH-SY5Y neuronal-like cells.

It is worth noting that although the transcription levels of many genes related to AD pathogenesis, autophagy, endocytosis, synaptic vesicle trafficking, and apoptosis were significantly altered among different groups, obvious morphology differences were not observed among these neuron-like cells after further treatment with Pls or $A\beta_{1-42}$. These results may be related to the short treatment time (24 h) applied in this study. Therefore, longer treatment time will be used in the next study, to further validate these transcriptional alterations by morphological changes. In addition, the protect effective of sea-food derived Pls needs to be further confirmed at the protein level.

CONCLUSION

In summary, the alteration of transcriptional profiles in AD model and AD_Pls cells were investigated, the relevance of PI3K-Akt/mTOR and MAPK signaling pathway, autophagy, endocytosis, synaptic vesicle trafficking, autophagy and apoptosis to the pathogenesis of AD, as well as the potential role of seafood-derived Pls in relieving AD progression were analyzed. The obtained data confirmed that the activation of autophagy in AD model cells is the first response to $A\beta_{1-42}$ impairment, while ATP depletion, deficient lysosomal proteolytic function are the second step. DEGs significantly identified among different groups suggested that therapeutic roles of seafood-derived Pls are mediated through accelerating the toxic $A\beta_{1-42}$ clearance, promoting neurotransmitter transport and synaptic transmission, and facilitating the formation of complex neural network and synaptic structures in AD model SH-SY5Y neuronal-like cells. Results of this work also provided evidence that the GPCRs implicate in Pls-signaling in neuronal cells. However, further *in vivo* studies are needed to validate the effect of Pls on AD proposed in this study at protein level, and to assess the potential use of seafood-derived Pls as an effective therapeutic agent for AD.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI under accession PRJNA728528.

AUTHOR CONTRIBUTIONS

JF and GS: study concept and design and participated in the drafting of the article. JF and XC: acquisition of data. QW and SG: analysis of samples and data interpretation. QS and MZ: critical revision of the manuscript for important intellectual content, and study

supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.773713/full#supplementary-material

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Mediterranean, DASH, and MIND Dietary Patterns and Cognitive Function: The 2-Year Longitudinal Changes in an Older Spanish Cohort

Stephanie K. Nishi[1,2,3,4,5], Nancy Babio[1,2,3,6]*, Carlos Gómez-Martínez[1,2,3], Miguel Ángel Martínez-González[3,7,8], Emilio Ros[3,9], Dolores Corella[3,10], Olga Castañer[3,11], J. Alfredo Martínez[3,12,13], Ángel M. Alonso-Gómez[3,14], Julia Wärnberg[3,15], Jesús Vioque[16,17], Dora Romaguera[3,18], José López-Miranda[3,19], Ramon Estruch[3,20], Francisco J. Tinahones[3,21], José Lapetra[3,22], J. Luís Serra-Majem[3,23], Aurora Bueno-Cavanillas[16,24,25], Josep A. Tur[3,26], Vicente Martín Sánchez[16,27], Xavier Pintó[3,28], Miguel Delgado-Rodríguez[16,29], Pilar Matía-Martín[30], Josep Vidal[31,32], Clotilde Vázquez[3,33], Lidia Daimiel[34], Cristina Razquin[3,7], Oscar Coltell[3,35], Nerea Becerra-Tomás[1,2,3], Rafael De La Torre Fornell[3,36], Itziar Abete[3,12], Carolina Sorto-Sanchez[3,14], Francisco Javier Barón-López[3,15], Antonio José Signes-Pastor[16,17], Jadwiga Konieczna[3,18], Antonio Garcia-Rios[3,19], Rosa Casas[3,20], Ana Maria Gomez-Perez[3,21], José Manuel Santos-Lozano[3,22], Ana García-Arellano[3,21], Patricia Guillem-Saiz[3,10], Jiaqi Ni[1,2,3], Maria Trinidad Soria-Florido[37], M. Ángeles Zulet[3,12], Jessica Vaquero-Luna[3,14], Estefanía Toledo[3,7], Montserrat Fitó[3,11] and Jordi Salas-Salvadó[1,2,3,6]*

[1] Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, and Hospital Universitari San Joan de Reus, Unitat de Nutrició Humana, Reus, Spain, [2] Institut d'Investigació Sanitária Pere Virgili (IISPV), Reus, Spain, [3] Consorcio CIBER, M.P. Fisiopatología de la Obesidad y Nutrición (CIBERObn), Instituto de Salud Carlos III (ISCIII), Madrid, Spain, [4] Toronto 3D (Diet, Digestive Tract and Disease) Knowledge Synthesis and Clinical Trials Unit, Toronto, ON, Canada, [5] Clinical Nutrition and Risk Factor Modification Centre, St. Michael's Hospital, Unity Health Toronto, Toronto, ON, Canada, [6] Nutrition Unit, University Hospital of Sant Joan de Reus, Reus, Spain, [7] Department of Preventive Medicine and Public Health, University of Navarra, IdiSNA, Pamplona, Spain, [8] Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, MA, United States, [9] Department of Endocrinology and Nutrition, Lipid Clinic, Institut d'Investigacions Biomèdiques August Pi Sunyer (IDIBAPS), Hospital Clínic, Barcelona, Spain, [10] Department of Preventive Medicine, University of Valencia, Valencia, Spain, [11] Unit of Cardiovascular Risk and Nutrition, Institut Hospital del Mar de Investigaciones Médicas Municipal d'Investigació Médica (IMIM), Barcelona, Spain, [12] Department of Nutrition, Food Sciences, and Physiology, Center for Nutrition Research, University of Navarra, IdiSNA, Pamplona, Spain, [13] Precision Nutrition and Cardiometabolic Health Program, IMDEA Food, CEI UAM + CSIC, Madrid, Spain, [14] Bioaraba Health Research Institute, Cardiovascular, Respiratory and Metabolic Area, Osakidetza Basque Health Service, Araba University Hospital, University of the Basque Country UPV/EHU, Vitoria-Gasteiz, Spain, [15] EpiPHAAN Research Group, School of Health Sciences, Instituto de Investigación Biomédica de Málaga (IBIMA), University of Málaga, Málaga, Spain, [16] CIBER de Epidemiología y Salud Pública (CIBERESP), Instituto de Salud Carlos III, Madrid, Spain, [17] Instituto de Investigación Sanitaria y Biomédica de Alicante, Universidad Miguel Hernández (ISABIAL-UMH), Alicante, Spain, [18] Health Research Institute of the Balearic Islands (IdISBa), Palma de Mallorca, Spain, [19] Department of Internal Medicine, Maimonides Biomedical Research Institute of Cordoba (IMIBIC), Reina Sofia University Hospital, University of Cordoba, Cordoba, Spain, [20] Department of Internal Medicine, Institut d'Investigacions Biomédiques August Pi Sunyer (IDIBAPS), Hospital Clinic, University of Barcelona, Barcelona, Spain, [21] Department of Endocrinology, Virgen de la Victoria Hospital, Instituto de Investigación Biomédica de Málaga (IBIMA), University of Málaga, Málaga, Spain, [22] Department of Family Medicine, Research Unit, Distrito Sanitario Atención Primaria Sevilla, Seville, Spain, [23] Research Institute of Biomedical and Health Sciences (IUIBS), University of Las Palmas de Gran Canaria and Centro Hospitalario Universitario Insular Materno Infantil (CHUIMI), Canarian Health Service, Las Palmas de Gran Canaria, Spain, [24] Department of Preventive Medicine and

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Edited by:
Yashi Mi,
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Rush University Medical Center, United States

***Correspondence:**
Stephanie K. Nishi
stephanie.nishi@urv.cat
Nancy Babio
nancy.babio@urv.cat

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Public Health, University of Granada, Granada, Spain, [25] Instituto de Investigación Biosanitaria ibs, GRANADA, Granada, Spain, [26] Research Group on Community Nutrition and Oxidative Stress, University of Balearic Islands, Palma de Mallorca, Spain, [27] Institute of Biomedicine (IBIOMED), University of León, León, Spain, [28] Lipids and Vascular Risk Unit, Internal Medicine, Hospital Universitario de Bellvitge, Hospitalet de Llobregat, Barcelona, Spain, [29] Division of Preventive Medicine, Faculty of Medicine, University of Jaén, Jaén, Spain, [30] Department of Endocrinology and Nutrition, Instituto de Investigación Sanitaria Hospital Clínico San Carlos (IdISSC), Madrid, Spain, [31] CIBER Diabetes y Enfermedades Metabólicas (CIBERDEM), Instituto de Salud Carlos III (ISCIII), Madrid, Spain, [32] Department of Endocrinology, Institut d' Investigacions Biomédiques August Pi Sunyer (IDIBAPS), Hospital Clinic, University of Barcelona, Barcelona, Spain, [33] Department of Endocrinology and Nutrition, Hospital Fundación Jimenez Díaz, Instituto de Investigaciones Biomédicas IISFJD, University Autonoma, Madrid, Spain, [34] Nutritional Control of the Epigenome Group, Precision Nutrition and Obesity Program, IMDEA Food, CEI UAM + CSIC, Madrid, Spain, [35] Department of Computer Languages and Systems, Universitat Jaume I, Castellon, Spain, [36] Integrative Pharmacology and Systems Neurosciences, Instituto Hospital del Mar de Investigaciones Médicas (IMIM), Barcelona, Spain, [37] Division of Pure and Applied Biochemistry, Lund University, Lund, Sweden

Background and Aims: Plant-forward dietary patterns have been associated with cardiometabolic health benefits, which, in turn, have been related to cognitive performance with inconsistent findings. The objective of this study was to examine the relationship between baseline adherence to three *a priori* dietary patterns (Mediterranean, DASH, and MIND diets) with 2-year changes in cognitive performance in older adults with overweight or obesity and high cardiovascular disease risk.

Methods: A prospective cohort analysis was conducted within the PREDIMED-Plus trial, involving 6,647 men and women aged 55–75 years with overweight or obesity and metabolic syndrome. Using a validated, semiquantitative 143-item food frequency questionnaire completed at baseline, the dietary pattern adherence scores were calculated. An extensive neuropsychological test battery was administered at baseline and 2-year follow-up. Multivariable-adjusted linear regression models were used to assess associations between 2-year changes in cognitive function *z*-scores across tertiles of baseline adherence to the *a priori* dietary patterns.

Results: Adherence to the Mediterranean diet at baseline was associated with 2-year changes in the general cognitive screening Mini-Mental State Examination (MMSE, β: 0.070; 95% CI: 0.014, 0.175, *P-trend* = 0.011), and two executive function-related assessments: the Trail Making Tests Part A (TMT-A, β: −0.054; 95% CI: −0.110, − 0.002, *P-trend* = 0.047) and Part B (TMT-B, β: −0.079; 95% CI: −0.134, −0.024, *P-trend* = 0.004). Adherence to the MIND diet was associated with the backward recall Digit Span Test assessment of working memory (DST-B, β: 0.058; 95% CI: 0.002, 0.114, *P-trend* = 0.045). However, higher adherence to the DASH dietary pattern was not associated with better cognitive function over a period of 2 years.

Conclusion: In older Spanish individuals with overweight or obesity and at high cardiovascular disease risk, higher baseline adherence to the Mediterranean dietary pattern may be associated with better cognitive performance than lower adherence over a period of 2 years.

Keywords: cognition, dietary pattern, Mediterranean diet (MedDiet), DASH diet, MIND diet

INTRODUCTION

Cognitive decline, associated with aging, is a serious public health concern, given the increasing prevalence of neurodegenerative diseases as people are living longer and the proportion of older persons worldwide continues to rise rapidly (United Nations et al., 2020). Globally, dementia affects an estimated 50 million people, and this prevalence is projected to increase over 130 million by 2050 (World Health Organization [WHO], 2019). Epidemiological studies further suggest a negative interaction of aging and obesity with cognitive dysfunction (Kanaya et al., 2009; Hassing et al., 2010).

With the prevalence of overweight and obesity affecting an estimated 30% of the adult population and more, there are additional adverse implications for cognition health (Ng et al., 2014; Balasubramanian et al., 2020). Cognitive decline carries a significant social and economic burden, given cognitive impairment and dementia are strong predictors of functional disability and dependence (Petersen et al., 2001). Cognitive decline is a normal part of the aging process; however, the rate of decline may vary depending on the differences in genetic and lifestyle-related factors (Wu et al., 2020).

The potential of modifiable lifestyle factors is important as there are no effective pharmacological agents identified for the improvement of cognition or delay of the progression of cognitive decline (Petersen et al., 2018). Diet is a key lifestyle risk factor. Individual nutrients and foods have been inconsistently associated with cognitive function, including some vitamins, carotenoids, long-chain *n*-3 polyunsaturated fatty acids (PUFAs), such as seafood, and whole foods rich in polyphenols, such as fruits and vegetables, nuts, olive oil, and coffee (Rutjes et al., 2018; Ammar et al., 2020; Brainard et al., 2020). As food is consumed as part of a dietary pattern, it is important to consider the interactions and associations of whole dietary approaches. Three dietary patterns, in particular, are hypothesized to have a beneficial impact on cognitive function: the Mediterranean diet (MedDiet), the Dietary Approaches to Stop Hypertension (DASH), and the MedDiet-DASH Intervention for Neurodegenerative Delay (MIND). The MedDiet and DASH are currently promoted for their cardiovascular benefits (Arnett et al., 2019) yet may also be advisable to benefit cognition in themselves and because of the association of vascular risk factors with dementia risk (Gottesman et al., 2017).

Epidemiological studies and clinical trials have shown a relationship between adherence to MedDiet and cognitive function (Loughrey et al., 2017; Wu and Sun, 2017), and the World Health Organization (WHO) has included this dietary pattern in their guidelines for risk reduction of cognitive decline and dementia; however, the strength of the recommendation is considered conditional (World Health Organization [WHO], 2019). A hybrid of the MedDiet and DASH diet, the MIND diet, is also being promoted for brain health, albeit it has been less extensively investigated in relation to cognition and other cardiometabolic health outcomes (van den Brink et al., 2019). At any rate, dietary recommendations for preventing cognitive decline are still not widely accepted in guidelines due to conflicting and limited evidence. The MedDiet, DASH, and MIND dietary patterns each represent a modifiable lifestyle practice that could aid cognitive performance, yet further evidence is needed to inform cognitive guideline recommendations, as well as assess whether changes may be observed in a period of 2 years.

The aim of this study was to prospectively examine the relationship between baseline adherence to *a priori* dietary patterns, assessed using the MedDiet, DASH, and MIND dietary patterns scores, with 2-year changes in cognitive performance in a large sample of community-dwelling older adults with overweight or obesity at high cardiovascular disease risk.

MATERIALS AND METHODS

Study Design

The present analyses were conducted within the framework of the PREvención con DIeta MEDiterránea (PREDIMED)-Plus trial, as an observational cohort, assessing the longitudinal (2-year) associations between baseline adherence to prespecified dietary patterns and cognitive performance. The PREDIMED-Plus study is an ongoing 6-year, multicenter, randomized, parallel-group and primary prevention trial conducted in Spain. The aim of the trial is to assess the effect of an intensive weight loss intervention program based on an energy-restricted traditional MedDiet and physical activity promotion and behavioral support, on clinical cardiovascular events, than usual care and dietary counseling intervention only with an energy unrestricted MedDiet (control group). More detailed information about the study protocol can be found at http://predimedplus.com/ and elsewhere (Martínez-González et al., 2019).

Participants

Participants were recruited between October 2013 and December 2016 in 23 Spanish health centers. Eligible participants were community-dwelling adults (55–75 years) with overweight or obesity (BMI: 27–40 kg/m^2) who met at least three criteria for metabolic syndrome, namely, without stroke, myocardial infarction, or diagnosis of neurodegenerative disease at baseline, according to the International Diabetes Federation and the American Heart Association (Alberti et al., 2009). Participants who had not completed the baseline dietary questionnaires or had reported energy intakes outside the prespecified limits of ≥800 to ≤4,000 kcal/day for men and ≥500 to ≤3,500 kcal/day for women were excluded from these analyses (Willett, 2012). If a given cognitive function assessment was missing, this test was not included in the analysis for that participant.

Exposure: Dietary Assessments

Trained dietitians assessed dietary intake *via* face-to-face interviews at baseline using a previously validated semiquantitative 143-item food frequency questionnaire (FFQ) (Fernández-Ballart et al., 2010). For each item, a portion size was established, and nine consumption frequencies were available, ranging from "never or almost never" to " ≥6 times/day". Energy and nutrient intakes were obtained using data from Spanish food composition tables and by multiplying the frequency by the portion size and accounting for the duration of the period assessed (Moreiras et al., 2018).

Dietary pattern adherence scores were computed from responses to the FFQ. In the case of the MedDiet, it was determined based on a Mediterranean Diet Adherence Screener (MEDAS) score, ranging from 0 to 14 points, which has been previously validated (Schröder et al., 2011; García-Conesa et al., 2020). The DASH diet was defined using the score developed by Fung et al. (2008), which ranges from 8 to 40 points. For the MIND diet, the score developed by Morris et al. (2015a; Berendsen et al., 2018), which ranges from 0 to 15 points, was used.

Outcome: Cognitive Assessments

Participants completed a battery of cognitive tasks at baseline and 2 years of follow-up. This battery of neuropsychological tests included Mini-Mental State Examination (MMSE), a commonly used cognitive screening test (Folstein et al., 1975; Blesa et al., 2001); clock-drawing test (CDT) for evaluating visuospatial and visuo-constructive capacity (del Ser Quijano et al., 2004; Aprahamian et al., 2009; Paganini-Hill and Clark, 2011); semantical and phonological verbal fluency tasks (VFT-a and VFT-p, respectively) for assessing verbal ability and executive function (Benton et al., 1994); Trail Making Tests (TMT) parts A and B for executive function assessment, where part A assesses attention and processing speed and part B further examines cognitive flexibility (Llinàs-Reglà et al., 2017); and forward recall and backward recall Digit Span Tests (DST-f and DST-b, respectively) of the Wechsler Adult Intelligence Scale-III (WAIS-III), where DST-f evaluates attention and short-term memory capacity and DST-b tests working memory (Wechsler, 1997; Rossi et al., 2008). Raw scores for each cognitive assessment were standardized using the mean and standard deviation from the baseline population scores, creating *z*-scores. Global cognitive function (GCF) was determined as a composite score of all eight assessments (Shah et al., 2013; Gómez Martínez et al., 2021), adding or subtracting each individual test value based on whether a higher score indicates higher or lower cognitive performance, respectively, using *z*-scores according to the following equation:

$$\mathrm{GCF} = \big(\mathrm{zMMSE} + \mathrm{zCDT} + \mathrm{zVFTa} + \mathrm{zVFTb} + (-\mathrm{zTMTA}) + (-\mathrm{zTMTB}) + \mathrm{zDSTf} + \mathrm{zDSTb}\big)/8.$$

Covariate Assessment

Trained staff collected information about sociodemographic (i.e., age, sex, education level, and civil status) and lifestyle (i.e., physical activity, dietary intake, and smoking habits) factors via interviewer-administered questionnaires. Physical activity was assessed using a Spanish validated version of the Minnesota leisure-time physical activity questionnaire (Elosua et al., 1994, 2000). Total daily energy intake was estimated according to data from the FFQ. Anthropometric variables, such as weight and height, were measured by trained staff using calibrated scales and wall-mounted stadiometers, respectively. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Personal history related to chronic diseases (e.g., hypertension, hypercholesterolemia, and type 2 diabetes) was self-reported or collected from the medical records of participants. Depressive symptomology was evaluated based on Beck's Depression Inventory-II (BDI-II) with the threshold for depressive status risk established as a score $\geq$ 14 (Beck et al., 1996; Sanz et al., 2003). The intervention group (treatment or control) and center size (<250, 250 to <300, 300 to <400, ≥400) of the PREDIMED-Plus study were also considered as covariates.

Statistical Analyses

All statistical analyses were performed using the latest PREDIMED-Plus study dataset generated on December 22, 2020. Data for dietary adherence scores (exposure variables) are presented as median (range). For the covariates and outcome variables, data are shown as percentages and mean ± standard deviation (SD), for qualitative and quantitative descriptive variables, respectively, and as β [95% confidence interval (CI)] for associations. Participants were classified according to tertiles of dietary pattern adherence, and the lowest tertile was used as the reference category. The chi-squared test and one-way ANOVA were used for qualitative and quantitative variables, respectively, to compare baseline characteristics according to dietary pattern adherence score.

Longitudinal associations between adherences to the *a priori* dietary patterns of participants who completed each of the neuropsychological function tests were analyzed separately using multivariate linear regression. All analyses were conducted with robust estimates of the variance to correct for intracluster correlation. Crude and two adjusted models were assessed. The first model was minimally adjusted using established non-modifiable risk factor-related confounders for cognitive function (age, sex) along with intervention arm, study center size, respective baseline cognitive function score, and corrected for clusters (to account for couples living in the same household being randomized as a single unit). The second model was further adjusted for baseline education level (i.e., primary school, secondary school, or college), civil status (i.e., single, divorced or separated, married, or widower), smoking status (i.e., former smoker, never smoked, or current smoker), BMI (kg/m^2), hypertension (yes/no), hypercholesterolemia (yes/no), diabetes (yes/no), depressive symptomology (yes/no), baseline physical activity (METs min/day), and total energy intake (kcal/day).

The probability *P* for trend across categories of dietary pattern adherence score was calculated using the median value of each category as a continuous variable, and a two-tailed *P*-value < 0.05 was considered statistically significant. Several sensitivity analyses were performed to test the robustness of the findings and identify significant exposure factors to aid in developing priorities for risk mitigation. First, the removal of participants with a baseline MMSE score $\leq$ 23 indicated possible mild dementia (Dementia Care Central, 2020). Second, alcohol was added as a potential confounder in the models (despite being a component of the MedDiet and MIND patterns) as excessive alcohol intake is considered a risk factor for cognitive decline and dementia (Livingston et al., 2020). Third, analyses were conducted assessing the impact of individual food components of each dietary pattern using linear regression accounting for multicollinearity, if present. Statistical analyses were performed using Stata (14.0, StataCorp LP, TX, United States).

RESULTS

Figure 1 provides the flow diagram of participants. This study included a total of 6,647 participants (mean age 65 years, 48% women). **Table 1** provides the baseline characteristics of the participants overall and shows the categories representing the lowest and highest adherence to each of the *a priori* dietary pattern scores at baseline. The median (range) of dietary

adherence scores for the lowest and highest tertiles of each of the three assessed dietary patterns were 6 (1–7) and 10 (10–14) for the MedDiet (lowest possible score 0, highest possible score 14), respectively; 19 (8–21) and 30 (27–38) for DASH (lowest possible score 8, highest possible score 40), respectively; and 8 (2.5–8.5) and 10.5 (10.0–13.5) for MIND (lowest possible score 0, highest possible score 15), respectively.

A higher percentage of women ($P \leq 0.001$), older age ($P < 0.001$), higher physical activity ($P < 0.001$), and a tendency toward lower BMI, although not clinically significant ($P \leq 0.05$), were observed in the highest adherence tertiles for all three dietary patterns. In the DASH and MIND patterns, lower percentages of daily energy intake and current smokers (both $P < 0.001$) were also observed in the highest adherence tertiles of these patterns. Furthermore, higher adherence to the DASH and MIND diets were associated with less alcohol intake ($P < 0.001$) and depression ($P = 0.001$), respectively. For the MedDiet, a lower percentage of participants with depressive symptoms, higher alcohol, and total energy (all $P < 0.01$) was observed in the highest adherence tertile compared with the lowest adherence tertile.

Figure 2 and **Supplementary Table 1** show the β coefficients (95% CIs) associated with 2-year changes in cognitive assessment *z*-scores across tertiles of *a priori* dietary pattern adherence scores. Results of the fully adjusted linear regression models show a significant association between highest adherence to the MedDiet and 2-year changes in MMSE (β: 0.070; 95% CI: 0.014, 0.175, *P-trend* = 0.011), TMT-A (β: −0.054; 95% CI: −0.110, −0.002, *P-trend* = 0.047), and TMT-B (β: −0.062; 95% CI: −0.116, −0.007, *P-trend* = 0.024). Adherence to the MIND diet was significantly associated with 2-year changes in DST-B (β: 0.058; 95% CI: 0.002, 0.114, *P-trend* = 0.045). No other significant beneficial associations with changes in cognitive performance measured by the different neuropsychological test batteries were observed between adherence to the MedDiet, MIND, or DASH dietary patterns. Conversely, significant associations were observed in the crude models with greater 2-year increases in the DASH diet being associated with lower performance in all nine cognitive tests. Sensitivity analyses, which included assessing only participants with baseline MMSE scores above 23 (as scores 23 and below suggest possible mild dementia or worse), or the addition of alcohol as a potential confounder in the model, did not significantly modify the findings (data not shown). The only modification observed was that including total alcohol intake in the model slightly, but non-significantly, mitigated any negative associations observed between adherence to the DASH diet and changes in cognitive function. **Figure 3** shows the impact of all 14 food components of the MedDiet on changes of each individual cognitive test. Of these components, olive oil used as the primary oil was found to be positively associated with changes in global cognitive function, as well as changes in the two DSTs (both forward recall $P = 0.007$ and backward recall $P \leq 0.001$). Nut intake was significantly and positively associated with an increase in CDT performance ($P = 0.034$) and trended toward beneficially impacting changes in MMSE score ($P = 0.069$). Red wine was also significantly associated with changes in various cognitive function assessments, yet indicating converse findings, where red wine intake may have a beneficial relationship with changes in TMT-A ($P = 0.004$),

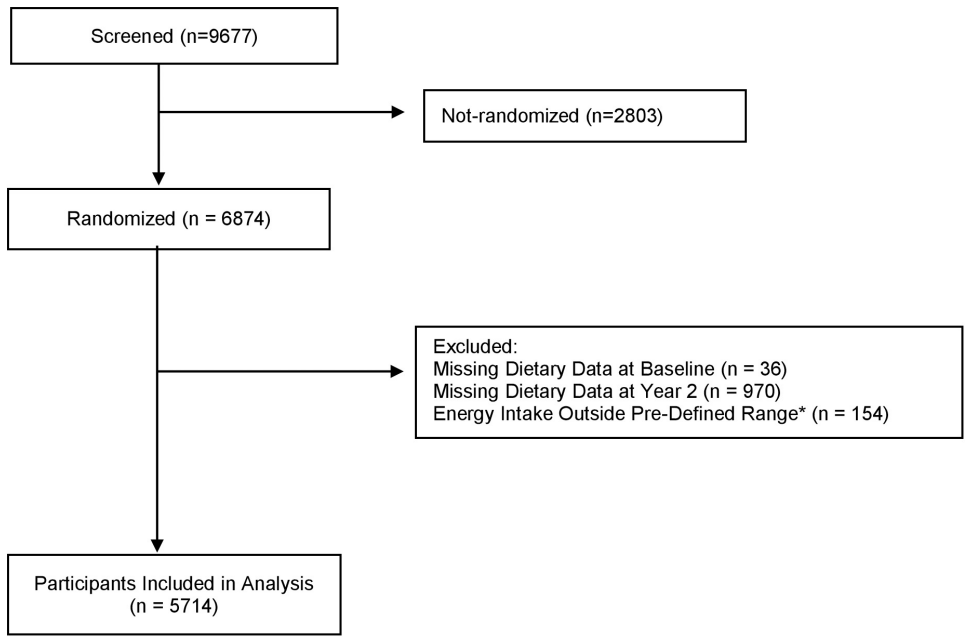

FIGURE 1 | Flow diagram of participants for the analysis of *a priori* dietary pattern adherence and cognitive performance in the PREDIMED-Plus trial. *Energy intakes outside pre-specified limits were identified as ≤800 to ≥4,000 Kcal/d for men and ≤500 to ≥3,500 Kcal/d for women.

TABLE 1 | Baseline characteristics of the PREDIMED-Plus participants according to categories of highest and lowest baseline adherence (based on tertile categorization[1]) to *a priori* dietary patterns.

Dietary patterns (score range)	**Total**	**MedDiet (0–14)**			**DASH (8–40)**			**MIND (0–15)**		
Trait		**Lowest**	**Highest**	***P*-value**	**Lowest**	**Highest**	***P*-value**	**Lowest**	**Highest**	***P*-value**
Diet score adherence, Median (Range)		6 (1–7)	10 (10–14)		19 (8–21)	30 (27–38)		8 (2.5–8.5)	10.5 (10.0–13.5)	
Frequency, *n*	6,647	2,415	1,621		2,674	2,141		3,021	1,652	
Socio-demographic data										
Age (years)	65.0 ± 4.9	64.5 ± 5.0	65.5 ± 4.8	<0.001	64.1 ± 5.0	65.9 ± 4.6	<0.001	64.7 ± 5.0	65.4 ± 4.9	<0.001
Sex (women)	3,218 (48.4)	1,086 (45.0)	831 (51.3)	<0.001	915 (34.2)	1,372 (64.1)	<0.001	1,374 (45.5)	854 (51.7)	0.001
Education level										
Primary school	3,270 (49.2)	1,201 (49.7)	803 (49.5)		1,213 (45.4)	1,138 (53.2)		1,431 (47.4)	822 (49.8)	
High school	1,918 (28.9)	727 (30.1)	425 (26.2)		849 (31.8)	540 (25.2)		922 (30.5)	447 (27.1)	
College	1,459 (22.0)	487 (20.2)	393 (24.2)	0.010	612 (22.9)	463 (21.6)	<0.001	668 (22.1)	383 (23.2)	0.014
Civil status										
Single, divorced, or separated	858 (12.9)	330 (13.7)	203 (12.5)		323 (12.1)	301 (14.1)		390 (12.9)	238 (14.4)	
Married	5,101 (76.7)	1,834 (75.9)	1,270 (78.4)		2,117 (79.2)	1,577 (73.7)		2,328 (77.1)	1,243 (75.2)	
Widower	688 (10.4)	251 (10.4)	148 (9.1)	0.192	234 (8.8)	263 (12.3)	<0.001	303 (10.0)	171 (10.4)	0.151
Disease risk factors										
Body mass index, kg/m^2	32.5 ± 3.4	32.9 ± 3.5	32.1 ± 3.3	<0.001	32.7 ± 3.4	32.4 ± 3.5	0.012	32.7 ± 3.5	32.4 ± 3.3	0.050
Physical activity (MET min/day)	351.9 ± 329.1	316.3 ± 312.7	408.8 ± 368.1	<0.001	325.7 ± 322.0	382.7 ± 339.9	<0.001	317.2 ± 305.1	396.9 ± 362.8	<0.001
Smoking status										
Never smoked	2,948 (44.4)	1,052 (43.6)	732 (45.2)		965 (36.1)	1,137 (53.1)		1,320 (43.7)	727 (44.0)	
Former smoker	2,876 (43.3)	1,036 (42.9)	715 (44.1)		1,286 (48.1)	801 (37.4)		1,267 (41.9)	753 (45.6)	
Current smoker	823 (12.4)	327 (13.5)	174 (10.7)	0.128	423 (15.8)	203 (9.5)	<0.001	434 (14.4)	172 (10.4)	<0.001
Diabetes	2,047 (30.8)	772 (32.0)	475 (29.3)	0.194	784 (29.3)	659 (30.8)	0.003	949 (31.4)	524 (31.7)	0.140
Hypertension	5,583 (84.0)	2,064 (85.5)	1,339 (82.6)	0.035	2,254 (84.3)	1,775 (82.9)	0.222	2,553 (84.5)	1,379 (83.5)	0.573
Hypercholesterolemia	4,649 (69.9)	1,679 (69.5)	1,116 (68.9)	0.281	1,829 (68.4)	1,513 (70.7)	0.072	2,069 (68.5)	1,198 (72.5)	0.016
Depressive symptoms	1,368 (20.6)	571 (23.6)	304 (18.8)	<0.001	533 (19.9)	477 (22.3)	0.059	683 (22.6)	307 (18.6)	0.001
Dietary intake										
Alcohol intake (g/day)	11.0 ± 15.0	11.1 ± 15.2	11.9 ± 16.0	0.004	15.2 ± 17.7	7.0 ± 10.7	<0.001	10.7 ± 15.3	11.6 ± 14.5	0.152
Energy intake (kcal/day)	2,365.2 ± 551.5	2,364.1 ± 597.2	2,434.4 ± 502.3	<0.001	2,467.3 ± 569.1	2,283.5 ± 506.0	<0.001	2,417.6 ± 578.6	2,310.8 ± 512.8	<0.001
Cognitive function tests										
GCF[2]	0.02 ± 0.65	0.02 ± 0.66	0.03 ± 0.63	0.852	0.10 ± 0.63	-0.08 ± 0.66	<0.001	0.04 ± 0.64	0.01 ± 0.64	0.543
MMSE	28.22 ± 1.91	28.17 ± 1.94	28.29 ± 1.83	0.154	28.38 ± 1.77	28.01 ± 2.02	<0.001	28.25 ± 1.86	28.18 ± 2.00	0.500
CDT	5.93 ± 1.23	5.91 ± 1.28	5.95 ± 1.20	0.578	6.02 ± 1.19	5.80 ± 1.31	<0.001	5.95 ± 1.22	5.91 ± 1.25	0.566
VFT-a	16.01 ± 4.90	15.88 ± 4.96	16.23 ± 4.94	0.080	16.42 ± 4.95	15.45 ± 4.82	<0.001	16.13 ± 4.92	15.95 ± 4.89	0.205
VFT-p	12.18 ± 4.53	11.96 ± 4.55	12.43 ± 4.57	0.004	12.42 ± 4.50	11.75 ± 4.47	<0.001	12.15 ± 4.56	12.14 ± 4.45	0.572
TMT-A	52.80 ± 28.56	53.01 ± 28.86	52.23 ± 27.33	0.649	49.95 ± 26.31	56.33 ± 31.62	<0.001	52.38 ± 28.22	53.55 ± 29.71	0.411
TMT-B	130.05 ± 72.36	131.96 ± 75.05	127.49 ± 68.05	0.158	122.47 ± 68.08	139.77 ± 72.36	<0.001	128.70 ± 71.89	131.59 ± 72.39	0.365
DST-f	8.79 ± 2.46	8.66 ± 2.44	8.89 ± 2.45	0.013	8.95 ± 2.46	8.56 ± 2.40	<0.001	8.79 ± 2.43	8.73 ± 2.50	0.579
DST-b	5.11 ± 2.22	5.13 ± 2.19	5.09 ± 2.26	0.834	5.33 ± 2.24	4.80 ± 2.15	<0.001	5.18 ± 2.19	4.99 ± 2.26	0.034

Data are n (%) or mean ± SD for categorical and quantitative variables, respectively. The exception is the diet score is represented as mean (range).
The chi-squared analysis was used to assess categorical variables and one-way ANOVA for quantitative variables.
[1]Highest and lowest dietary pattern adherence categories were determined based on tertiles of baseline data with the highest and lowest adherence groups being tertile 3 and 1, respectively.
[2]A composite of z-scores was used to calculate GCF using the formula: GCF = $(Z_{MMSE} + Z_{CDT} + Z_{VFT-a} + Z_{VFT-b} + (-Z_{TMT-A}) + (-Z_{TMT-B}) + Z_{DST-f} + Z_{DST-b})/8$.
CDT, clock drawing test; DASH, Dietary Approaches to Stop Hypertension; DST-b, digit span test-backward; DST-f, digit span test -forward; GCF, global cognitive function; MedDiet, Mediterranean dietary pattern; MIND, Mediterranean-DASH Intervention for Neurodegenerative Delay; MMSE, Mini-Mental State Examination; TMT-A, Trail Making Test Part A; TMT-B, Trail Making Test Part B; VFT-a, verbal fluency tasks semantical; VFT-p, verbal fluency tasks phonological.

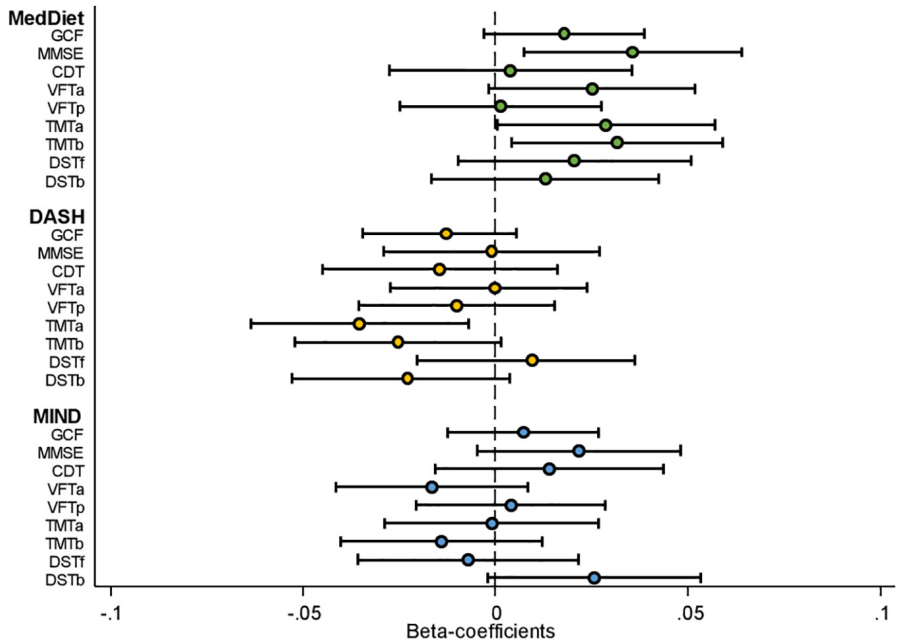

FIGURE 2 | Cognitive function assessment by dietary pattern adherence [standardized beta-coefficients (95% confidence intervals)]. CDT; clock drawing test; DASH; Dietary Approaches to Stop Hypertension; DST-b, digit span test backward; DST-f, digit span test forward; GCF, global cognitive function; MedDiet, Mediterranean dietary pattern; MIND; Mediterranean-DASH Intervention for Neurodegenerative Delay; MMSE, Mini-Mental State Examination; TMT-A, Trail Making Test Part A; TMT-B, Trail Making Test Part B; VFT-a; verbal fluency tasks semantical; VFT-b; verbal fluency tasks phonological. Model presented adjusted for age (in Years), sex, intervention group, centre size (<250, 250 to <300, 300 to <400, ≥400), respective cognitive test score at baseline, baseline education level (primary school, secondary school collage), civil status (single, divorced, or separated, married, and windower), smoking habits (smoker, former smoker, and never smoked), corrected for clusters (to account for couples living in the same household being randomized as a single unit), BMI (kg/m^2), hypertension (yes/no), baseline physical activity (MET min/week) and total energy intake (kcal/day). For the neurological tests, a positive beta-coefficient value in the figure indicates better cognitive performance according to the associated test.

TMT-B (P = 0.006), and DST-b (P = 0.020), and a negative association with changes in VFT-a (P = 0.048). Likewise, preferably consuming white meat as opposed to red or processed meat showed conflicting findings where a beneficial association was observed with changes in DST-f (P = 0.001) and negatively associated with changes in CDT (P = 0.001). Fish and shellfish intake was negatively associated with changes in DST-b (P = 0.008). Analyses investigating the DASH and MIND diets showed similar associations with nut consumption and wine intake. Additionally, when assessed within the context of the MIND diet, lower consumption of confectionery products was associated with improvements in 2-year changes in GCF (P = 0.007), VFT-a (P = 0.019), TMT-a (P = 0.011), DST-b (P = 0.042), and higher red meat intake was associated with worsening changes in TMT-a (P = 0.001) and TMT-b (P = 0.014) scores.

Table 2 presents the quantity of intake of various dietary components overall and by tertile of dietary pattern adherence score and shows differences between the dietary patterns. When assessing the MedDiet adherence, 94.7% of participants within the highest adherence tertile used olive oil as their primary oil with a mean intake level of 47.4 ± 14.7 g/day, nut intake was 19.9 ± 18.2 g/day, and red wine consumption was on average 59.4 ± 98.1 g/day.

DISCUSSION

This study examined the PREDIMED-Plus trial as a longitudinal, observational cohort to evaluate the relationship between adherence to *a priori* dietary patterns and changes in cognitive performance in community-dwelling older adults with overweight or obesity and at high cardiovascular disease risk. Findings suggested that the MedDiet may support cognitive function in older age as significant beneficial associations were observed between greater adherence to the MedDiet with favorable cognitive changes in MMSE, TMT-A, and TMT-B assessments over the follow-up period of 2 years. This represented better general cognitive function, as well as executive function specifically attention and processing speed and cognitive flexibility in those with higher adherence to a MedDiet. Findings also indicated that the MIND diet may be associated with better working memory based on higher adherence being related to higher DST-b assessment. However, the DASH diet was not beneficially associated with 2-year changes in cognitive function in this population with overweight or obesity at high cardiovascular disease risk. The observed advantageous associations between adherence to the MedDiet and cognition align with previous findings presented in systematic reviews and meta-analyses of observational studies suggesting associations between MedDiet adherence with slower

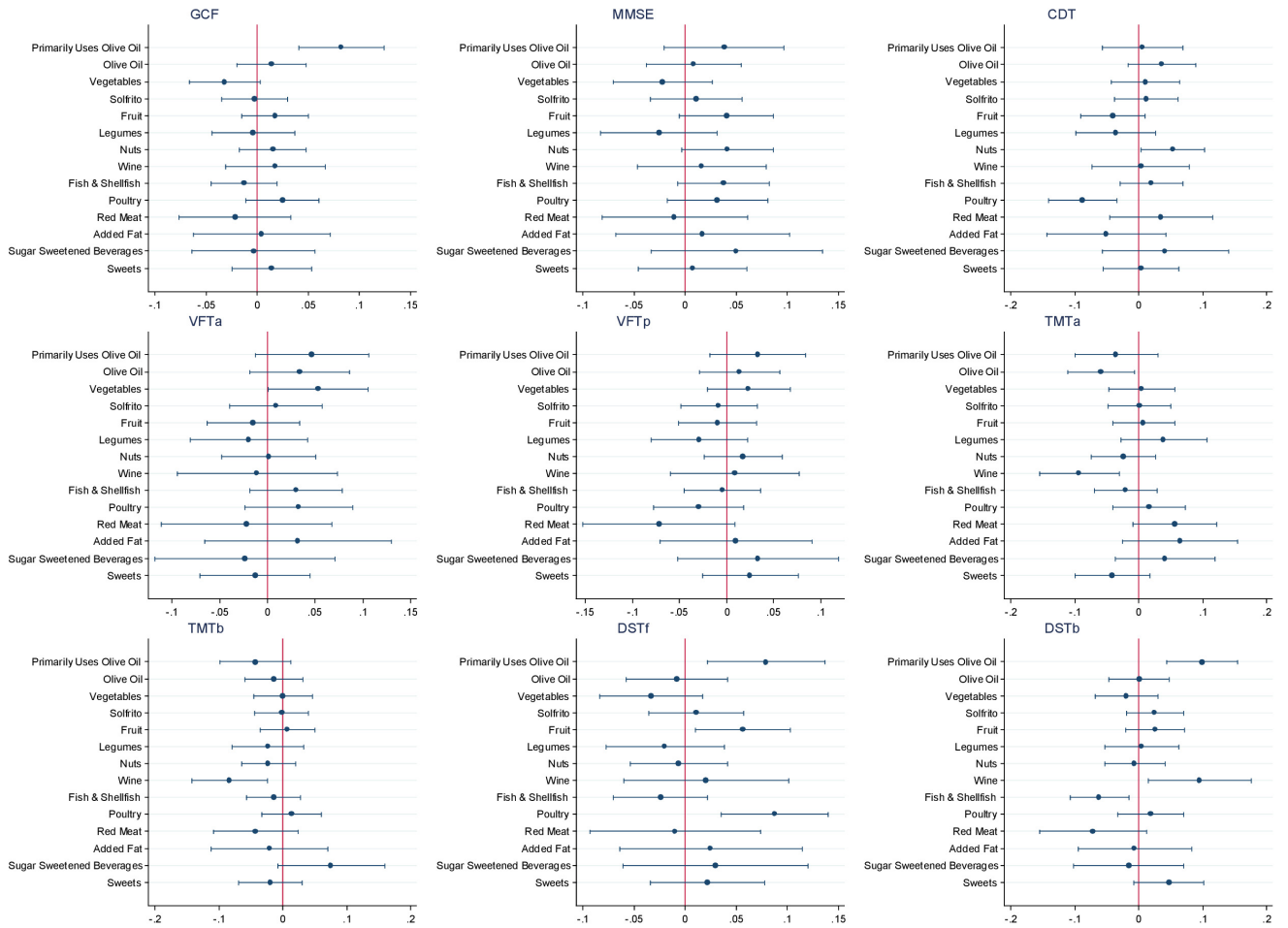

FIGURE 3 | Association of specific dietary components of the Mediterranean dietary pattern (MedDiet) on the cognitive function battery of tests. CDT; clock drawing test; DST-b, digit span test backward; DST-f, digit span test forward; GCF, global cognitive function; MMSE, Mini-Mental State Examination; TMT-A, Trail Making Test Part A; TMT-B, Trail Making Test Part B; VFT-a; verbal fluency tasks semantical; VFT-b; verbal fluency tasks phonological.

cognitive decline, lower risk of dementia (especially Alzheimer's disease), and reduced conversion of mild cognitive impairment to Alzheimer's disease (Singh et al., 2014; Loughrey et al., 2017; Wu and Sun, 2017). Recently, a meta-analysis including nine prospective cohort studies reported that high adherence to the MedDiet was associated with a 21% risk reduction in pooled cognitive disorders, in addition to a dose-response with positive findings almost exclusively limited to higher MedDiet adherence (Wu and Sun, 2017). Furthermore, neuroimaging evaluations have found evidence in favor of a protective effect (Karstens et al., 2019). Nevertheless, it is interesting to highlight that previous prospective cohort studies generally had 4 or more years of follow-up, many of which were conducted in relatively healthy, non-Mediterranean populations, with many of the cognitive assessments using screening tests based on criteria to discriminate overall mild cognitive impairment or dementia. Our study also found a positive association between higher adherence to MedDiet and a screening test (MMSE) but also showed specific beneficial associations for executive functioning, including attention and processing speed (TMT-A) and cognitive flexibility (TMT-B). Conversely, a systematic review of randomized controlled trials (nine reports, five unique trials) showed inconsistent findings when comparing a MedDiet with either a waiting list, usual diet, or a low-fat control group for a duration ranging from 10 days to 6.5 years on cognition or brain morphology and function (Radd-Vagenas et al., 2018). However, the authors stated that significant and clinically meaningful effect sizes were found for cognitive composites in the largest and most robust trial, with a duration of 4.1 years, conducted within the context of the PREDIMED trial (Valls-Pedret et al., 2015). Furthermore, in a more recent analysis of a smaller sub-cohort of the PREDIMED-Plus trial evaluating cognition, higher adherence to an energy-reduced MedDiet was associated with greater improvements in memory; however, interpretation of these findings was related to the interplay with weight loss (Soldevila-Domenech et al., 2021). In this study population, we did not find an association for the GCF, which may be explained by the short duration (2 years) and by the broad

TABLE 2 | Quantity of dietary intake by tertile of dietary pattern adherence score.

Dietary pattern	Total	MedDiet			DASH			MIND		
Baseline intake		**Lowest adherence**	**Moderate adherence**	**Highest adherence**	**Lowest adherence**	**Moderate adherence**	**Highest adherence**	**Lowest adherence**	**Moderate adherence**	**Highest adherence**
Score, median (range)		6 (1–7)	8 (8–9)	10 (10–14)	19 (8–21)	24 (22–26)	30 (27–38)	8 (2.5–8.5)	9 (9.0–9.5)	10.5 (10.0–13.5)
Olive oil as the primary oil used, *n* (%)	5,266 (79.2)	1,516 (62.8)	2,214 (84.8)	1,536 (94.8)	2,050 (76.7)	1,454 (79.4)	1,762 (82.3)	2,299 (76.1)	1,578 (79.9)	1,389 (84.1)
Olive oil, g/d	39.9 ± 17.0	33.9 ± 16.3	40.7 ± 17.0	47.3 ± 14.7	41.4 ± 16.8	39.3 ± 16.9	38.5 ± 17.2	39.2 ± 17.7	40.1 ± 16.5	40.7 ± 16.4
Vegetables, g/d	328.1 ± 139.9	295.2 ± 132.5	332.1 ± 137.4	370.8 ± 142.5	268.6 ± 111.0	329.1 ± 125.7	401.7 ± 148.4	280.0 ± 123.1	342.9 ± 128.7	398.7 ± 147.7
Green leafy vegetables, g/d	77.8 ± 42.5	68.4 ± 39.3	78.5 ± 42.4	90.5 ± 44.0	64.1 ± 35.4	78.0 ± 40.4	94.6 ± 46.2	63.7 ± 35.4	81.0 ± 39.9	99.7 ± 47.1
Other vegetables, g/d	301.4 ± 125.8	276.3 ± 121.0	303.2 ± 123.4	335.9 ± 128.3	253.8 ± 104.7	303.3 ± 115.5	359.1 ± 133.8	264.4 ± 116.7	314.8 ± 117.5	352.9 ± 130.2
Sofrito, *n* (%)	3,772 (56.8)	985 (40.8)	1,531 (58.6)	1,256 (77.5)	1,528 (57.1)	1,027 (56.1)	1,217 (56.8)	1,707 (56.5)	1,136 (57.6)	929 (56.2)
Fruit, g/d	359.2 ± 206.8	315.2 ± 190.5	365.9 ± 203.8	414.0 ± 220.2	271.5 ± 163.2	365.7 ± 191.6	463.3 ± 218.2	323.7 ± 190.8	367.2 ± 196.1	414.6 ± 232.6
Fruit juice (natural), g/d	59.2 ± 94.5	61.8 ± 101.5	56.6 ± 91.7	59.6 ± 87.6	55.5 ± 92.7	55.6 ± 94.6	66.9 ± 96.1	57.9 ± 94.4	57.9 ± 87.4	63.2 ± 102.3
Berries, g/d	6.0 ± 8.6	5.6 ± 8.8	5.8 ± 8.1	7.0 ± 8.9	4.1 ± 5.7	6.0 ± 8.4	8.4 ± 10.8	4.7 ± 6.7	6.1 ± 7.9	8.3 ± 11.4
Whole grains, g/d	41.3 ± 63.4	33.4 ± 57.1	43.8 ± 64.2	49.0 ± 69.4	19.3 ± 49.2	38.4 ± 60.4	71.2 ± 69.5	22.6 ± 44.3	41.4 ± 62.1	75.3 ± 78.6
Legumes, g/d	20.7 ± 11.2	19.0 ± 10.3	20.8 ± 11.3	23.1 ± 12.1	17.8 ± 9.0	20.3 ± 10.5	24.7 ± 13.1	18.4 ± 9.9	21.3 ± 11.0	24.3 ± 12.7
Nuts, g/d	14.9 ± 17.1	11.1 ± 15.2	15.5 ± 16.8	20.2 ± 18.8	9.8 ± 13.1	14.0 ± 15.8	22.1 ± 20.0	10.5 ± 14.0	15.9 ± 17.3	21.8 ± 19.6
Red Wine, g/d	46.9 ± 90.2	40.4 ± 85.5	44.9 ± 87.2	59.9 ± 100.0	61.5 ± 108.6	43.1 ± 82.0	32.1 ± 65.5	38.3 ± 84.6	49.1 ± 91.7	60.1 ± 96.5
Low-fat dairy, g/d	256.6 ± 201.6	244.0 ± 195.7	258.7 ± 202.1	271.9 ± 208.1	189.3 ± 171.7	267.3 ± 201.1	331.4 ± 208.3	249.2 ± 200.0	256.6 ± 202.0	269.9 ± 203.2
Cheese, g/d	346.2 ± 201.3	348.2 ± 194.3	343.8 ± 202.4	347.0 ± 209.7	300.4 ± 179.4	351.8 ± 203.9	399.5 ± 211.4	355.4 ± 197.1	338.8 ± 203.8	338.0 ± 205.3
Fish and seafood, g/d	102.2 ± 47.7	89.9 ± 46.0	104.1 ± 46.7	117.5 ± 46.8	95.4 ± 46.2	103.0 ± 48.0	110.0 ± 48.1	94.4 ± 47.0	105.6 ± 45.2	112.5 ± 49.5
Poultry, g/d	51.0 ± 28.7	48.3 ± 29.9	51.5 ± 27.2	54.3 ± 28.8	48.0 ± 28.2	51.7 ± 29.0	54.1 ± 28.7	42.3 ± 28.9	54.6 ± 26.6	62.5 ± 25.4
Red meat, g/d	47.4 ± 33.7	55.3 ± 37.8	44.9 ± 31.6	39.8 ± 27.3	60.2 ± 35.9	45.1 ± 30.8	33.4 ± 26.2	52.2 ± 35.2	45.9 ± 32.4	40.5 ± 30.9
Processed meat, g/d	36.4 ± 24.1	40.4 ± 27.7	34.7 ± 22.3	33.3 ± 19.9	44.9 ± 27.4	34.4 ± 21.7	27.5 ± 17.0	39.8 ± 24.2	35.8 ± 23.0	30.9 ± 24.0
Added fats[1], g/d	2.5 ± 6.4	3.9 ± 9.0	2.0 ± 4.3	1.4 ± 3.4	1.7 ± 3.9	1.5 ± 3.5	1.2 ± 2.9	1.9 ± 4.3	1.2 ± 2.9	0.9 ± 2.3
Sugar-sweetened beverages, ml/d	39.1 ± 89.3	60.6 ± 118.3	29.5 ± 68.9	22.4 ± 55.3	66.3 ± 114.1	30.9 ± 79.2	12.0 ± 37.4	48.9 ± 101.8	33.9 ± 79.2	27.3 ± 72.7
Confectionary/Pastries, g/d	13.7 ± 17.6	18.3 ± 20.9	12.3 ± 16.2	9.0 ± 11.7	15.9 ± 19.5	13.1 ± 16.3	11.5 ± 15.6	18.0 ± 20.8	11.6 ± 14.5	8.5 ± 11.5
Fast/fried foods, g/d	23.8 ± 24.7	28.8 ± 28.4	21.8 ± 22.5	19.5 ± 20.7	30.5 ± 28.4	21.9 ± 22.2	16.9 ± 19.1	31.2 ± 28.6	20.5 ± 20.3	14.1 ± 16.5
Sodium, mg/d	3,281.9 ± 1,012.9	3,350.98 ± 1,064.2	3,237.8 ± 990.4	3,250.3 ± 964.1	3,637.4 ± 1,046.4	3,190.1 ± 957.6	2,916.5 ± 857.5	3,407.1 ± 1,050.5	3,247.2 ± 973.2	3,094.6 ± 956.2

[1]*Added fats include butter, margarine, lard, and cream.*

Cells filled in gray represent food groupings that are not a main component of the specified dietary pattern.

Cells with no fill (i.e., have a white background) represent food groupings that are a main component of the specified dietary pattern.

DASH, Dietary Approaches to Stop Hypertension; MedDiet, Mediterranean dietary pattern; MIND, Mediterranean-DASH Intervention for Neurodegenerative delay.

neuropsychological battery utilized compared with other studies. However, the present findings further support the MedDiet for better cognition while suggesting that beneficial associations may be observable within a shorter timeframe and have applicability for populations at greater risk of cognitive decline (older, with overweight or obesity, and at high risk of cardiovascular disease), which could have implications for improving quality of life. In particular, obesity and its comorbidities are associated with accelerated cognitive decline and impaired cognitive performance including neurodegenerative pathologies, such as dementia, in later life (Dye et al., 2017).

While a significant association was seen in the present analyses with the MedDiet and MIND diet within a shorter follow-up duration with some cognitive function assessments compared with other prospective cohort studies that have been conducted, this relationship was not found with the DASH diet. While MedDiet has been associated with a lower risk of cognitive impairment, it has not always been associated with a slower decline in cognitive function (Keenan et al., 2020). Inconsistent findings have previously been observed with the DASH diet (van den Brink et al., 2019). However, MIND dietary adherence has been associated with better cognitive function across various domains in a systematic review of 13 MIND studies (9 cohorts, 3 cross-sectional, and 1 RCT) evaluating cognitive functioning in older adults (Kheirouri and Alizadeh, 2021). The observed discrepancies with present findings may be related to differences in the types of foods consumed by the study population, and a potential threshold effect related to the amount of each food component consumed, as well as the cognitive tests performed.

The analyzed three dietary patterns each have plant-based foundations, with moderate to high amounts of fish and dairy products, yet they differ in the types and amounts of each dietary component. The MedDiet is typically characterized by high consumption of olive oil, fruits, vegetables, legumes, nuts, cereals, and unsaturated fatty acids; low consumption of meat and saturated fatty acids; low to moderate consumption of dairy products; moderate to high consumption of fish; and a regular, but moderate, intake of wine (Sánchez-Sánchez et al., 2020). The DASH diet shares many similarities yet differs in recommending low-fat dairy and low sodium, besides having fewer specifications (Fung et al., 2008). Based on these two dietary patterns, the MIND diet was developed combining Mediterranean and DASH aspects and incorporating purported neuroprotective foods such as green leafy vegetables and berries (Morris et al., 2015a,b). A potential explanation for our discordant findings may be due to differences in the use of olive oil as the primary oil between tertiles of adherence. In our sample, the use of olive oil was clearly linked with a beneficial association in the GCF composite, as well epidemiological and clinical evidence have suggested improved cognition with olive oil (Millman et al., 2021; Theodore et al., 2021). With any dietary pattern, in addition to observing associations with specific food components, there is also the potential for synergistic effects (Schulz et al., 2021). The present findings suggest such effects given the observed associations for MedDiet with MMSE and TMT-A and TMT-B or the MIND diet with DST-b did not appear to be fully explained by any one component of the dietary pattern; however, further investigation is warranted.

Potential Mechanisms

The antioxidant, vitamin, probiotic, plant protein, and unsaturated fatty acid content along with low glycemic index/load components of the *a priori* dietary patterns studied have been proposed to possibly affect biological mechanisms of neurocognitive aging (Frisardi et al., 2010). These factors are thought to potentially lead to improved cognition through influencing vascular health and direct promotion of neuroprotection via anti-inflammatory mechanisms and reducing oxidative stress, ameliorating glycemic control, and supporting a favorable microbiome (Caracciolo et al., 2014). Specifically, the observations with the MedDiet and changes in cognitive function may be related to synergistic or individual associations of specific foods, such as olive oil and nuts, due to associations of these foodstuffs with the above-mentioned mechanisms (Viguiliouk et al., 2014; Marcelino et al., 2019; Creedon et al., 2020).

Limitations and Strengths

There are several limitations to this research. First, the demographic profile of the PREDIMED-Plus cohort, which is composed of predominantly white, older Spanish individuals with metabolic syndrome and overweight or obesity, may limit the generalizability of the results to other populations. However, the homogeneity and the large sample size of the cohort increase the internal validity of the findings by avoiding potential confounding effects of socioeconomic status, educational level, and access to health care. Second, FFQs tend to be limited concerning the variety of foods assessed, as compared to 24-h recalls and food records but are often more likely to reflect usual intake (Willett, 2012). The certainty in the dietary pattern scoring systems utilized may also be limited and experience restrictions due to a potential lack of direct alignment of food items and questions noted in the FFQ with each of the diet score components. FFQs are also prone to misclassification and recall bias as they rely on the memory of participants. This is particularly important in a study of cognition when there could be a decline in cognitive function and memory deficits in the population. However, due to the prospective nature of the study, baseline diet recall is unlikely to have been influenced by cognitive outcomes over the follow-up period, and baseline cognitive function was considered as a confounding variable. Another limitation is that the categorization of dietary pattern adherence was based on sample-specific cutoffs, and there are methodological differences among the various dietary scoring systems available, limiting comparability (van den Brink et al., 2019). Likewise, differences in the types of foods consumed by the study population, and the narrow range in scores between lowest and highest tertiles of intake, may have limited the ability to

discern a difference, especially for the MIND pattern. Given that optimal or absolute minimum amounts of key foods for cognitive performance are still unclear, threshold amounts of foods for a neuroprotective effect may not have been reached in the present analyses especially in those consuming lower overall energy intake levels. In terms of cognitive assessments, while the use of a composite domain *z*-score may provide an overall global assessment of cognitive function, the component tests used to create these scores in other studies vary, thus making comparisons difficult. Also, a global screening tool may be less sensitive to detect possible associations due to a potential ceiling effect (Franco-Marina et al., 2010), hence potentially explaining the null associations observed with GCF. Presenting this composite score in addition to the individual cognitive test assessments in this study provides a broader picture of the relationship of these dietary patterns with overall cognition and specific cognitive functions. Additionally, as an observational study, our analysis may be limited by the relatively short duration and is prone to residual confounding from factors not assessed in our models. Specifically, possible genetic interactions, such as with the apolipoprotein E E4 (*ApoEε4*) genotype, which has been associated with cognition, especially in the presence of hypercholesterolemia (Perna et al., 2016), was not able to be accounted for in the current analyses. Finally, a cause-effect relationship could not be determined due to the nature of the study design, as an observational cohort.

Nonetheless, this study is strengthened by the longitudinal analysis conducted in a large cohort using a comprehensive and thoroughly measured battery of cognitive tests that assess various function areas, as well as the use of an FFQ developed and validated for an older Spanish population. The statistical models were also adjusted for multiple sociodemographic, economic, anthropometric, lifestyle, and biological confounders of the association between diet and cognition, while evaluation of the three distinct *a priori* dietary patterns within the same study cohort minimizes the effects of population-specific confounders or effect modifiers.

Future Directions

Considering the limitations of the present analyses and inconsistencies observed in the literature, studies, especially randomized controlled trials, accounting for relevant genotypes, use of dietary compliance biomarkers such as *via* the development of diet-specific metabolomes, and undertaking standardized neuropsychological assessments including biomarkers and neuroimaging would be useful for future research.

CONCLUSION

In older Spanish adults with overweight or obesity, higher adherence to the MedDiet may help mitigate the risk of cognitive decline, specifically as it relates to general and executive cognitive functioning, even over a short (2-year) period.

DATA AVAILABILITY STATEMENT

Data described in the manuscript, code book, and analytic code will be made available upon request pending application and approval of the PREDIMED-Plus Steering Committee. There are restrictions on the availability of data for the PREDIMED-Plus trial, due to the signed consent agreements around data sharing, which only allow access to external researchers for studies following the project purposes. Requestors wishing to access the PREDIMED-Plus trial data used in this study can make a request to the PREDIMED-Plus trial Steering Committee chair: (JS-S, jordi.salas@urv.cat). The request will then be passed to members of the PREDIMED-Plus Steering Committee for deliberation.

ETHICS STATEMENT

The study was conducted in accordance with the principles of the Declaration of Helsinki. The respective Institutional Review Board (IRB) of all study centres approved the study protocol. The trial was registered at the International Standard Randomized Controlled Trial in 2014 (ISRCTNwww.isrctn.com/ISRCTN8989887089898870). All participants provided written informed consent.

AUTHOR CONTRIBUTIONS

MAM-G, DC, JS-S, MF, JAM, AMA-G, JW, JV, DR, JL-M, RE, FJT, JL, JLS-M, AB-C, JAT, VMS, XP, MD-R, PM-M, JV, CV, LD, and ER (all the principal PREDIMED-Plus investigators) contributed to the study concept and design and to data extraction from the participants in the PREDIMED-Plus trial, with SKN, NB, and JS-S, contributing to the study concept and design of the present analyses. SKN, NB, CG-M, NB-T and JS-S performed the statistical analyses. SKN, NB and JS-S drafted the manuscript. All authors reviewed the manuscript for important intellectual content and approved the final version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.782067/full#supplementary-material

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Dynamic Changes of Endogenic or Exogenic β-Carboline Alkaloid Harmine in Different Mammals and Human *in vivo* at Developmental and Physiological States

*Ning Cao[1], Shuping Li[1], Aimin Xu[2], Manlin Li[1], Xiaoguang Zou[2], Zunji Ke[3], Gang Deng[1], Xuemei Cheng[1] and Changhong Wang[1]**

[1] The MOE Key Laboratory for Standardization of Chinese Medicines, Shanghai Key Laboratory of Chinese Compound Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai, China, [2] Kashi Prefecture First People's Hospital, Kashi, China, [3] School of Basic Medicine, Shanghai University of Chinese Medicine, Shanghai, China

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Yashi Mi,
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Ghulam Hussain,
Government College University, Faisalabad, Pakistan
Dan Zhang,
Shaanxi University of Technology, China

***Correspondence:**
Changhong Wang
wchcxm@shutcm.edu.cn;
wchcxm@hotmail.com

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Objective: Several β-carboline alkaloids (βCBs), such as harmine, harmaline, harmane, and nor-harmane, are effective for Alzheimer's disease mouse models. They can be found in some plants, common foodstuffs, and blank plasma of various mammals. However, whether these compounds in mammals are exogenous or endogenous remain unclear.

Methods: The exposure levels of βCBs and of neurotransmitters in plasma and tissues of pup rats, aging rats, mice of different physiological states, and healthy volunteers were detected by using UPLC-MS/MS. Plasma and tissue samples from 110 newborn rats up to 29 days old at 11 sampling points were collected and were analyzed to determine the concentration variation of βCBs in the developmental phase of newborn rats. The plasma of rats aged 2 to 18 months was used to detect the variation trend of βCBs and with some neurotransmitters. The plasma samples of normal C57BL/6 mice, APP/PS1 double transgenic mice, and scopolamine-induced memory impairment mice were collected and were analyzed to compare the difference of βCBs in different physiological states. The exposure levels of βCBs such as harmine, harmaline, and harmane in plasma of 550 healthy volunteers were also detected and analyzed on the basis of gender, race, and age.

Results: Results showed that harmine was the main compound found in rats, mice, and human, which can be detected in a newborn rat plasma (0.16 ± 0.03 ng/ml) and brain (0.33 ± 0.14 ng/g) without any exogenous consumption. The concentration of harmine in rat plasma showed a decreasing trend similar to the exposure levels of neurotransmitters such as 5-hydroxytryptamine, acetylcholine chloride, glutamic acid, tyrosine, and phenylalanine during the growth period of 18 months. The harmine exposure in rats and human indicates high dependence on the physiological and pathological status such as aging, gender, and race.

Conclusion: The dynamic changes of harmine exposure in different animals and human, *in vivo,* at developmental and physiological states indicate that harmine is a naturally and widely distributed endogenous substance in different mammals and human. In addition to exogenous ingestion, spontaneous synthesis might be another important source of harmine in mammals, which should be verified by further experiment.

Keywords: harmine, β-carboline alkaloids, exposure levels, endogenous substance, Alzheimer's disease

INTRODUCTION

The β-carboline alkaloids (βCBs), such as harmine, harmaline, harmane, and nor-harmane, are active components of *Peganum harmala* (Li et al., 2017a). Traditionally, *P. harmala* is used to treat diseases, such as cough, asthma, rheumatoid arthritis, and swelling pain in regions such as the Middle East, central Asia, and South America (Zhao et al., 2012). In addition, such βCBs show various pharmacological effects, including hypoglycemic effect, antineoplastic activity, and acetylcholinesterase (AChE) inhibitory activity (Li et al., 2017a). These βCBs are also present in other plants, including *Banisteriopsis caapi*, *Tribulus terrestris*, and *Ayahuasca* (Li et al., 2016). Such βCBs can also be found in common foodstuffs, such as plant-derived foods (e.g., grapes, rice, corn, barely, bean, and rye), processed foods (e.g., wine, beer, whiskey, brandy, sake, coffee, vinegar, and tobacco), and meat products (e.g., barbecue, smoked fish, and smoked sausages) (Xie et al., 2021). Moreover, both harmane and nor-harmane are widely distributed in the brain, liver, blood, and urine of human and many other mammals (Louis et al., 2005, 2010, 2013). Based on previous reports, plasma contents of harmane in patients with tremor and Parkinson's disease were higher than those in healthy individuals (Louis et al., 2005, 2014). Harmine, harmaline, and other βCBs have been found in the blank plasma of adult rats and mice even without consuming such compounds (Li et al., 2016). Consequently, whether harmine, harmaline, harmane, and nor-harmane are endogenous or exogenous in mammalian tissues and plasma, and whether such βCBs in mammals are useless or functional, remain unclear.

The Pictet–Spengler reaction (P–S reaction) in plants synthesized the βCBs (Cao and Wang, 2021). Strictosidine synthase is a vital enzyme in the reaction (Stockigt et al., 2011). In addition, a protein similar to strictosidine synthase is found in the human brain, which may indicate that the P–S reaction may also occur in human (Fabbri et al., 2000). Tryptamine is a synthetic precursor of the P–S reaction. Moreover, tryptamine is widely distributed in the brain (Abu Ghazaleh et al., 2015). Thus, harmaline, harmine, harmane, and nor-harmane may be self-synthesized in mammals. Furthermore, the function of such βCBs in mammals remains unknown.

Based on previous reports, βCBs can play various pharmacological effects, such as anticoagulant activity, hypoglycemic effect, antineoplastic activity, antioxidative, and anti-inflammatory activity (Li et al., 2017a). These βCBs are either AChE and butyrylcholinesterase inhibitors or monoamine oxidase-A (MAO-A) inhibitors (Li et al., 2017a). In addition, the four abovementioned alkaloids can bind with opioid receptors, imidazoline I_2 receptors, and 5-hydroxytryptamine (5-HT) receptors, which contribute to the analgesic effect, reduction of withdrawal syndrome, and regulation of neurotransmitters (Li et al., 2017a). Moreover, neurotoxicity of harmine, harmaline, harmane, and nor-harmane is an indivisible side effect, which leads to essential tremor (Wang et al., 2019).

Based on our published research and results, harmine, harmaline, harmane, and nor-harmane are effective in mice with nervous system diseases, such as Alzheimer's disease (AD) (Li et al., 2018). AD is a common neurodegenerative disease, which is characterized by a decline in memory, language, and other cognitive skills (Association, 2016). Although no conclusion has been found on the pathogenesis of AD, several recognized hypotheses have been carried out. A popular mechanism is self-replication and spreading of the Aβ and Tau aggregates (Kumar et al., 2015). Not labeled by thioflavin or Congo red-based probes, Aβ deposition in transgenic mice is similar to brain tissue of patients with AD (Hampel et al., 2018). In addition, the cholinergic system of the brain plays an important role in AD. Cholinesterase inhibitors can increase the availability of acetylcholine ACh at synapses in the brain (Hampel et al., 2018). The effective cholinesterase inhibitors such as donepezil, galantamine, and rivastigmine have been proven to be clinically useful in AD treatment (Perng et al., 2018). Moreover, neurotransmitters play an indispensable role in AD development, and aberrant neurotransmitter release at synapses can cause cognitive decline in AD (Tang, 2019). Researchers have found that AD is associated with inadequate levels of various neurotransmitters (Kumar et al., 2015). The AChE inhibitory activity and regulation of neurotransmitters are the potential underlying mechanisms of harmine, harmaline, harmane, and nor-harmane in the treatment of AD mouse models. Thus, such alkaloids, which are widely distributed in the body fluids of the mammals, may improve the prevention and treatment of AD.

Therefore, harmaline, harmine, harmane, and nor-harmane may be endogenous substances, and a potential relationship can be found between such endogenous alkaloids and AD.

The plasma of rats, mice, and human at different ages were tested, particularly the newborn pup rats, to verify whether

Abbreviations: βCBs, β-Carboline alkaloids; 5-HT, 5-Hydroxytryptamine; 5-HIAA, 5-Hydroxyindole-3-acetic acid; ACh, Acetylcholine chloride; AChE, Acetylcholinesterase; AD, Alzheimer's disease; Ch, Choline chloride; L-Glu, L-Glutamic acid; L-Phe, L-Phenylalanine; L-Trp, L-Tryptophan; L-Tyr, L-Tyrosine; MAO-A, Monoamine oxidase A; P–S reaction, Pictet–Spengler reaction.

harmaline, harmine, harmane, and nor-harmane are endogenous and naturally present in mammals. The AD is a cognitive impairment disease, which is closely associated with age. Brain development in childhood, particularly born within a month, is crucial to cognitive function (Mollon et al., 2018). Memory and learning ability have developed from infancy to adulthood based on processing speed, working memory, language, and visuospatial test (Mollon et al., 2018). However, cognition decreases with age (Guerreiro and Bras, 2015). According to research published in 2016, most people with AD are aged 65 or older, and 15% of people with AD are aged 65–74, whereas 44% are aged 75–84 (Association, 2016). Growth and aging are important processes of cognitive level development. Thus, the contents of harmine, harmaline, harmane, and nor-harmane in different developmental states of mammals are determined. In this article, the developmental stage of rats is defined as less than 29 days old, whereas aging is defined as between 2 and 18 months of age.

Plasma of AD mouse models is tested and compared with those of the control animals' in accordance with the possible mechanism of AD to confirm whether the content variation of harmine, harmaline, harmane, and nor-harmane in mammal plasma implies the occurrence of AD. Exposure levels of these βCBs in healthy volunteers were also detected to explore the relationship between βCBs exposure of patterns and age, as well as of gender and race.

In general, the abovementioned research is important to determine the origin and functions of harmine, harmaline, harmane, and nor-harmane in mammal plasma. Studies on the relationship among plasma contents of such βCBs in different developmental and physiological states of mammals can provide a comprehensive understanding of the endogenous physiological effects of such alkaloids. Furthermore, the results may provide insights into the anti-AD functional foods and drugs.

MATERIALS AND METHODS

Reagents and Materials

Harmine, harmane, and harmaline (purity > 98%) were isolated by HPLC from the seeds of *P. harmala* in our laboratory. Nor-harmane was purchased from Sigma Aldrich Co. (St. Louis, MO, United States). Scopolamine hydrobromide was purchased from TCI (Shanghai) Development, Co., Ltd. (Shanghai, China). The L-tryptophan (L-Trp), 5-hydroxytryptamine (5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), acetylcholine chloride (ACh), choline chloride (Ch), L-glutamic acid monosodium salt monohydrate (L-Glu), L-phenylalanine (L-Phe), L-tyrosine (L-Tyr), theophylline, tacrine (internal standard), and heparin sodium were purchased from Sigma Aldrich Co. (St. Louis, MO, United States). Perchloric acid and sodium hydroxide were purchased from Meilunbio® Biotech, Co., Ltd. (Dalian, China). Bovine serum albumin was purchased from YEASEN Biotechnology, Co., Ltd. (Shanghai, China). The HPLC-grade acetonitrile, methanol, and formic acid were purchased from Fisher Scientific, Co. (Santa Clara, CA, United States). Deionized water (>18 mΩ) was purified by Milli-Q Academic System (Millipore, Corp, Billerica, MA, United States).

Animals

Twenty adult and 11 pregnant Sprague-Dawley rats, and 10 C75BL/6 mice were obtained from the Drug Safety Evaluation and Research Center of Shanghai University of Traditional Chinese Medicine. Ten APP/PS1 double transgenic mice in C57BL/6 background aged 5–6 months with their age-matched littermates were obtained from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). Animals were housed in a well-lighted air-conditioned room under standard environmental conditions (room temperature and relative humidity were kept at 25°C ± 1°C and 60–65%, respectively) and given free access to rodent chow and tap water prior to the study. All procedures using animals were in accordance with the regulations for animal experimentation issued by the State Committee of Science and Technology of the People's Republic of China on 14 November 1988 and approved by the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine (NO. PZSHUTCM190912018; Approval date: 12 September, 2019).

Voluntary Subjects

Participants were recruited from two cohorts: Shanghai University of Traditional Chinese Medicine (i.e., the SH cohort) and Kashi Prefecture First People's Hospital, Xinjiang, China (i.e., the XJ cohort). This study focused on the exposure rules of harmine, harmaline, harmane, and nor-harmane in plasma of non-AD patients with different ages, genders, and races. Therefore, all participants were normal individuals, with a low likelihood of clinical AD. During recruitment, cognitive function can be assessed using a variety of simple, informal techniques in accordance with the National Institute on Aging-Alzheimer's Association Guidelines (Albert et al., 2011). A total of 131 participants were recruited from the SH cohort. Most of them were students aged 24 to 30, and 15 volunteers were staffs aged 31 to 55. The other 419 participants were enrolled for the XJ cohort. All of them went to a hospital for physical examination. The 419 healthy volunteers were in the age range of 19–81 years old. All subjects were given an informed consent and asked no intervention before and throughout the study period. Exclusion criteria were not available. The study was approved by the Research Ethics Committee of Kashi Prefecture First People's Hospital, and all participants provided an informed consent (NO. 2017-03; Approval date: February 28, 2017).

Analysis of Target βCBs in Plasma and Tissues of Mammals by UPLC-ESI-MS/MS

The concentrations of alkaloids were quantified using the SHIMADZU LC-30AD UPLC system (Shimadzu, Kyoto, Japan) connected to an AB Sciex QTRAP® 6500 triple quadrupole mass spectrometer (SCIEX, United States) equipped with an ESI source using positive ion detection mode for multiple reaction monitoring. Based on a previously validated method,

chromatographic separation was conducted using a UPLC BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm, Waters, United States) (Zhao et al., 2011). Given the difference in the linearity range, the sample pre-treatment was slightly adjusted, and the methods and results are shown in **Supplementary Figure 1** and **Supplementary Tables 1–3**, respectively. The mobile phase consisted of an aqueous solution of 0.1% formic acid (solvent A) and acetonitrile (solvent B) with a flow rate of 0.4 ml/min. The gradient elution was established as follows: 0–2.5 min, 9–13% B; 2.51–3 min, 14–14.5% B; 3–4 min, 14.5–15.5% B; 4–5 min, 15.5–90% B; 5–6 min, 90–90% B; 6–7 min, 90–9% B; 7–8 min, 9% B. All other instrumental parameters were set in accordance with previous studies, and the method was well validated and successfully applied to determine the concentrations of alkaloids (Zhao et al., 2011).

Analysis of Neurotransmitters in Plasma of Mammals by UPLC-ESI-MS/MS

The concentrations of neurotransmitters, including L-Trp, 5-HT, 5-HIAA, ACh, Ch, L-Glu, L-Phe, and L-Tyr, were determined using a SHIMADZU LC-30AD UPLC system (Shimadzu, Kyoto, Japan) connected to an AB Sciex QTRAP® 6500 triple quadrupole mass spectrometer (SCIEX, United States) equipped with an

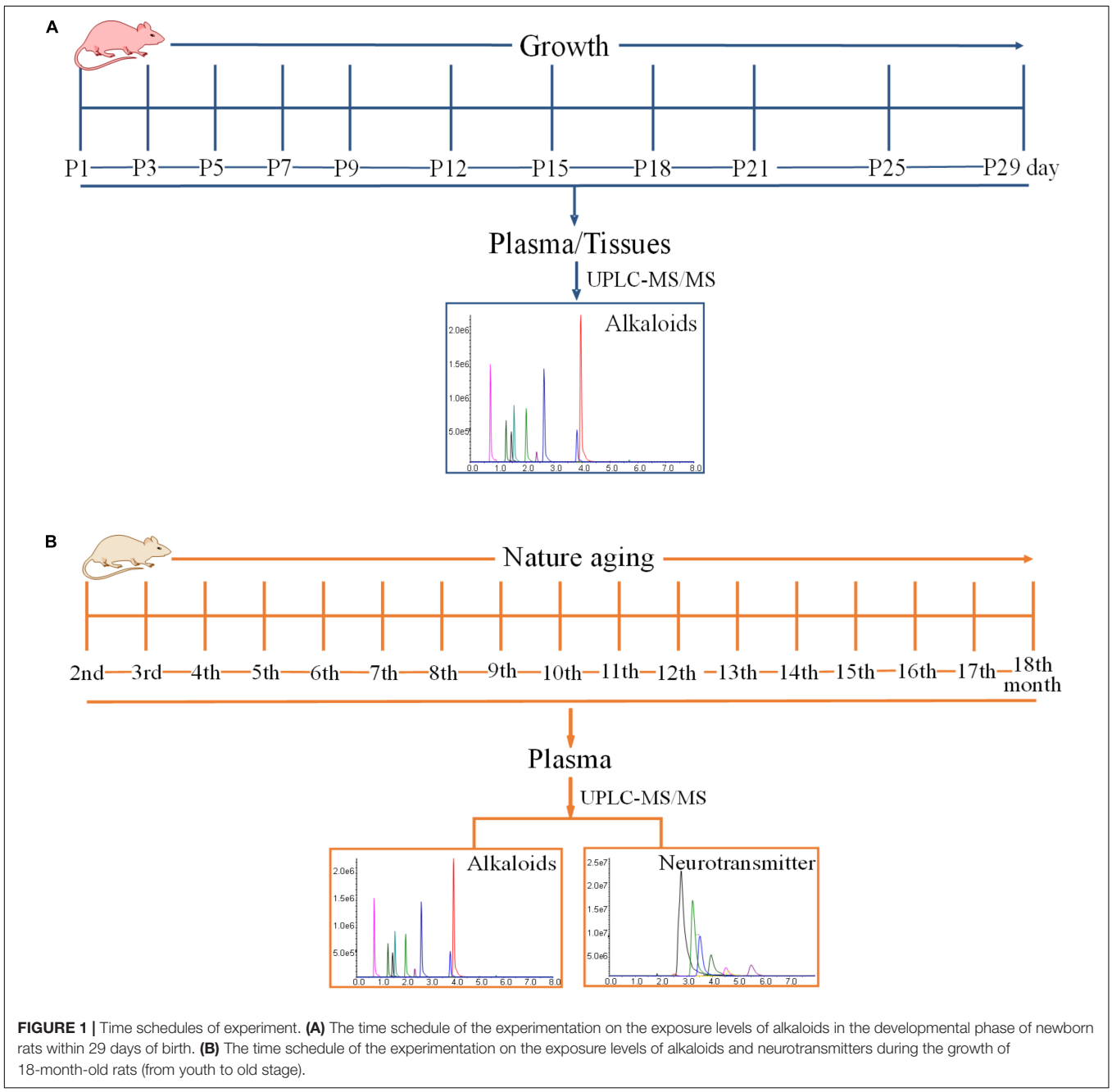

FIGURE 1 | Time schedules of experiment. **(A)** The time schedule of the experimentation on the exposure levels of alkaloids in the developmental phase of newborn rats within 29 days of birth. **(B)** The time schedule of the experimentation on the exposure levels of alkaloids and neurotransmitters during the growth of 18-month-old rats (from youth to old stage).

ESI source. Chromatographic separation was conducted using a ZIC-cHILIC column (150 mm × 2.1 mm, 3 μm) with a SeQuant ZIC-cHILIC guard column (20 mm × 2.1 mm, 5 μm, Merck-Sequant, Germany). The mobile phase consisted of an acetonitrile (A)–water mixture containing 0.1% formic acid (B), and the gradient elution was established as: 0–8 min, 65% A. All other instrumental parameters and sample pre-treatment were set in accordance with previous studies, and the method was well validated and successfully applied to determine the concentrations of neurotransmitters (Jiang et al., 2019).

Exposure Levels of Target Alkaloids in the Developmental Phase of Newborn Rats Within 29 Days

Eleven Sprague-Dawley pregnant rats were used in this study. The pregnant rats were raised under an environmentally controlled room with free access to food and water all throughout the experiment. After parturition, pup rats were grouped into 11 on the basis of mother rats. The individual group indicated one sampling point and contained 10 pup rats obtained from 10 different mother rats. The pup rats were sacrificed at postnatal day 1 (P1), 3 (P3), 5 (P5), 7 (P7), 9 (P9), 12 (P12), 15 (P15), 18 (P18), 21 (P21), 25 (P25), and 29 (P29) for sample collection, in which 11 sampling points were collected. The specific time is shown in **Figure 1A**. Each pregnant rat could give birth to around 10 pup rats, and every pup rat was sacrificed at certain time to ensure that each point in time contained 10 samples (five female and five male). The sex of pup rats was determined based on the anogenital distance. Pup rats were anesthetized at specified time for sample collection, and the grouping and sampling time is shown in **Supplementary Table 4**. Approximately 0.5 ml of blood sample was collected from the angular vein of each rat and transferred into a 1.5 ml heparinized tube. The supernatant plasma (100 μl) was transferred into another 1.5 mL centrifuge tube after the centrifugation of blood at 3,000 × *g* and 4°C for 10 min. The tissues of the brain, heart, liver, spleen, lung, kidney, genital organ, muscle, white fat, and brown fat of each pup rat were also collected. Various tissues were weighted and stored at a suitable tube. All plasma and tissue samples were stored at −80°C until analysis.

Exposure Levels of Alkaloids in Fodder and Bedding

The concentrations of harmine, harmaline, harmane, and norharmane in the fodder and bedding were detected. The fodder and bedding were obtained from the cages of pregnant rats and their pup rats. The detection method was referred to a quality specification established by Yang et al. (2014). The fodder and bedding were accurately powdered and weighted. The powders were added to methanol, which was 25 times its volume, and were ultrasound-treated for 25 min. In addition, power and frequency were kept at 250 W and 30 kHz, respectively. Afterward, supernatant (5 mL) was transferred to another tube and evaporated to dry at 37°C under a slight stream of nitrogen. The dried residue was reconstituted with 100 μl of 9% acetonitrile and vortexed for 2 min. After centrifugation at 13,000 × *g* and 4°C for 10 min, 20 μl of supernatant was injected into the UPLC-ESI-MS/MS system for alkaloid analysis.

Exposure Levels of Alkaloids and Neurotransmitters During the Growth of 18-Month-Old Rats (From Youth to Old Stage)

Twenty Sprague-Dawley rats (200–220 g), with 10 males and 10 females, were used in this study. The rats were raised under an environmentally controlled room with free access to food and water all throughout the experiment. The experimentation lasted for 16 months. Blood samples were collected once a month, and blood was drawn at around 9:00 am to 11:00 am. The schematic diagram is shown in **Figure 1B**. Blood samples were collected from the angular vein for hematological analyses every month after rats were anesthetized with isoflurane. The blood samples were promptly centrifuged at 3,000 × *g* and 4°C for 10 min, and all of the supernatant plasma was transferred into a new 1.5 ml centrifuge tube. Plasma samples were stored at −80°C until analysis. After sample collection, plasma was used to analyze some alkaloids and neurotransmitters after pretreatment.

Alkaloid Exposure Levels in Different Physiological States of Mice

Two AD mouse models were used, including 10 male APP/PS1 double transgenic mice and 10 male scopolamine molding mice. All mice were housed under an environmentally controlled room with free access to food and water before the experiment. Based on the results of the Morris Water Maze test, the APP/PS1 double transgenic mice had an impaired spatial learning and memory compared with C57BL/6 mice (He et al., 2015). Mice were anesthetized with isoflurane, and blood was collected from the right orbital vein (He et al., 2015). The blood samples were centrifuged at 3,000 × *g* and 4°C for 10 min. Then, supernatant plasma was collected and stored at −80°C until analysis. Another AD mouse model was composed of male C57BL/6 mice molded through intraperitoneal injection of scopolamine (1 mg/kg) for 7 days (Li et al., 2018). Compared with C57BL/6, the scopolamine-molded mice had an impaired memory based on the results of the Morris Water Maze test (Li et al., 2018). After molding, the model mice and control were anesthetized with isoflurane, and blood was collected from the right orbital vein. The blood samples were centrifuged at 3,000 × *g* and 4°C for

TABLE 1 | Characteristics of healthy participants.

Characteristics of subjects	SH cohort	XJ cohort
Number	131	419
Gender (women/men)	79/52	101/318
Age (mean ± SD)	28 ± 4	41 ± 12
Race (Han/Uyghur)	124/7	274/145
Drinking (yes/no)	13/118	199/220
Smoking (yes/no)	9/122	162/257

-no relevant statistics.

10 min. Then, supernatant plasma was collected and stored at −80°C until analysis.

Exposure Levels of Alkaloids in Different Developmental and Health Conditions in Human

All 550 individuals were given written informed consents before blood was drawn. In addition, the participants and their relatives were informed that the anonymized data might be used in clinical research studies. Moreover, health examination and questionnaires were necessary. The guardian was requested to answer the questionnaires if participant could not answer. The survey involved various aspects, including name, age, nationality, gender, living habits, and contact information. Living habits included drinking and smoking. Detail information is shown in **Table 1**. Health examination about physiological states included neurodegeneration and any other disease. This experiment aimed to detect exposure levels of harmine, harmaline, harmane, and nor-harmane in various developmental and physiological states. Furthermore, the difference of alkaloid exposure levels by gender, race, and lifestyle was detected. Blood sampling and numbering were directed by nurses in Kashi Prefecture First People's Hospital and Shuguang Hospital affiliated to Shanghai University of Traditional Chinese Medicine. Serial numbers corresponded to the detailed information about every participant. The numbers were randomly allocated by sequence of arrival at hospital. Analysis about alkaloids in plasma was developed without further information except for serial numbers. Approximately 1 ml of venous blood was collected from participants who have fasted for at least 12 h. The blood samples were centrifuged at 3,000 × *g* and 4°C for 10 min to obtain plasma. Then, plasma samples were stored at −80°C until analysis.

Statistical Analysis

Statistical evaluation was performed using SPPS version 18, and the data were presented as mean ± SD. Unadjusted *P* values were reported as this study used an exploratory rather than confirmatory analysis. This study aimed to confirm whether harmine, harmaline, harmane, and nor-harmane were endogenous and compare their exposure levels in different gender, nationality, and lifestyle. Thus, four separate stepwise logistic regression analyses were conducted, which is one analysis each for harmine, harmaline, harmane, and nor-harmane, serving as the dependent variable. Data distribution was graphically evaluated using histograms and Q–Q plots. Non-parametric and parametric tests were used in the study. The *T* test was used to compare the concentrations of alkaloids among the different groups. A non-parametric test was applied as part of the statistical analyses for non-normal distribution. Participants were divided into two groups, and 60 years old was selected as the division point. Kruskal–Wallis H non-parametric test was used to compare the two groups. The threshold for statistical significance was set at $P < 0.05$ (2-tailed).

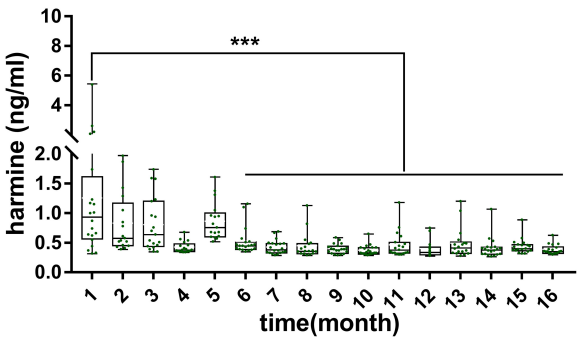

FIGURE 2 | Concentration of harmine in rat plasma and various tissues during the development of pup rats. Significant difference: ***$P < 0.001$.

RESULTS

Alkaloid Exposure Levels in the Developmental Phase of Newborn Rats Within 29 Days After Birth

The validated analytical method was applied to study the exposure levels of alkaloids in plasma and various tissues with the growth of pup rats within a month. The plasma concentrations of harmine were calculated by using the calibration curve. However, the concentrations of harmaline, harmane, and nor-harmane were difficult to count when the contents were below the lower limit of detection. As shown in **Figure 2**, the contents of harmine in plasma and in other organs showed different variations. First, harmine was detected in plasma and all tissues. The contents were listed as follows: plasma, 0.16 ± 0.03 ng/ml; brain, 0.33 ± 0.14 ng/g; heart, 0.34 ± 0.15 ng/g; liver, 0.26 ± 0.11 ng/g; spleen, 0.37 ± 0.12 ng/g; lungs, 0.46 ± 0.11 ng/g; kidney, 0.44 ± 0.13 ng/g; genital organ, 0.39 ± 0.12 ng/g; white fat, 0.33 ± 0.07 ng/g; brown fat, 0.36 ± 0.17 ng/g; and muscle, 0.31 ± 0.18 ng/g. The concentrations of harmine remarkably changed with time because of the various developmental characteristics of different organs. The concentrations of harmine in the plasma, brain, white fat, and genital organ decreased, whereas other organs (heart, liver, spleen, lungs, kidney, muscle, and brown fat) showed no significant trend with the growth of pup rats. Plasma content stabilized on the ninth day ($P > 0.05$), whereas the concentrations in the brain and white fat stabilized on the fifteenth and twenty-first day, respectively ($P > 0.05$). The contents of harmine in plasma, brain, and white fat of 3-day-old rats were significantly lower than those of 1-day-old rats but higher than those at the plateau stage ($P < 0.05$). No plateau was observed in the content of harmine in the genital organ, and it was decreasing everyday ($P < 0.001$). The brain was a crucial functional organ of harmine, whereas the liver was a vital metabolic organ. Then, the amounts of harmine in various tissues were calculated, and the amount–time curves were drawn (**Supplementary Figure 2**). The absolute amount of harmine increased in tissues of the brain, heart, liver, spleen, lungs, and kidney with the growth of pup rats. In tissues of the heart, lungs, and kidney, the absolute amount of harmine increased with the development of organs until the third week after birth. Notably,

the absolute amount of harmine in the brain increased faster than the development of body weight in the second week after birth.

Harmine, harmaline, harmane, and nor-harmane were not detected in fodder and bedding. A weak signal response was detected at the same peak time in harmine, and all of the responses were below the lower limit of quantification after the supernatant was concentrated 50 times. The detail dates are shown in the **Supplementary Table 5**.

Exposure Levels of Alkaloids and Neurotransmitters During the Growth of 18-Month-Old Rats (From Youth to Old Stage)

The alkaloids in plasma during the aging of rats were determined by using a validated analytical method. As shown in **Figure 3**, the concentrations of harmine in plasma decreased gradually with aging. The contents of harmine reached 1.80 ± 1.51 ng/mL in the first month and reduced to 0.35 ± 0.04 ng/ml in the sixteenth month. The concentrations of harmine in the first month were significantly higher than those in the sixth to sixteenth month ($P < 0.01$). Moreover, in the second to fifth month, the contents of harmine showed a decreasing trend with slight fluctuation. The concentrations stabilized in the sixth month and maintained at a low concentration ($P > 0.05$).

Meanwhile, neurotransmitter concentration in plasma during aging of rats was determined by using a validated analytical method. As shown in **Figure 4**, various neurotransmitters showed different trends (concentration range, mean ± SD): 5-HIAA (154.7–691, 384.3 ± 151.3 ng/ml), 5-HT (45.4–9850.2, 2,653 ± 2,644 ng/ml), ACh (30.1–137.7, 63.4 ± 25.4 ng/ml), Ch (112.1–1,526.1, 613 ± 295.1 ng/ml), Glu (778.2–5,998.6, 1,878.9 ± 914.8 ng/ml), $_{L}$-Trp (11,006.5–44,853.4, 21,091.9 ± 5,915.5 ng/ml), Phe (5,734.4–16,919.3, 9,169.1 ± 1,703.7 ng/ml), and Tyr (10,098–32,219.6, 20,339.2 ± 4,809.8 ng/ml). The exposure levels of 5-HT, ACh, Glu, L-trp, and Phe reduced with aging, but the contents of 5-HIAA, Ch, and Tyr showed no significant tendency over time.

Exposure Levels of Alkaloids in Different Physiological States of Mice

As shown in **Figure 5**, in different physiological states of mice, the contents of harmine, harmane, and harmaline were different. As shown in **Figure 5A**, the contents of harmine and harmane in wild-type mice plasma (harmine: 0.43 ± 0.26 ng/ml, harmane: 0.095 ± 0.078 ng/ml) were higher than those in APP/PS1 double transgenic mice (harmine: 0.28 ± 0.060 ng/ml, harmane: 0.085 ± 0.028 ng/ml). However, the concentrations of harmaline in wide-type mice plasma (0.20 ± 0.22 ng/ml) were lower than those in APP/PS1 double transgenic mice (0.26 ± 0.38 ng/ml). As shown in **Figure 5B**, the contents of harmine and harmane in normal mice plasma (harmine: 0.60 ± 0.28 ng/ml, harmane: 0.23 ± 0.15 ng/ml) were higher than those in scopolamine-mode mice (harmine: 0.54 ± 0.37 ng/ml, harmane: 0.17 ± 0.11 ng/ml). By contrast, harmaline showed opposite results (control: 0.11 ± 0.034 ng/ml, model: 0.15 ± 0.080 ng/ml).

Exposure Levels of Alkaloids in Different Developmental and Physiological States of Human

In human plasma, the concentrations of nor-harmane were below the lower limit of detection, which could not be determined in most samples, and the results of the other three alkaloids are summarized in **Figure 6**. The content of harmine in plasma was higher than that of harmane and harmaline. The participants were divided into groups on the basis of age, gender, ethnicity, and living habit. Harmine (1.90 ± 3.27 ng/ml) and harmaline (0.36 ± 0.90 ng/ml) concentrations in individuals below 60 years old were significantly higher than those above 60 years old (harmine: 0.99 ± 0.54 ng/ml; harmaline: 0.13 ± 0.07 ng/ml; **Figure 6A**, $P < 0.001$). The plasma contents of harmine and harmaline in female and male were significantly different, among which the contents in female were higher (**Figure 6B**, $P < 0.05$). Detail information were shown as follows: harmine (female: 2.03 ± 3.07 ng/ml; male: 1.57 ± 2.16 ng/ml) and harmaline (female: 0.57 ± 1.32 ng/ml; male: 0.24 ± 0.53 ng/ml). In plasma samples obtained from participants with different ethnicities, the plasma concentrations of harmine and harmaline of Han nationality (harmine: 1.82 ± 2.74 ng/ml; harmaline: 0.42 ± 1 ng/ml) were higher than those of Uighur (harmine: 1.47 ± 1.72 ng/ml; harmaline: 0.17 ± 0.38 ng/ml), which showed significant difference (**Figure 6C**, $P < 0.05$). Drinking was a factor affecting the contents of harmaline, and the harmaline level of individuals who do not drink (0.42 ± 1.02 ng/ml) were higher than those who drink (0.22 ± 0.57 ng/ml; **Figure 6D**, $P < 0.05$). However, harmine was not affected ($P > 0.05$). Smoking did not affect the concentration of the three compounds in plasma (**Figure 6E**, $P > 0.05$). The concentrations of harmane showed no difference among different ages, genders, and ethnicities ($P > 0.05$).

DISCUSSION

The content of harmine, harmaline, harmane, and nor-harmane in different developmental and physiological states of rat was detected to explore the origin and function of such alkaloids. The concentrations of harmaline and harmane in some rat plasma samples were below the LLOQ, which were limited by the detecting condition. As for nor-harmane, this result was not determined in many samples; thus, nor-harmane had no results. Harmine was detected in newborn pup rat plasma and tissues. Harmine was discovered in each tissue of pup rats, including the brain, heart, liver, spleen, lungs, kidney, muscle, white fat, brown fat, and genital organ. In addition, researchers found harmine in pig and rat brain (Li et al., 2016). The exposure level of harmine in rat plasma decreased with age. Notably, harmine was found in the whole life of rat. In human plasma, harmine concentration changed with age, gender, race, and physiological and pathological status. Therefore, harmine is a naturally and widely distributed endogenous substance in mammals. The concentrations of harmaline and harmane were lower than that of harmine in most plasma of rats, mice, and human. Harmaline

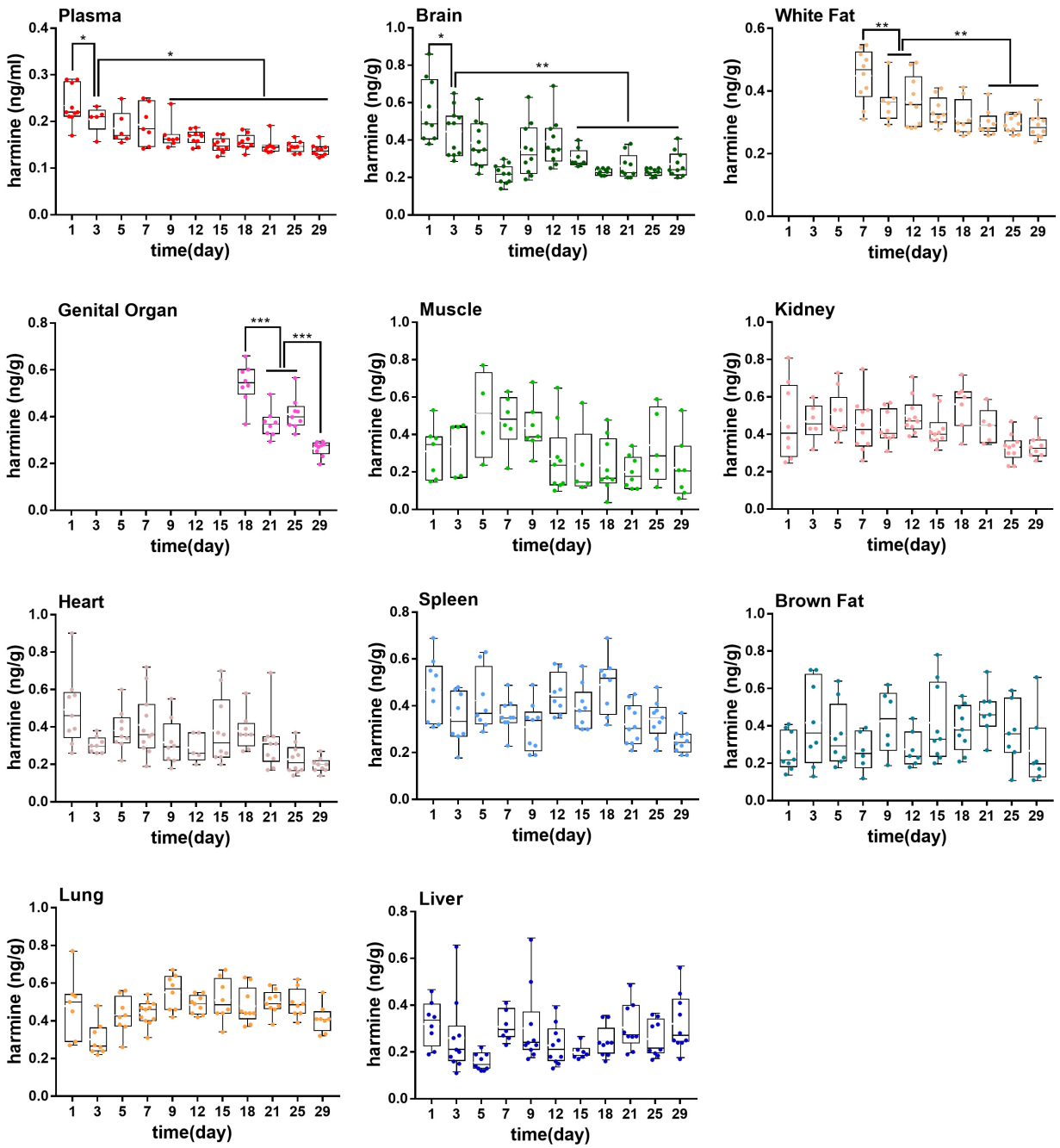

FIGURE 3 | Concentrations of harmine in rat plasma during the growth of 18-month-old rats (from youth to old stage). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

could turn to harmine *in vivo* with the presence of heme peroxidase (Wang et al., 2021), which might explain the contents of harmine were higher than that of harmaline under normal conditions. Based on the aforementioned research, harmine was more likely to be endogenous compounds than the other three alkaloids because harmine was detected in each rat, mouse, and human plasma, whereas the other three compounds were not.

Notably, harmine could be detected in plasma and other tissues of newborn rats without consuming any foodstuffs containing target alkaloids. Meanwhile, the fodder and bedding were free of harmine, and the exogenous disturbances from the growth environment of rats could be further excluded. Despite a weak signal response at the same peak time in harmine, the response was below the lower limit of detection. Thus, the trace amount of harmine, harmaline, harmane, and nor-harmane in fodder and bedding could be ignored. On the contrary, if the concentration of harmine was below the lower limit of detection, then the concentrations of harmine in fodder would reach

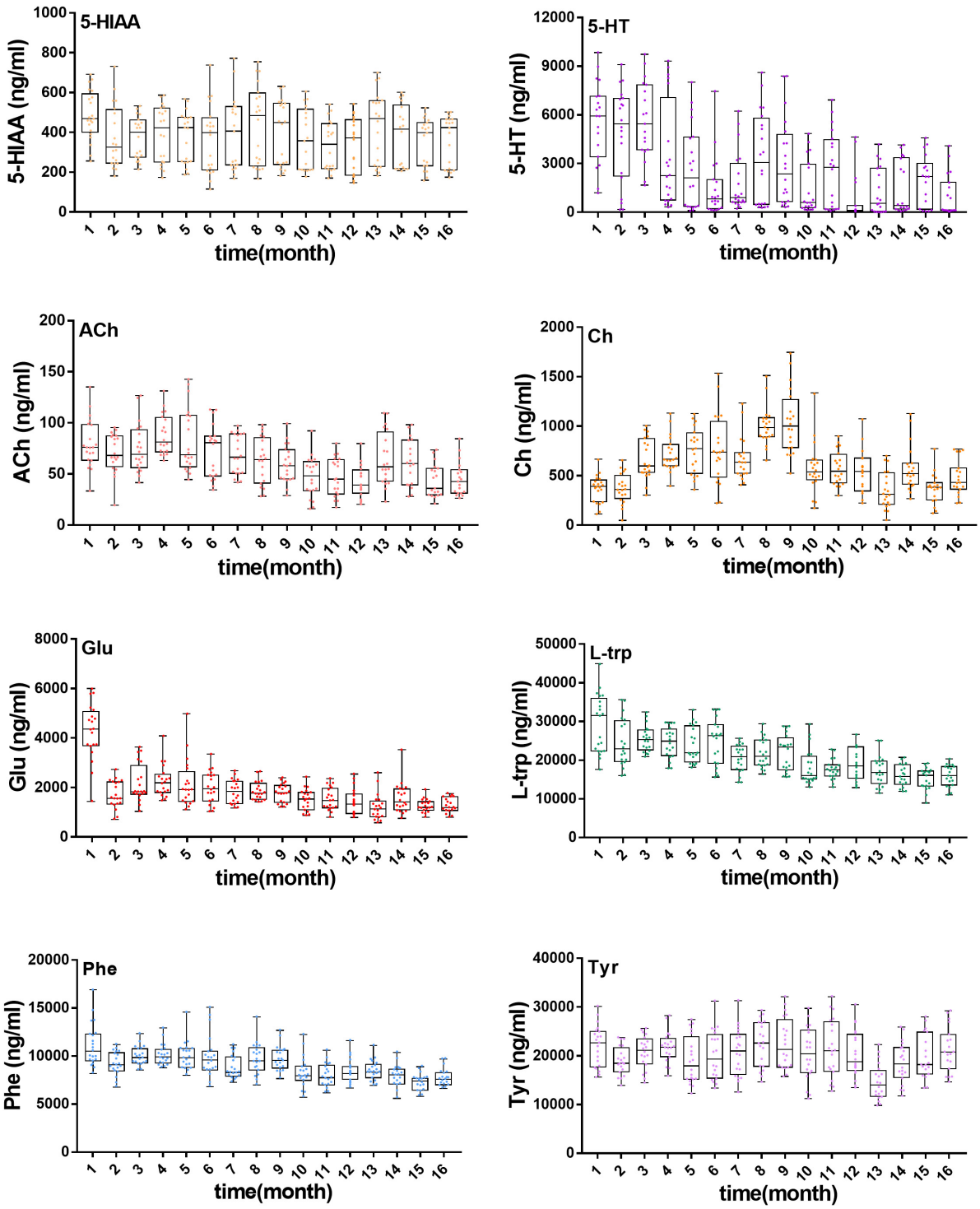

FIGURE 4 | Concentration of eight neurotransmitters in rat plasma during the growth of 18-month-old rats (from youth to old stage), including 5-HIAA, 5-HT, ACh, Ch, Glu, L-Trp, Phe, Tyr.

0.012 ng/g. The maximum daily food consumption of SD rats was approximately 20 g, and the bioavailability of harmine was 17% (Li et al., 2016; Wang et al., 2019). Considering that the blood volume accounted for 8% of the body weight of rats, combined with the daily intake of 20 g of each rat and the bioavailability of about 17% of harmine, the exogenous intake of harmine would

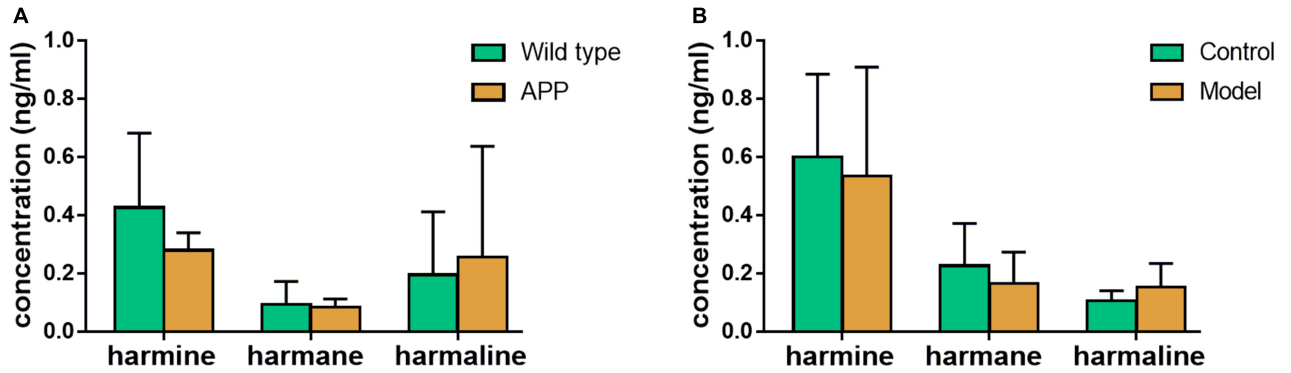

FIGURE 5 | Exposure regulation of different healthy states. **(A)** wild-type mice compared with APP knock-in mice; **(B)** scopolamine-mode mice compared with normal mice.

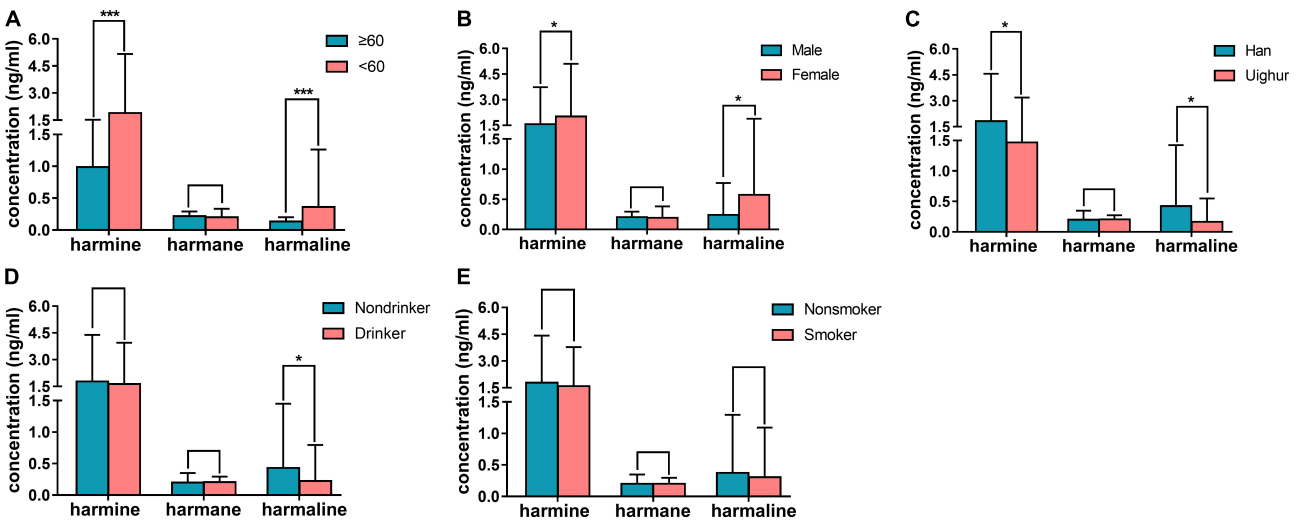

FIGURE 6 | Results of human plasma determination. **(A)** contents of harmine, harmane, and harmaline in plasma of healthy individuals who were below 60 years old compared with those above 60 years old; **(B)** concentrations of the abovementioned alkaloids in plasma of healthy male individuals compared with female; **(C)** concentrations of the abovementioned alkaloids in plasma of Han compared with Uighur; **(D)** concentrations of the abovementioned alkaloids in plasma of healthy individuals who drink in daily life compared with those who were not; **(E)** concentrations of the abovementioned alkaloids in plasma of individuals who smoke in daily life compared with those who were not; Significant difference: $^{*}P < 0.05$, $^{***}P < 0.001$.

only account for 1.3% of the total blood drug concentration. Therefore, based on the results of this experiment, harmine in newborn rat plasma and other tissues is natural rather than exogenous uptake.

Based on previous research of the synthesis of βCBs in plants, the P–S reaction was an essential part in the whole synthetic route, in which the strictosidine synthase was the vital enzyme in life (Stockigt et al., 2011; Zhu et al., 2015). The P–S reaction easily occurred without enzyme catalysis during cooking, including roasted coffee, barbecue, and toasted bread (Xie et al., 2021). The structure and function of strictosidine synthase in plants have been determined (Ma et al., 2006). Except for tryptamine and secologanin, other amines and aldehydes could be a substrate based on substrate specificity studies (Ma et al., 2006). Furthermore, a protein similar to strictosidine synthase was found in other life forms, including human (Cao and Wang, 2021). In addition, tryptamine, one of the substrates, is a popular compound in mammals (Abu Ghazaleh et al., 2015). Therefore, harmine may be synthesized *in vivo* in mammals by using tryptamine as a substrate under catalysis by synthesizing proteins similar to strictosidine in plants. Protein dysfunction may explain the decrease in harmine with aging. Age-dependent proteostasis decreases protein aberrant folding and aggregation, thereby leading to protein dysfunction (Hipp et al., 2014). Researchers found that the oxidative damage of membrane proteins and cytoplasmic proteins in the brain of an older person was higher than that of younger person (Granold et al., 2015). However, more experiments are needed to confirm the underlying mechanism.

The concentration of harmine in rats decreased with growth. Based on previous reports, harmine could stimulate proliferation of human neural progenitor cells and inhibit

the dual specificity tyrosine-phosphorylation-regulated kinase, which regulated brain development (Dakic et al., 2016). Harmane could activate the firing and burst activity of dopamine neurons, and the increase in firing rate produced by harmane was greater than that produced by nicotine (Arib et al., 2010), which may imply that harmine has a similar effect because of the similar structure. In addition, tryptamine, the synthetic precursor of harmine, was widely distributed in the brain (Abu Ghazaleh et al., 2015). Therefore, harmine could play a role in the development of the nervous system. The sharp reduction of harmine in a developmental state may indicate that harmine may play an important role in primary functions in the early stage of nervous system growth and development.

The developmental and physiological states of newborn rats experienced great changes in a month, and pup rats from different maternal rats usually had inborn differences (Jungner et al., 2019). Thus, the sampling time and grouping are important factors. The dates of each sampling point were obtained from 10 different maternal rats to avoid inborn differences and make the experiment more accurate. The sampling time was different because the samples were not delivered on the same day. Thus, the experiment eliminated congenital difference of pup rats and reflected the variation trend of harmine in various tissues and plasma with the development of pup rats.

The concentration of harmine in rat plasma decreased with age. The brain exhibited signs of compromised bioenergetics with aging, including inflammation, accrual of oxidatively modified molecules, and other impairments. In addition, aging individuals were vulnerable to AD or other neurodegenerative diseases (Corriveau et al., 2017). Alzheimer's disease (AD) was a deadly and progressive neurodegenerative disorder, and the pathogenesis of the disease had no clear conclusion at present (Hampel et al., 2018). Researchers directly associated the disease with aging but not specifically with the theories of aging in general. Distinguishing normal aging from AD is difficult because aging is a main risk factor for acquiring AD (Hargis and Blalock, 2017). Researchers showed the relationship between aging and AD, and they regarded the aging rodent models as an AD model (Hargis and Blalock, 2017). Many factors of aging could alleviate AD phenotypes, and drugs and treatment for AD could slow the aging phenotypes (Xia et al., 2018). Harmine is a compound that enhanced the spatial cognition of AD mouse models and regulated the concentration of various neurotransmitters (Li et al., 2018). Based on the pharmacological effects of harmine on AD treatment, the lack of harmine might cause or accelerate the development of AD during aging. Moreover, harmine is the inhibitor of dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A) (Sitz et al., 2008). The expression of DYRK1A increases with age, whereas DYRK1A could promote the formation of characteristic pathological hallmarks of AD by direct phosphorylation of tau and Aβ (Sitz et al., 2008; Chaves et al., 2020).

The contents of 5-HT, ACh, Glu, $_{L}$-Trp, and Phe in rats reduced with aging. In addition, the contents of such neurotransmitters were lower in plasma of AD mouse models with intraperitoneal injection of scopolamine than those of normal mice (Li et al., 2018). The AD was associated with inadequate levels of a variety of neurotransmitters (Kumar et al., 2015). Neurotransmitters played a significant role in brain circuit involved in many aspects of learning and memory, particularly serotonergic, glutamatergic, and cholinergic neurotransmitters (Kumar et al., 2015). This result indicated the relationship between aging and AD.

The content of harmine, harmaline, and harmane in two mouse models, including APP/PS1 double transgenic mice and scopolamine-induced memory impairment model mice, showed no significant difference compared with the control. The AD model mice were determined to clarify the relationship between AD and alkaloids. Scopolamine was a non-selective antagonist of the muscarinic cholinergic receptor, which led to cognitive deficits associated with the reduction of cholinergic neurotransmission (Tang, 2019). The APP/PS1 double transgenic mice showed typical Aβ pathology and memory impairment in an age-dependent manner (Esquerda-Canals et al., 2017). Although harmine, harmaline, and harmane could enhance the cognitive ability of AD mouse models, the occurrence of disease played a little role in the regulation of these alkaloids.

Harmaline and harmine showed a significant difference among young and old people. Based on previous statistics, old people may suffer from AD (Association, 2016). The reduction of harmine and harmaline may account for the easy attack because of an efficient pharmacology in AD (Li et al., 2017a). In addition, when the neuronal cell of the central nervous system was senescent, aged neurons showed signs of impaired cellular signaling, which exhibited a memory decline (Swenson et al., 2019). The results were consistent with the reduction of harmine in an aging rat plasma.

Based on the results, the contents of harmaline and harmine in female plasma were higher than those of male plasma ($P < 0.05$). The result may be due to the different expression levels of cytochrome enzyme in women and men. Harmaline and harmine were metabolized by CYP2D6 (Li et al., 2016). The expression level of CYP2D6 in female was lower than that of male based on research, and the response to opioids showed gender differences (Bebia et al., 2004; Lopes et al., 2020). The difference in the expression of CYP2D6 accounted for the significant difference in the plasma contents of harmine and harmaline in female and male. On the contrary, no gender difference was associated with CYP1A2 activity (Bebia et al., 2004). Thus, no difference in gender was found among the plasma concentrations of harmane.

The concentrations of harmaline and harmine in plasma of Han subjects were higher than those of Uyghur ($P < 0.05$). The CYP2D6 was the main metabolic enzyme of harmaline and harmine (Li et al., 2017b). A reduced functional allele CYP2D6 ∗ 10 was important in Asian population, and Asians have a high frequency of reduced functional allele (median = 41%), CYP2D6 ∗ 10 contributed to the population shift to the right of the metabolic rates indicating slower metabolism (Bradford, 2002). Researchers compared the expression of CYP2D6 ∗ 10 in the Han and Uyghur blood. The result showed that the expression of CYP2D6 ∗ 10 in Han was higher than that in Uyghur's (Zuo et al., 2012). Consequently, the metabolic rate of CYP2D6 in Uyghur was faster than that in Han's, and the concentrations of harmaline

and harmine in Han plasma were higher than those of Uyghur. Different from harmine and harmaline, CYP1A2 was the main metabolic enzyme of harmane (Herraiz et al., 2008). Moreover, no significant difference of CYP1A2 was found between Han and Uyghur. Therefore, the plasma concentrations of harmane showed no difference between Han and Uyghur.

No significant differences in such alkaloids were found among smokers and drinkers, except for harmaline. The plasma concentration of harmaline in drinkers was lower than that of non-drinker ($P < 0.05$). This finding was consistent with various reports, that is, ethanol consumption could adversely affect AD and increase the risk of AD development (Huang et al., 2018). Increasing consumption of hard liquor could increase the rate of cognitive decline compared with mild to moderate drinks (Rehm et al., 2019). Such studies showed the correlation between drinking and disease, but no finding indicated a causal impact of alcohol on AD. Thus, the degree of drinking and standardization of alcohol may be a vital element for further research.

CONCLUSION

Harmine is a widespread βCBs in mammals, and it could be discovered in the plasma of rat, mouse, and human. Harmine was found in newborn rat plasma and many tissues without any consumption. In addition, harmine showed a high correlation with growth (aging), gender, race, and physiological status. These results revealed that harmine might be a potential biomarker in diagnosis of AD and effective remedy of AD. However, further studies were warranted to determine the underlying mechanism of endogenous synthesis and the specific function of harmine in mammals. This study was the first to investigate the variation of harmine concentration in mammals in different developmental and health states. This study laid the foundation for the discovery of novel AD biomarkers and provided insights into the development of functional foods and drugs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee of Kashi Prefecture First People's Hospital, and all participants provided informed consent (No. 2017-03; Approval date: February 28, 2017). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine (No. PZSHUTCM190912018; Approval date: 12 September, 2019).

AUTHOR CONTRIBUTIONS

NC performed most of the experiments, analyzed the data, and wrote the manuscript. SL detected the concentrations of harmine, harmaline, and harmane in plasma of AD mode mice and control. AX and XZ collected plasma and basic information of healthy volunteers and AD patients. ZK provided the idea of the experiment about pup rats. ML helped to conduct the experiment. GD collected plasma samples of aging rats. XC helped to develop the UPLC-MS/MS detection. CW proposed the project and gave suggestions on the revision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.773638/full#supplementary-material

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The Food Additive β-Caryophyllene Exerts Its Neuroprotective Effects Through the JAK2-STAT3-BACE1 Pathway

Yujia Zhang[1†], Qiaoyan Huang[1†], Sichen Wang[1], Ziqian Liao[2], Haichao Jin[1], Shuo Huang[1], Xiao Hong[1], Yiming Liu[1], Jie Pang[3], Qing Shen[4], Qingcheng Wang[5]*, Changyu Li[6]* and Liting Ji[1]**

[1] School of Pharmaceutical Sciences, Zhejiang Chinese Medical University, Hangzhou, China, [2] The First School of Clinical Medicine, Zhejiang Chinese Medical University, Hangzhou, China, [3] Department of Cardiology, Zhejiang Provincial People's Hospital, Affiliated People's Hospital of Hangzhou Medical College, Hangzhou, China, [4] Collaborative Innovation Center of Seafood Deep Processing, Zhejiang Province Joint Key Laboratory of Aquatic Products Processing, Institute of Seafood, Zhejiang Gongshang University, Hangzhou, China, [5] Department of Cardiology, Hangzhou Linping Hospital of Traditional Chinese Medicine, Linping, China, [6] Academy of Chinese Medical Sciences, Zhejiang Chinese Medical University, Hangzhou, China

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Tiantian Zhang,
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Bing Chun Yan,
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Zhiqiang Wang,
Hebei University, China

***Correspondence:**
Qing Shen
leonqshen@163.com
Qingcheng Wang
wqc198477@sina.com
Changyu Li
lcyzcmu@sina.com
Liting Ji
jltzcmu@sina.com

[†] These authors have contributed equally to this work

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Despite extensive research on Alzheimer's disease (AD), its diagnosis and treatment remain challenging, and no effective therapies are currently available. Amyloid β (Aβ) extracellular plaques and intracellular neurofibrillary tangles are the histological characteristics of AD that have been directly linked to neuropathological events such as synaptic and neuronal cell loss. In this study, we explored whether the "JAK2-STAT3-BACE1" pathway is involved in neuroprotection conferred by the food flavouring agent β-caryophyllene (BCP). PC-12 cells with overexpressed amyloid-β protein precursor (APP) were utilised to construct an AD model *in vitro*, which was then split into four groups, namely control, empty vector, APP overexpression, and BCP (5, 10, and 20 μM). CCK-8 was used to evaluate cell viability, immunofluorescence was utilised to examine synaptic morphology, and quantitative real-time polymerase chain reaction and western blot were used to examine gene and protein expression levels. The relative expression levels of JAK2, STAT3, and BACE1 mRNA in the transfected PC-12 cells were found to be significantly upregulated. The cell morphology altered dramatically 72 h after transfection, becoming rounder, with a decrease in cell number. BCP exhibited the potential to dramatically increase PC-12 cell viability while protecting cell morphology. BCP inhibited APP, JAK2, STAT3, BACE1 mRNA and BACE1 protein overexpression, as well as JAK2 and STAT3 hyperphosphorylation. Molecular docking simulated the docking of BCP with JAK2, STAT3, BACE1, CB2. And JAK2 was found to be the most stable protein. In conclusion, inhibition of the "JAK2-STAT3-BACE1" signalling pathway may be one of the mechanisms through which BCP protects neurons and antagonises Aβ's neurotoxicity.

Keywords: Alzheimer's disease, amyloid-β protein precursor, amyloid β, PC-12 cell, β-caryophyllene, JAK2-STAT3-BACE1

INTRODUCTION

Currently, approximately 50 million people worldwide suffer from dementia, and the majority of these people have Alzheimer's disease (AD). AD is a neurodegenerative disease whose major risk factor is age (Nguyen and Endres, 2021). Despite decades of research in this field, diagnosing AD remains challenging, and no effective therapies are available to date. According to a recent pathological study, patients with AD exhibit neuropathological features such as synaptic dysfunction, neuron loss, an inflammatory microglial response to dying cells, and brain microvasculature damage. Amyloid β (Aβ) extracellular plaques and intracellular neurofibrillary tangles are the histopathological characteristics of AD that have been directly linked to neuropathological events such as synaptic and neuronal cell death (Cisternas et al., 2020; Fracassi et al., 2020). Aβ oligomers can exert their toxic effect through multiple mechanisms by interacting with the synapses (Zolochevska and Taglialatela, 2020). For instance, a study found severe AD symptoms such as a defect in basal neurotransmission and the spatial memory damage in tau-knockout and amyloid precursor protein (APP) overexpression mice (Puzzo et al., 2020). In addition, the mice with reduced Aβ plaque load in the hippocampus performed significantly better in cognitive tests (Cone et al., 2021). Many studies have proved that Aβ exerts a significant impact on AD onset and progression, although drugs to combat this mechanism are still lacking.

β-Caryophyllene (BCP) is a spicy, peppery terpene found in various spices and edible plants, and it has a dry and sweet flavour. It has been approved by the Food and Drug Administration as a 'generally recognised as safe' food additive or ingredient in cosmetics due to its distinct flavour and noteworthy safety profile (FDA, 2020). According to research, BCP confers neuroprotection in several animal models of cognitive impairment. Yang et al. (2017) found that BCP can reduce the cerebral infarction volume, cerebral oedema, and neurological deficits in mice, in addition to exerting neuroprotective effects. Hu et al. (2017) found that BCP can inhibit neuroinflammation caused by Aβ oligomers in BV-2 microglia. Ojha et al. (2016) discovered that BCP inhibits proinflammatory cytokines and inflammatory mediators such as COX-2 and iNOS. However, the mechanism of its neuroprotective action remains to be investigated further.

Recently, our group found that BCP may protect SH-SY5Y cells against Aβ treatment, while inhibiting JAK2 expression (Zhang et al., 2020). BACE1, an essential protein in APP synthesis, can be activated by the "JAK2-STAT3" pathway (Bera et al., 2020). Therefore, BCP's neuroprotective effect may be related to the "JAK2-STAT3-BACE1" pathway. We studied the effects of BCP on neuronal injury caused by APP overexpression and excessive activation of "JAK2-STAT3-BACE1" *in vitro* to provide preliminary data for the neuroprotective effect of BCP and drug design.

MATERIALS AND METHODS

Cell Culture

Highly differentiated rat pheochromocytoma cells (PC-12) purchased from the Center for Excellence in Molecular Cell Science, CAS, were grown in Dulbecco's modified Eagle's medium (C11995500BT, Gibco, New York, NY, United States), containing 10% foetal bovine serum (1966174C, Gibco, New York, NY, United States) and 1% penicillin streptomycin (15140-122, Gibco, New York, NY, United States), and incubated at 37°C under 5% CO_2 atmosphere. Cell medium was replaced every 2 days, and the cells were sub-cultured once they reached 80% confluence. The cells were divided into four groups: control group (CN) without special treatment; empty vector group (EVG), with PC-12 cells transfected with empty vectors plasmid; overexpression group (OE), with PC-12 cells transfected with human APP plasmid; and BCP, with PC-12 cells transfected for 48 h and incubated with different concentrations of BCP (5, 10, and 20 μM) for 24 h.

Transient Transfections

According to the manufacturer's instructions, 2.5 μg of human APP plasmid (500 ng/μl, GCPE0196596, Shanghai Genechem, Shanghai, China) or empty vector plasmid (3894 ng/μl, P18111400, Shanghai Genechem, Shanghai, China) was mixed with Lipofectamine 3000 (L3000008, Invitrogen, Carlsbad, CA, United States) in Opti-MEM I (31985062, Gibco, New York, NY, United States) and incubated for 15 min at room temperature before transfection in 1 mL of the culture medium for 48 h.

Viability Assay of PC-12 Cells

The viability of PC-12 cells was determined using the Cell Counting Kit-8 (CCK-8, HY-K0301-500T, Dojindo, Japan). According to the specifications, CCK8 reagent was added to the cells, and the cells were incubated for 1 h after treating the cells as required. Cell death/proliferation was evaluated using a configurable multi-mode microplate reader (BioTek Synergy H1, Santa Clara, CA, United States) to measure the optical density values.

Observation of Neurite Morphology of PC-12 Cells by Immunofluorescence

The cells were seeded in a 24-well plate with poly-L-lysine glass slides and cultured in an incubator for 24 h, after which the medium was removed, and the cells were washed with phosphate-buffered saline (PBS, No. P1010, Solarbio, Beijing, China). The cells were then sequentially treated with 4% paraformaldehyde (C11325432, Macklin, Shanghai, China), 0.5% Triton X-100 (MKBQ0896V, Sigma-Aldrich, St. Louis, MO, United States), and 5% BSA (A8020, Solarbio, Beijing, China). The cells were gently washed with PBS each time when the reagent was changed. The treated cells were incubated with β-actin (13E5) Rabbit mAb (4970, CST, MA, United States) overnight. The antibody was recovered, washed with PBS, and then incubated with goat anti-rabbit IgG (RS23220, Immunoway, Shanghai, China) mixed with DAPI (097M4085V, Sigma, St. Louis, MO, United States) for 2 h

in the dark. After washing with PBS, the slides were removed, placed on the slides with anti-fluorescence attenuation mounting tablets (10142592, Dako, Copenhagen, Denmark), and observed under a fluorescence inverted microscope.

Detection of Gene Expression Through Quantitative Real-Time PCR

We strictly followed the RNA Extraction Kit (AJ31039A, TaKaRa, Japan) instructions for total cell RNA extraction. We prepared a 30 μl reverse transcription system according to the TaKaRa Reverse Transcription Kit (AK22355A, TaKaRa, Japan) and the TaKaRa Fluorescence Quantitative Kit (AI71774A, TaKaRa, Japan) instructions. The transcription reaction conditions were as follows: 37°C, 15 min; 85°C, 5 s. Real-time PCR reactions were carried out in the CFX96 Real-Time System (BIO-RAD, CA, United States) by using TB green^{TM} Premix Ex Taq^{TM} II (Tli RNaseH Plus) (RR820A, TaKaRa, Japan). Cycling conditions were: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s. Gene expression was quantified using the comparative cycle threshold method ($\Delta\Delta$CT). SANGON biotech (Shanghai, China) designed and synthesised the primer used, and the primer sequence is given in **Table 1**.

Analysis of JAK2, STAT3, and BACE1 Peptide Levels by Western Blotting

The quantities of JAK2, STAT3, and BACE1 were estimated through western blotting. After removing the medium, the cells were rinsed with PBS two times. Then, total proteins from cultured cells were extracted with RIPA lysis (P0013C, Beyotime, Shanghai, China) buffer mixed with protease inhibitors (CW2200, CWBIO, Beijing, China), phenylmethanesulfonyl fluoride (ST506, Beyotime, Shanghai, China) and Phosphatase Inhibitor Cocktail (CW2383, CWBIO, Beijing, China). The protein concentrations in the cell lysates were determined using a BCA protein assay kit (P0012, Beyotime, Shanghai, China). Then 20 μg of the extracted protein was subjected to 10% SDS-PAGE. The blots were transferred onto the polyvinylidene fluoride membranes (1620177, BIO-RAD, CA, United States) and blocked for 2 h with 10% non-fat milk blocking buffer. Then, the blots were incubated overnight at 4°C with purified anti-β-amyloid, 1-16 antibody (1:1000, #SIG-39320, Biolegend, CA, United States), JAK2 antibody (1:1000, AF6022, Affinity, Jiangsu, China), phospho-JAK2 (tyr1007) antibody (1:1000, AF3022, Affinity, Jiangsu, China), phospho-STAT3 (tyr705) antibody (1:1000, AF3293, Affinity, Jiangsu, China), STAT3 antibody-c-terminal (1:1000, AF6294, Affinity, Jiangsu, China), anti-GAPDH antibody (1:5000, ET1601-4, HuaAn, Shandong, China) or rabbit anti-BACE1 polyclonal antibody (1:2000, bs0164r, BIOSS, Beijing, China). After incubation with horse radish peroxidase (HRP)-conjugated second antibodies, the blots were visualised using the ECL reagent (#KF005, Affinity, Jiangsu, China) under standard conditions and quantified using the ChemiDoc Touch System (BIO-RAD, CA, United States). After stripping, the membranes were re-probed for GAPDH, which was used as a loading control.

TABLE 1 | Primer sequences used in the study.

Gene	Primer Sequence (5′ -3′)
JAK2-F	AAGTGCGTGCGAGCGAAGATC
JAK2-R	ACTGCTGAATGAACCTGCGGAATC
STAT3-F	AGGGCTTCTCGTTCTGGGTCTG
STAT3-R	CTCCCGCTCCTTGCTGATGAAAC
BACE1-F	GTCCTTCCGCATCACCATCCTTC
BACE1-R	ACTGTGAGACGGCGAACTTGTAAC
GAPDH-F	GACATGCCGCCTGGAGAAAC
GSPDH-R	AGCCCAGGATGCCCTTTAGT
APP-F	AGGACTGACCACTCGACCAG
APP-R	CGGGGGTCTAGTTCTGCAT

Molecular Docking

Molecular docking study was performed to investigate the binding mode between the compound and proteins by using Autodock vina 1.1.2 (Trott and Olson, 2010). The three-dimensional (3D) structure of the proteins was downloaded from Pubchem (pubchem.ncbi.nlm.nih.gov). The 2D structure of the compound was drawn using ChemBioDraw Ultra 14.0 and then converted to the corresponding 3D structure by using ChemBio3D Ultra 14.0 software. The AutoDockTools 1.5.6 package (Sanner, 1999; Morris et al., 2009) was employed to generate the docking input files. The ligand was prepared for docking by merging the non-polar hydrogen atoms and defining rotatable bonds. For Vina docking, the default parameters were used, if not mentioned otherwise. The best-scoring pose judged by the Vina docking score was selected and visually analysed using PyMoL 1.7.6 software.[1]

Statistical Analysis

Statistical significance of the experiments involving two groups was assessed using the Student's *t*-test. One-way ANOVA was employed for determining differences in the mean values among multiple groups. For all statistical analyses, GraphPad Prism 5.04 software was used.

RESULTS

Amyloid-β Protein Precursor Overexpression Induced Apoptosis of PC-12 Cells

After 48 h of transfection, the PC-12 cells displayed a high fluorescence expression and a normal cell state (**Figure 1A**); however, the PC-12 cells with APP gene overexpression displayed more Aβ and low cell viability. Since the antibody $A\beta_{1-16}$ recognises the $A\beta_{1-42}$ protofibril (Colvin et al., 2017), we selected this antibody for western blot detection. No difference was observed in the APP mRNA and protein levels between the EVG group and the control group. We discovered that the

[1]www.pymol.org

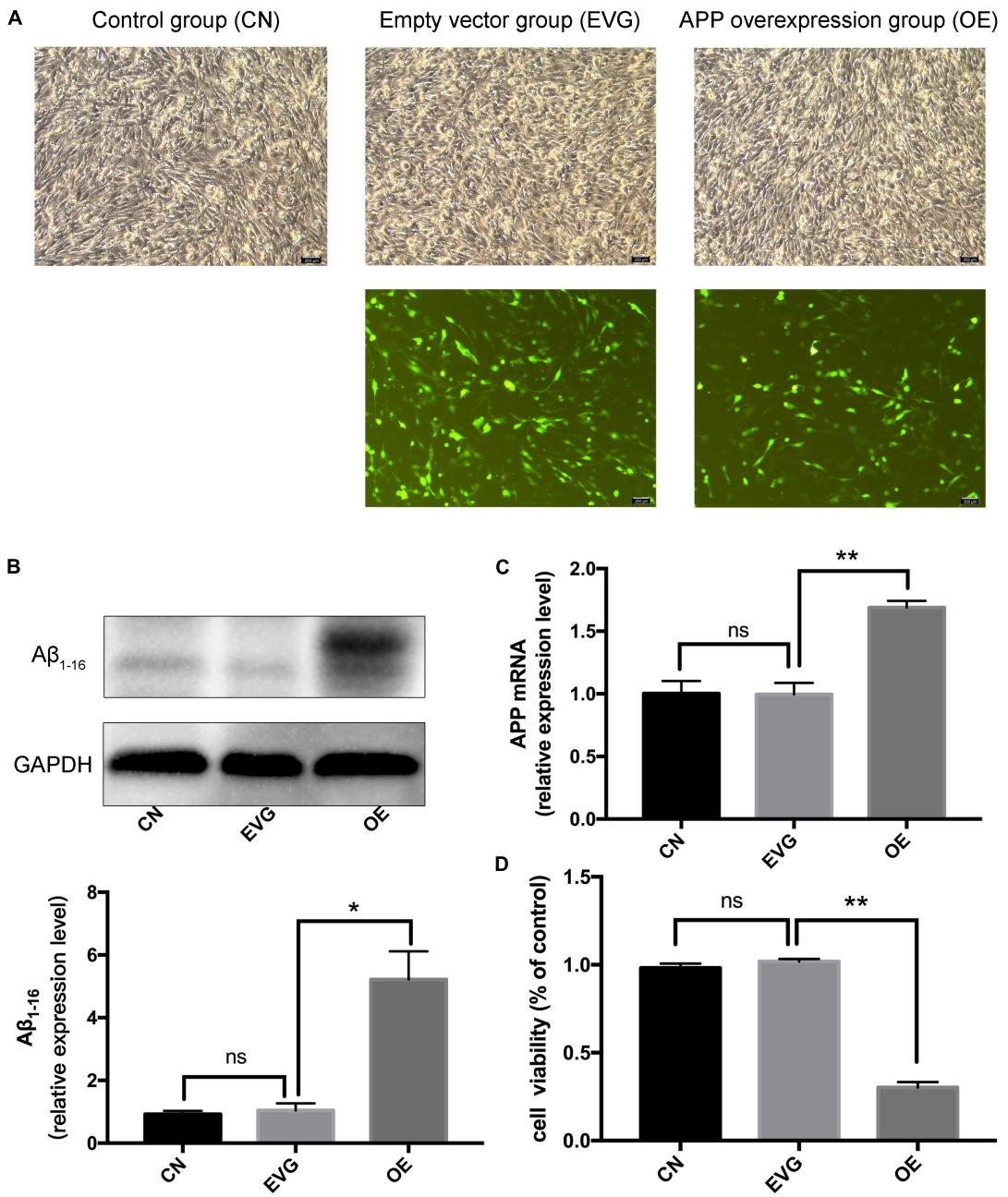

FIGURE 1 | The expression of APP and viability of PC-12 cells after transfection. CN denotes control group without special treatment; EVG denotes the empty vector group in which the PC-12 cells were transfected with empty vectors plasmid; while OE denotes the group in which the PC-12 cells were transfected with human APP plasmid. **(A)** Observation of the morphology of PC-12 cells transfected for 48 h by using a fluorescence inverted microscope. **(B,C)** The expression level of $A\beta_{1-16}$ protein and APP mRNA in PC-12 cells 48 h after transfection. **(D)** Cell viability of PC-12 cells 48 h after transfection. Compared with CN, ^{ns} $p > 0.05$. Compared with EVG, $^{*}p < 0.05$, $^{**}p < 0.01$. One-way ANOVA with Bonferroni's *post hoc* test. Scale bar = 200 μm.

mRNA and protein levels of APP were significantly higher in PC-12 cells of the OE group than in those of the EVG group (**Figures 1B,C**). Cell viability of the OE group was significantly lower than that of the EVG group 72 h after transfection, as measured by CCK-8 (**Figure 1D**). These results indicated that the nerve cell injury model caused by APP gene overexpression was effectively constructed.

β-Caryophyllene Attenuated PC-12 Cell Apoptosis in the Amyloid-β Protein Precursor Overexpression Group

Cell viability of PC-12 cells incubated with BCP for 24 h was detected using CCK-8. The results indicated no significant difference between the 5 μM BCP group and control group.

TABLE 2 | Effects of different concentrations of BCP on the 24-h viability of PC-12 cells.

Treatment	Neuronal survival (% CCK-8 reduction)
CN[a]	98.06 ± 0.64
5 μM BCP[b]	100.2 ± 0.65[ns]
10 μM BCP[b]	102.8 ± 0.62*
15 μM BCP[b]	103.3 ± 0.86*
20 μM BCP[b]	104.2 ± 0.76*
25 μM BCP[b]	106.5 ± 0.79**

[a] *The control group without special treatment.*
[b] *The group incubated with different concentrations of BCP for 24 h.*
ns: no significant difference vs. CN.
***$p < 0.01$, * $p < 0.05$ vs. CN.*

The groups with 10 and 20 μM of BCP displayed significantly improved cell viability compared with the control group. Compared with the control group, the 25 μM BCP group was found to have significantly enhanced cell viability (**Table 2** and **Figure 2A**), indicating that BCP can increase the viability of PC-12 cells.

Furthermore, our results indicated that BCP can improve the viability of PC-12 cells following transfection. Compared with that in the OE group, the number of cells in the 5, 10, and 20 μM BCP groups increased by 3.1, 14.6, and 17.8%, respectively. The viability of PC-12 cells overexpressing APP gene was significantly reduced when compared with that of the vector group. Compared with the OE group, the 10 and 20 μM BCP groups displayed substantially improved cell viability (**Table 3** and **Figure 2B**).

β-Caryophyllene Improved the Neurite Morphology of Amyloid-β Protein Precursor Overexpressed PC-12 Cells

By using immunofluorescence to examine the synaptic morphology of PC-12 cells, researchers have discovered the phenomena of cell rounding and synaptic loss after 72 h of PC-12 cell transfection with a plasmid containing APP cDNA. As shown in **Figure 3**, when the EVG group was compared with the OE group, the OE group cells were found to be significantly rounded, with the neurite being lost; however, the BCP group's neurite morphology was found to be improved.

TABLE 3 | After 48 h of transfection, the effect of BCP on the viability of PC-12 cells for 24 h.

Treatment	Neuronal survival (% CCK-8 reduction)
CN[a]	100.0 ± 1.09
EVG[b]	91.25 ± 5.6[ns]
OE[c]	30.23 ± 1.8**
5 μM BCP[d]	31.18 ± 1.6
10 μM BCP[d]	34.66 ± 0.3[Δ]
20 μM BCP[d]	35.61 ± 0.5[Δ]

[a] *The control group without special treatment.*
[b] *The empty vector group, in which the PC-12 cells were transfected with plasmid vectors without gene fragments.*
[c] *The group in which the PC-12 cells were transfected with human APP plasmid.*
[d] *The group in which the PC-12 cells were transfected for 48 h and then incubated with different concentrations of BCP for 24 h.*
ns: no significant difference vs. CN.
***$p < 0.01$ vs. EVG.*
[Δ] $p < 0.05$ vs. OE.

β-Caryophyllene Inhibited the Expressions of JAK2, Amyloid-β Protein Precursor, STAT3, and BACE1 in Amyloid-β Protein Precursor Overexpressed PC-12 Cells

After applying BCP (5, 10, and 20 μM) to the nerve injury model for 24 h, the expression levels of JAK2, APP, STAT3, and BACE1 mRNA were determined using the qRT-PCR assay; the results are depicted in **Figure 4**. APP, JAK2, BACE1, and STAT3's mRNA

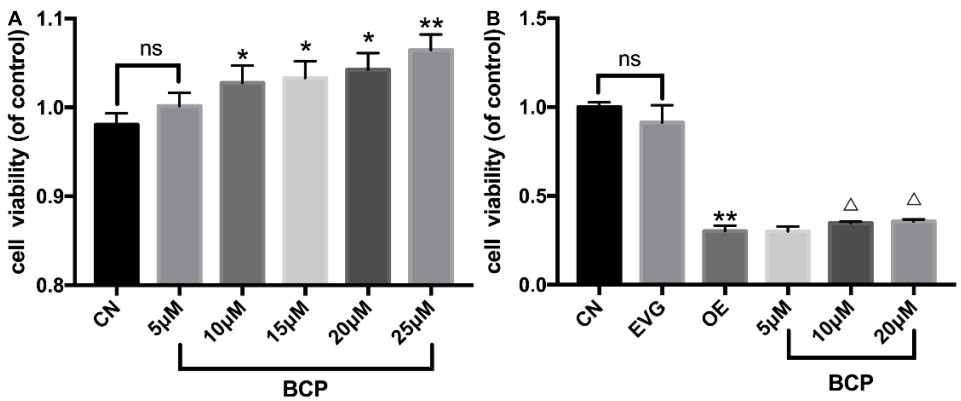

FIGURE 2 | Effect of different doses of BCP on the viability of PC-12 cells. CN denotes the control group without special treatment; EVG denotes the empty vector group in which the PC-12 cells were transfected with plasmid vectors without gene fragments; OE means the group in which the PC-12 cells were transfected with human APP plasmid; and BCP denotes the group in which the PC-12 cells were transfected for 48 h and then incubated with different concentrations of BCP for 24 h. **(A)** Effects of different concentrations of BCP on the viability of PC-12 cells for 24 h. **(B)** After 48 h of transfection, the effect of BCP on the viability of PC-12 cells for 24 h. Compared with CN, ^{ns} $p > 0.05$. Compared with EVG, $^{*}p < 0.05$, $^{**}p < 0.01$. Compared with OE group, $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$. One-way ANOVA with Bonferroni's *post hoc* test.

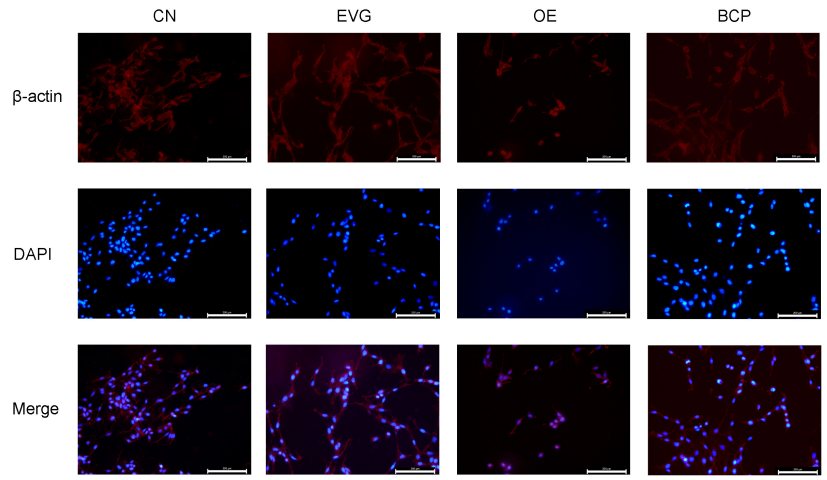

FIGURE 3 | Immunofluorescence detection of the effect of β-caryophyllene on the neurites of PC-12 cells with APP overexpression. CN denotes the control group without special treatment; EVG denotes the group in which the PC-12 cells were transfected with empty vectors plasmid for 72 h; and OE denotes the group in which the PC-12 cells were transfected with human APP plasmid for 72 h. In the BCP group, the PC-12 cells were transfected for 48 h to overexpress APP and 10 μM BCP acted on the PC-12 cells for 24 h. Scale bar = 200 μm.

levels in the OE group increased significantly compared with those in the EVG group.

Compared with the OE group, the 5 μM BCP group exhibited no significant difference, the 10 μM BCP group exhibited significantly downregulated APP, JAK2, BACE1, and STAT3 expressions at the mRNA level, and the 20 μM BCP group exhibited significantly downregulated JAK2 and BACE1 expressions at the mRNA levels (**Figure 4**).

The phosphorylation level of JAK2 protein in the OE group was significantly higher than that in the EVG group. The phosphorylation level of JAK2 protein was significantly lower in the 5, 10, and 20 μM BCP groups than in the OE group. **Figure 5A** depicts the aforementioned results.

The phosphorylation level of STAT3 protein in the OE group was significantly higher than that in the EVG group. The phosphorylation level of STAT3 protein in the BCP (5, 10, and 20 μM) groups was lower than that in the OE group, whereas the phosphorylation level of STAT3 protein in the 10 and 20 μM BCP groups was more significant (**Figure 5B**).

The expression level of BACE1 protein in the OE group was upregulated compared with that in the EVG group. The 5 and 20 μM BCP groups displayed a considerably downregulated BACE1 protein expression level, whereas the 10 μM BCP group displayed significantly downregulated BACE1 protein expression (**Figure 5C**).

Molecular Docking Simulation of β-Caryophyllene With JAK2, STAT3, BACE1, and Cannabinoid Receptor Type 2 Proteins

The estimated ΔG values of the docking model were obtained through molecular docking, and the estimated ΔG values of JAK2, STAT3, and BACE1 were compared with those of the positive protein CB2 of BCP to predict if the protein interacts with BCP. The larger the negative value, the more stable is the docking model. **Table 4** displays the docking data, indicating that (+)-BCP has the best docking stability with JAK2.

Molecular docking analysis revealed that (−)-BCP binds to the JAK2 water accessible cavity's hydrophobic region (**Figure 6**). In this model, (−)-BCP was docked into the hydrophobic binding cavity of the docking pocket calculated by Autodock Vina to form alkyl hydrophobic interactions with the amino acid residues val-911, lys-882, ala-880, met-929, leu-983, val-863, and leu-855, as well as van der Waals interactions with the amino acid residues asp-994, gly-993, leu-332, gly-935, and gly-856.

DISCUSSION

Alzheimer's disease has been causing a substantial health burden, and its prevalence is increasing with global population ageing (Urfer et al., 2021). After decades of research, scientists have concluded that Aβ extracellular plaques are directly linked to the neuropathological events such as synaptic and neuronal cell death (Cisternas et al., 2020; Fracassi et al., 2020). According to research, increasing Aβ oligomer formation triggers neuronal dysfunction and network alternations in learning and memory circuitry prior to the clinical onset of AD, and inhibition of Aβ accumulation improves cognitive behaviour and neuroinflammation in AD mice (Jiang et al., 2021). We utilised PC-12 cells overexpressing the APP gene to construct an *in vitro* AD model. At 72 h after transfection, the cell morphology altered significantly, becoming rounder, and the number of cells decreased. Moreover, we found that the relative expression levels of JAK2, STAT3, and BACE1 mRNA were considerably upregulated in the transfected PC-12 cells of human APP cDNA.

Activation of the "p-JAK2-p-STAT3-NF-κB-BACE1" pathway has been linked to Aβ accumulation and neurotoxicity (Park et al., 2016). The "JAK2-STAT3" pathway can mediate

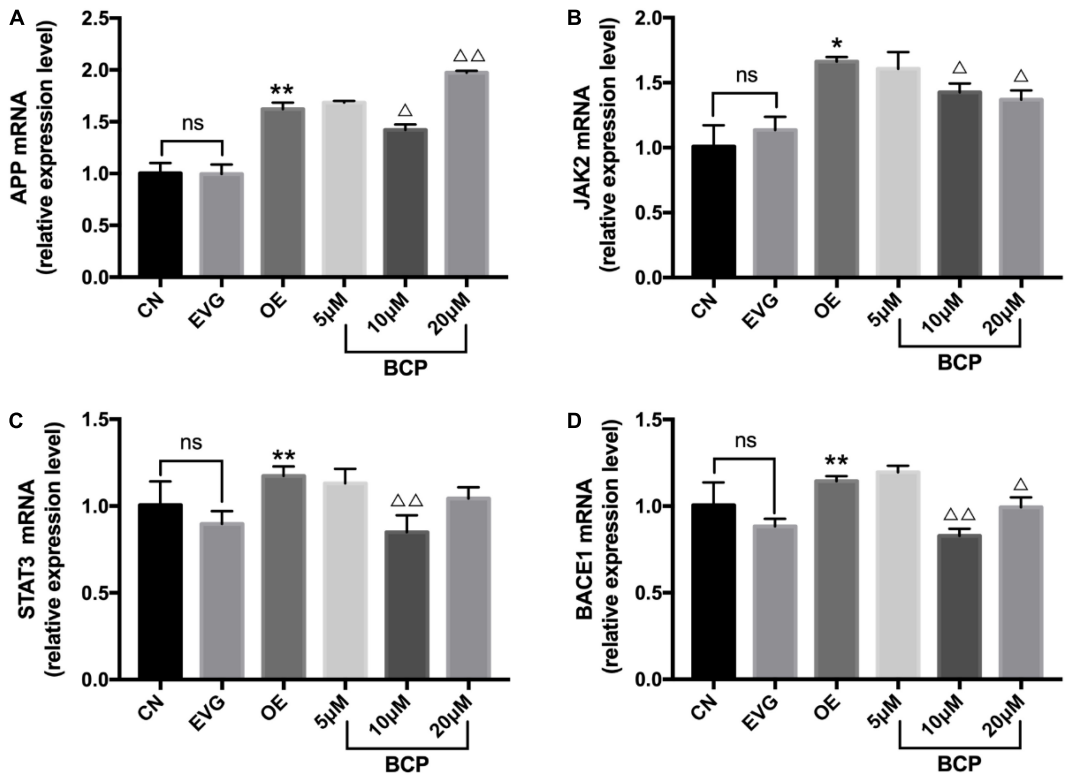

FIGURE 4 | Expressions of JAK2, APP, STAT3, and BACE1 mRNA in the PC-12 cells. CN denotes the control group without special treatment; EVG denotes the empty vector group in which the PC-12 cells were transfected with plasmid vectors without gene fragments; OE denotes the group in which the PC-12 cells were transfected with a plasmid containing APP cDNA; and BCP denotes the group in which the effect of BCP was assessed on 24-h viability of PC-12 cells transfected for 48 h. **(A)** Relative expression level of APP mRNA. **(B)** Relative expression level of JAK2 mRNA. **(C)** Relative expression level of STAT3 mRNA. **(D)** Relative expression level of BACE1 mRNA. Compared with CN, $^{ns}p > 0.05$. Compared with EVG, $^{*}p < 0.05$, $^{**}p < 0.01$. Compared with OE group, $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$. One-way ANOVA with Bonferroni's *post hoc* test.

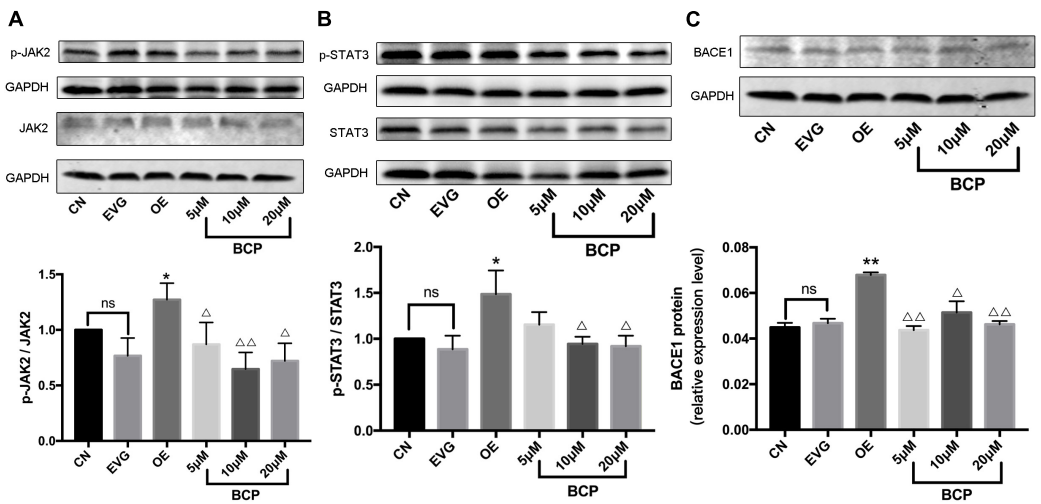

FIGURE 5 | Effect of β-caryophyllene on JAK2 and STAT3 phosphorylation and BACE1 protein expression in PC-12 cells. CN denotes the control group without special treatment; EVG denotes the empty vector group in which the PC-12 cells were transfected with plasmid vectors without gene fragments; OE denotes the group in which the PC-12 cells were transfected with a plasmid containing APP cDNA; and BCP denotes the group in which the effect of BCP on the 24-h viability of PC-12 cells transfected for 48 h was assessed. **(A)** Phosphorylation level of JAK2 protein. **(B)** Phosphorylation level of STAT3 protein. **(C)** Relative expression level of BACE1 protein. Compared with CN, $^{ns}p > 0.05$. Compared with EVG, $^{*}p < 0.05$, $^{**}p < 0.01$. Compared with OE group, $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$. One-way ANOVA with Bonferroni's *post hoc* test.

TABLE 4 | Results of molecular docking.

NO.	Target	uniprot entrez id	pdb id	Ligand	Estimated ΔG kcal/mol
1	JAK2	O60674	5L3A	(+)-β-Caryophyllene	−6.87
2	CB2[a]	P34972	6KPC	(+)-β-Caryophyllene	−6.75
3	CB2[a]	P34972	6KPC	(−)-β-Caryophyllene	−6.74
4	STAT3	P40763	5AX3	(−)-β-Caryophyllene	−6.62
5	JAK2	O60674	5L3A	(−)-β-Caryophyllene	−6.60
6	STAT3	P40763	5AX3	(+)-β-Caryophyllene	−6.49
7	BACE1	P56817	7DCZ	(+)-β-Caryophyllene	−6.49
8	BACE1	P56817	7DCZ	(−)-β-Caryophyllene	−6.45

[a]*cannabinoid receptor type 2.*

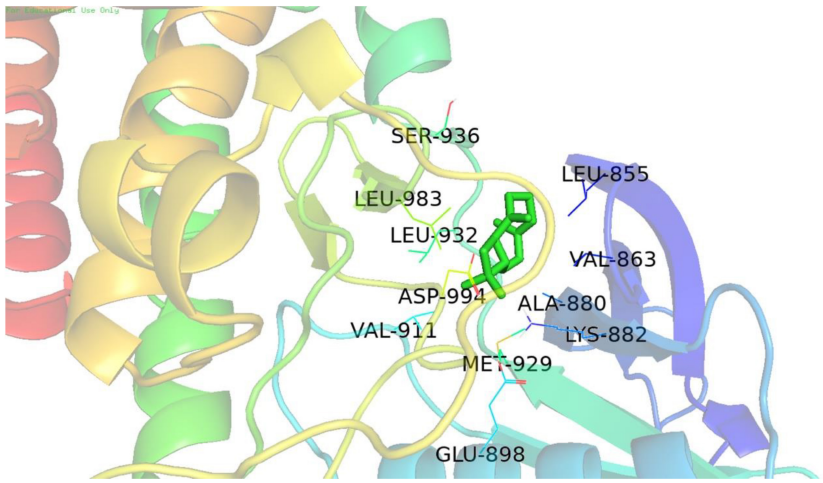

FIGURE 6 | The best docking stability model. Model of the putative interaction of (+)-β-caryophyllene with the JAK2 receptor determined.

the expression of endogenous BACE1. BACE1 is an APP cleavage protein that is required for APP processing to Aβ (Ye et al., 2017) and is a promising therapeutic target for reducing Aβ production in early AD. BACE1 has other substrates outside the amyloidogenic pathway that may be essential for synaptic plasticity and synaptic homeostasis (Hampel et al., 2021), which has been one of the most crucial research topics for scientists.

Our team investigated the neuroprotective effects of BCP and discovered that it may considerably improve PC-12 cell viability while also protecting the cell morphology. Further investigation showed that BCP can inhibit APP, JAK2, STAT3, BACE1 mRNA and protein overexpression, as well as JAK2 and STAT3 hyperphosphorylation. We discovered that 10 and 20 μM BCP may lower BACE1 protein expression, implying that BCP has great potential in AD treatment.

β-Caryophyllene has been shown to inhibit neuronal death. In a study by Yujie Cheng, the oral administration of BCP prevented cognitive impairment in APP/PS1 mice, and this positive cognitive effect was associated with reduced β-amyloid burden in both hippocampus and cerebral cortex (Cheng et al., 2014). The Rui Wang team discovered that miR-433, which targets JAK2, was downregulated in both AD serum and SH-SY5Y cells treated with Aβ (Wang and Zhang, 2020). In addition, a study reported that the "JAK2-STAT3" pathway activation inhibits NSC neurogenesis, whereas inhibition of the "JAK2-STAT3" pathway improves memory deficit in AD mice (Kong et al., 2019). To investigate whether the neuroprotective effect of BCP is related to the "JAK2-STAT3-BACE1" pathway, we utilised molecular docking simulation, a well-established *in silico* structure-based approach, which is extensively used in drug discovery (Pinzi and Rastelli, 2019). The molecular docking field has been advancing, with new algorithms and methods appearing at an exponential rate, making it helpful in accurately determining the mechanism of ligand-protein interaction. Because BCP exhibits selective full agonism on cannabinoid receptor type 2 (CB2) (Hashiesh et al., 2020), we chose CB2 as a positive protein for molecular docking and discovered that BCP and JAK2 have good docking stability. Prediction of the interaction between biological targets and ligands through molecular docking provides ideas for the follow-up study on the mechanism of BCP protecting nerve cells, which can provide more possibilities for AD treatment.

The results suggested that BCP might reduce BACE1 expression by inhibiting JAK2 phosphorylation, which is consistent with western blot results. Recent studies suggest that BCP has anti-inflammatory and antioxidant effects that apromote neuroprotection in different cognitive damage animal models (Chávez-Hurtado et al., 2020). In addition, we found that BCP

exerts a positive effect on reducing Aβ load, which indicates its significance in the treatment of AD. To explore the function mechanism of BCP antagonizing Aβ, it is necessary to further study and clarify the relationship between BCP and receptors of the "JAK2-STAT3-BACE1" signalling pathway.

CONCLUSION

Modern research mostly focuses on the action mechanism of BCP in improving neuroinflammation and cognitive decline (Lindsey et al., 2019). This study investigated the notable effect of BCP on protecting nerve cells and APP processing to Aβ. The essential protein BACE1 and the phosphorylation level of the "JAK2-STAT3" pathway decreased significantly when BCP was incubated with the cells transfected with human APP plasmid. Therefore, exploring the potential of BCP in reducing the damage to nerve cells caused by Aβ holds great significance. Based on the outcome of various experiments, we concluded that "JAK2-STAT3-BACE1" pathway inhibition might be one of the avenues for investigating BCP's neuroprotective effects and antagonism of Aβ's neurotoxicity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

YZ conducted experiments and wrote manuscript. SW, HJ, and SH analyzed the data. CL and LJ designed the study. QS and QW guided the experiment. Other authors helped revise the text to the final form. The final manuscript has been read and approved by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2022.814432/full#supplementary-material

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ORIGINAL RESEARCH
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Mediation Effects of IL-1β and IL-18 on the Association Between Vitamin D Levels and Mild Cognitive Impairment Among Chinese Older Adults: A Case–Control Study in Taiyuan, China

*Le Cheng[1], Ruirui Dong[1], Chenmeng Song[1], Xuemin Li[2], Luping Zhang[1], Mengqian Shi[1], Chenhui Lv[1], Lili Wang[1], Jie Kou[1], Haoran Xie[1], Wenjuan Feng[1] and Haifeng Zhao[1]**

[1] Department of Nutrition and Food Hygiene, School of Public Health, Shanxi Medical University, Taiyuan, China, [2] Center for Disease Control and Prevention in Shanxi Province, Taiyuan, China

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***Correspondence:**
Haifeng Zhao
haifengzao75@163.com

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Objective: Mild cognitive impairment (MCI) is a common, chronic, and complex disease in the elderly, which is often influenced by a variety of factors that include nutrition and inflammation. This study was undertaken to evaluate the mediation effects of inflammation on the association between vitamin D levels and MCI.

Methods: We explored the associations of inflammation and cognitive impairment related to 25(OH)D_3 deficiency among 360 older people from the communities in China. Demographic characteristics, lifestyle, and health status were investigated by questionnaire, cognitive function was detected by MoCA, and plasma 25(OH)D_3, interleukin-1β (IL-1β), and interleukin-18 (IL-18) were measured by ELISA. Spearman's correlation analysis and logistic regression analysis were used to analyze the relationship among 25(OH)D_3, IL-1β, and IL-18 in the MCI group and the control group and further to analyze the relationship between 25(OH)D_3 and inflammatory factors in the MCI group. Finally, mediation analysis was performed to evaluate whether inflammation mediated the effect of 25(OH)D_3 deficiency on cognitive impairment.

Results: There were lower plasma 25(OH)D_3 concentration and higher IL-1β and IL-18 levels in the MCI group compared with the controls. The levels of 25(OH)D_3 were positively correlated with the MoCA scores and scores of different domains; the levels of IL-1β and IL-18 were negatively correlated with them ($p < 0.05$). In multivariate logistic analysis, there were significant associations among 25(OH)D_3, IL-1β, IL-18, and MCI after adjusted. Further analysis revealed the significant association between the subjects with VD deficiency and the highest quartile of IL-18 in MCI (OR = 4.066), not with IL-1β after adjusting the confounding variables in MCI group. Ultimately, mediation analysis suggested that IL-1β and IL-18 could explain 25.4 and 17.5% of effect of the risk of cognitive impairment related to 25(OH)D_3 deficiency.

Conclusion: Our findings suggested that 25(OH)D_3 deficiency could increase the risk of cognitive impairment by a mechanism partly involving inflammation. Therefore, vitamin D supplementation may improve or delay the decline in cognitive function caused by inflammation in the elderly.

Keywords: mild cognitive impairment, 25(OH)D_3, inflammatory factors, IL-1β, IL-18, mediation effect, case-control study

INTRODUCTION

Mild cognitive impairment (MCI) is characterized by a subtle decline in cognitive function and influenced by multiple factors that include pathophysiology, lifestyle, eating habits, and so on, and it is now also recognized as a risk factor for Alzheimer's disease (AD) (Langa and Levine, 2014). In China, the latest report showed that the overall prevalence of dementia was 6.0% and of MCI was 15.5% in Chinese adults aged 60 years or older (Jia et al., 2020). This higher prevalence makes them an urgent public health problem in China, accompanied that the population has aged. But, the pathologic substrates of MCI and AD are equally complex and must take into account not only conventional the loss of neurons, plaque, and tangle pathology but also a wide range of cellular, biochemical, and molecular mechanisms, such as inflammation and so on (Mufson et al., 2012). Several population-based studies had also reported an association between peripheral inflammatory factors and the risk of MCI or dementia (Bossù et al., 2008; Scarabino et al., 2020). However, it is still unclear how much of the risk of cognitive impairment is caused by inflammation. Furthermore, there are also a lot of inflammation-related mechanisms, which also involve different inflammatory factors. Therefore, in this study, we selected interleukin-1β (IL-1β) and interleukin-18 (IL-18) as the inflammatory factors, which were not only related to inflammation, but also related to the loss of neurons.

Interleukin-1β and IL-18 can regulate or participate in neuronal damage through different mechanisms in the blood–brain barrier or in brain before or during AD occurred including in the brain and the periphery (Heppner et al., 2015; He et al., 2016; Heneka et al., 2018). Meanwhile, the high levels of IL-1β and IL-18 may be due to the activation of pyroptosis, a proinflammatory form of cell death. When pyroptosis has occurred, NOD-like receptors such as protein 1 (NLRP1), NLRP3, and other inflammasomes were activated by AD-related markers or cellular damage danger signals, which in turn activated caspase-1 and the pyroptosis execution protein gasdermin-D (GSDMD). These caused cell membrane damage and released large amounts of mature IL-1β and IL-18 (Heppner et al., 2015; He et al., 2016; Heneka et al., 2018).

Vitamin D (VD), as an essential micronutrient for the body, is mainly obtained through skin synthesis and dietary intake. Before exerting biological effects, VD must undergo hydroxylation in the liver and kidneys to be converted into 25-hydroxy-vitamin D [25(OH)D] and 1,25-dihydroxy-vitamin D [1,25$(OH)_2$D] (Cui et al., 2017). As a neurosteroid hormone, VD also plays a certain neuroprotective effect in the brain. Some studies suggest that the lower 25(OH)D_3 is strongly associated with cognitive decline and neurodegenerative disease (Brouwer-Brolsma and de Groot, 2015; Jones et al., 2015; Keeney and Butterfield, 2015). A meta-analysis found that serum 25(OH)D levels were significantly lower in subjects with MCI and dementia than in healthy controls (Annweiler et al., 2016). Some observational studies had been found that VD deficiency [25(OH)D < 25 ng/mL] and inflammation factors [interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), IL-1β, C-reactive protein (CRP)] had a significant correlation (Laird et al., 2014). Additionally, a study by Briones also found that in patients with AD, the serum levels of IL-1β and 25(OH)D_3 showed a strong correlation (Briones and Darwish, 2012). Another study also explored the effect of VD supplementation on ameliorating cognitive function through the antiinflammatory mechanism *in vivo* (Medhat et al., 2020). Since deficiency in VD can be treated, VD may have an important public health inference in the prevention of age-related neurodegenerative diseases such as MCI and AD.

However, a challenge remains to fully understand the molecular mechanism of inflammation between VD and cognitive function. In other words, it was unclear what proportion of VD exerted neuroprotective effects through antiinflammatory mechanisms, or whether and how much the risk of increased cognitive impairment related to VD deficiency is explained by inflammation. Therefore, mediation analysis can be used to evaluate the mediating variable and further to reveal the internal mechanism and role of the causal association. So, we hypothesized that the 25(OH)D_3 deficiency could increase the risk of cognitive impairment by a mechanism involving inflammation. To prove our hypothesis, we conducted a case–control study to detect the levels of VD and inflammatory factors in the elderly and analyze the correlation. We used mediation analysis to explore the potential role of inflammatory factors in the association between the levels of 25(OH)D_3 and MCI.

MATERIALS AND METHODS

Study Design and Participants

The data of the case–control study were obtained from a population-based epidemiological study on cognitive impairment among elderly population. The research subjects were local residents ≥65 years old who come from six major districts of Taiyuan, Shanxi, China. The interviews were conducted by face-to-face, and data collection and investigations were performed by trained staffs during the period from March 2016 to July 2017. Subjects were excluded from the study if they exhibited: cognitive dysfunction caused by other non-vascular factors such as ischemic cerebrovascular disease, systemic

disease, taking drugs that affect cognitive function, degenerative disease, etc.; consciousness disturbances and patients with paranoia and mental illness; severe aphasia, hearing, visual impairment, severe movement, sensory impairment, etc.

All participants were informed of the objective of the study and their consent to participate in the study was obtained. The research protocol was approved by the Medical Ethics Committee of Shanxi Medical University, China.

Sample Size

The population of this investigation was the elderly in the communities of Taiyuan city. The sample size was determined according to the sample size formula of the 1:1 ratio case–control study. According to the 2010–2013 China National Nutrition and Health Survey (CNNHS), the VD deficiency rate among the elderly was 39.15% (Chen et al., 2017). The odds ratio (OR) of the expected exposure to the research factor was estimated to be 2. The sample size was estimated for the two-sided test with error probabilities of $\alpha = 0.05$ and 90% power ($\beta = 0.10$). Gender, age ($\pm$2 years), and education year ($\pm$2 years) as the matching factors and 180 aged people with MCI and 180 aged people with normal cognition served as the controls were included according to the calculation result.

Data Collection

All subjects were interviewed with their caregivers present by trained interviewers. The questionnaire was designed to obtain the following information regarding the patients' general characteristics: name, gender, age, height, weight, education level, whether to exercise, smoking, drinking, etc. The body mass index (BMI) based on the data of height and weight was calculated and divided into weight loss (<18.5), normal (18.5–23.9), overweight (24.0–27.9), and obesity ($\geq$28.0) according to the Chinese adult BMI standard. The definition of education level was illiteracy, education period of 0–6 years, education period of 6 years or more. Smokers were defined as those who smoked at least 1 cigarette per day in the past 6 months or longer. Drinkers were who drank at least two times a week and drank continuously for more than one year. The definition of the exercise was in the past 6 months, at least five times a week, each time lasting at least 10 min or more of sports, exercise, or recreational activities.

Assessment of Mild Cognitive Impairment

Participants underwent cognitive evaluation in a quiet room carried out by technicians with formal training. Montreal Cognitive Assessment (MoCA) was used to assess cognitive function, which includes the assessment of seven cognitive domains that include executive, naming, memory, attention, language, abstraction, and orientation (Ciesielska et al., 2016). The scoring standards are as follows: illiterate elderly with MoCA score $\leq$ 13 are classified as patients with MCI, $\geq$14 are classified as normal cognition; elderly people with education $\leq$ 6 years are classified as patients with MCI with MoCA score $\leq$ 19, and $\geq$20 are classified as normal cognition; elderly people with education years > 6 years with MoCA score $\leq$ 24 are classified as patients with MCI, $\geq$25 with normal cognition (Petersen, 2004).

Inclusion Criteria for Case Group and Control Group

Case group: (1) the subjects were aged $\geq$ 65 years and were in good health; (2) the MoCA score belongs to patients with MCI; (3) Taiyuan residents with long-term residence in the urban area or suburb of Taiyuan city; (4) cognitive test can be completed.

Control group: (1) the age, gender, and education of the subjects were matched with case group, and they were healthy; (2) the MoCA score belonged to the elderly with normal cognition; (3) Taiyuan residents with long-term residence in the urban area or suburb of Taiyuan city; (4) cognitive test can be completed.

Blood Sampling and Plasma Vitamin D and Inflammatory Factors Measurement

The blood samples were collected in the same season and drawn by venipuncture into 5-mL plain evacuated tubes and then centrifuged at 2,000 $\times$ g for 10 min from each participant after overnight fasting (8–12 h). All specimens were collected and analyzed within 1 h or stored at –80°C until use. Plasma vitamin D levels and levels of IL-1β and IL-18 were measured in duplicate using a commercially available enzyme-linked immunosorbent assay kit (Human 25-hydroxy vitamin D_3, England; Human IL-1β and IL-18, Enzyme-linked Biotechnology, Shanghai, China) (Samochocki et al., 2013).

The international and Chinese recommended classification standards for the degree of VD deficiency are $25(OH)D_3$ < 10 ng/mL (<25 nmol/L) for severe deficiency, <20 ng/mL (<50 nmol/L) for deficiency, 21–29 ng/mL (52–72 nmol/L) is insufficient, and $\geq$30 ng/mL ($\geq$75 nmol/L) is sufficient (de Oliveira et al., 2017).

Statistical Analysis

Mean and standard deviations (SDs) or median (interquartile range) were used as descriptive statistics for continuous variables, and percentage was used for categorical variables. For continuous variables, the Student's *t*-test or Mann–Whitney U test was used for between-group comparisons and chi-square test for categorical variables. Spearman's correlation was conducted to analyze the relationship between VD, inflammatory factors, and cognitive impairment. Logistic regression was used to assess the association among VD, inflammatory factors, and risk of MCI. OR and corresponding 95% confidence intervals (CIs) were calculated. Model 1 was used to calculate the crude OR, and model 2 was adjusted age, gender, education, economic status, BMI, smoking status (yes/no), drinking status (yes/no), exercising status (yes/no), hypertension (yes/no), diabetes (yes/no), and hyperlipoidemia (yes/no). Furthermore, we conducted a mediating effect model to determine that the inflammatory factor changes could explain the cognitive impairment associated with VD status. The mediation effect analysis has used three linear equations to analyze the association among independent variables (VD), mediator variables (inflammatory factors), and dependent variables

(MoCA). Variables such as age, gender, education, economic status, BMI, smoking, drinking, exercising, hypertension, diabetes, and hyperlipoidemia were included as confounders in the equation, as shown in **Figure 1**. The mediation proportion was used to evaluate the mediation effect in this study. All statistical analyses were performed using SPSS 22.0 and SAS 9.4. All reported *p*-values were two-sided and $p < 0.05$ were considered a statistically significant difference.

RESULTS

Characteristics of Study Population

This study involved 180 aged people with MCI and 180 age-, sex- and education-matched controls. The mean age was approximately 73 years, with 50% being female sex. **Table 1** shows the characteristics of the study participants. The results generally showed that there was no statistical difference between the two groups in terms of demographic characteristics, lifestyle, and health status ($p > 0.05$). Compared with the control group, the MCI group had lower scores in MoCA total scores and domains of executive, naming, memory, attention, language, abstraction, and orientation ($p < 0.001$). In this study, we found that 16 subjects (4.4%) were severe deficiency, 271 subjects (75.3%) were deficiency, 39 subjects (10.9%) were insufficient, and 34 subjects (9.4%) were sufficient about the level of plasma $25(OH)D_3$. There were fewer people in the MCI group who were sufficient, and more people who were insufficient, deficiency, and severe deficiency compared with the control group ($p < 0.001$).

Comparisons of Plasma 25(OH)D3 and Inflammatory Factors Between Mild Cognitive Impairment and Control Groups

In this case–control study, the median value of plasma $25(OH)D_3$ concentration of all subjects was 14.43 (11.58, 18.17) ng/mL, of which the median value of plasma $25(OH)D_3$ was 14.78 (11.81, 20.46) ng/mL in the control group, 14.16 (11.31, 17.38) ng/mL in the MCI group. Compared with the control group, the median value of plasma $25(OH)D_3$ concentration in the MCI group was lower ($p = 0.031$). Compared with the control group, the median values of plasma inflammatory factors IL-1β and IL-18 levels in the MCI group were significantly higher ($p < 0.001$; **Figure 2**).

The Association Among Vitamin D, Inflammatory Factors, and Mild Cognitive Impairment

We used Spearman's correlation and logistic regression to analyze the association among VD, inflammatory factors, and MCI (**Tables 2**, **3**). **Table 2** shows that the plasma $25(OH)D_3$ levels of the elderly were weak positively correlated with the total MoCA scores ($r = 0.257$) and were weak positively correlated with the language and abstraction ($r = 0.121$, 0.131, respectively). The plasma levels of IL-1β and IL-18 were negatively correlated with MoCA scores ($r = -0.440$, -0.434, respectively), and there was also a negative correlation in each domain ($p < 0.001$).

Logistic regression was performed on the association among cognitive function, $25(OH)D_3$, and inflammatory factors. The

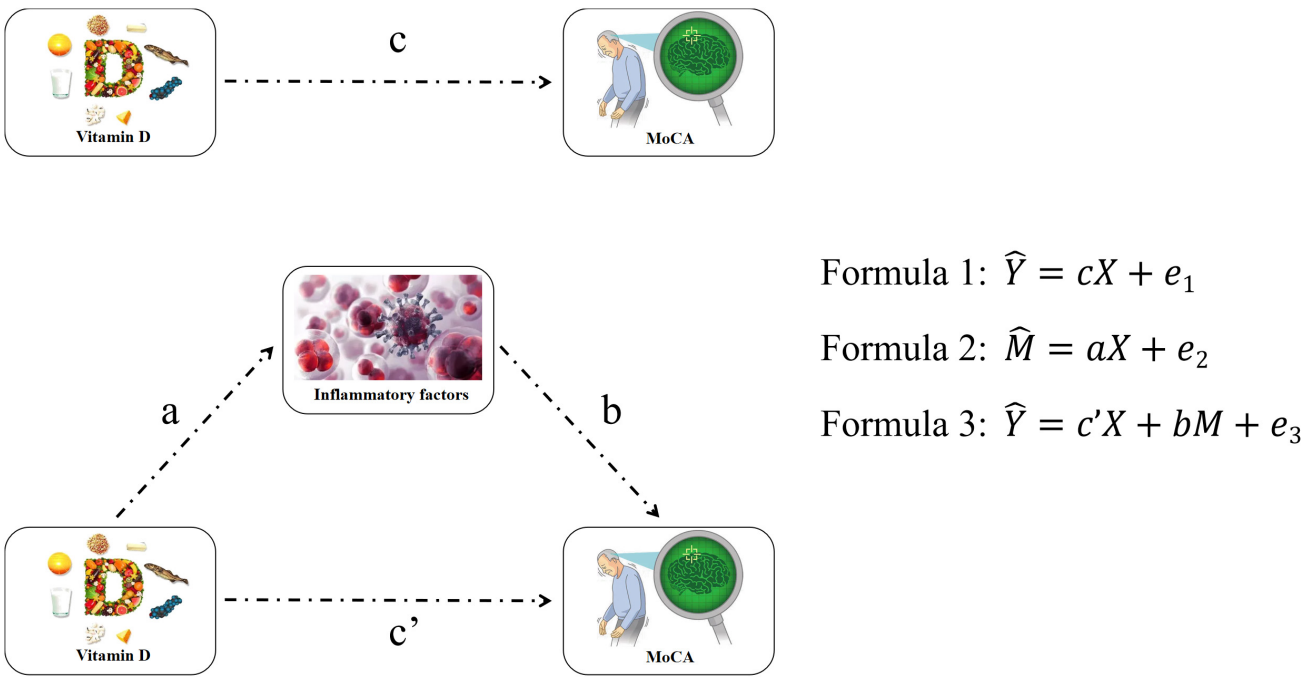

FIGURE 1 | Flow of mediation effect analysis. *Y*, *X*, and *M* are MoCA scale score, VD, and inflammatory factors concentration, respectively. a, b, c, c′, e_1, e_2, and e_3 are equation parameters.

TABLE 1 | Characteristics of the study population.

Characteristics*		All subjects, *n* = 360	MCI, *n* = 180	Control, *n* = 180	*p*-Value#
Sex	Males	179 (49.7%)	90 (50.0%)	89 (49.4%)	0.916
	female	181 (50.3%)	90 (50.0%)	91 (50.6%)	
Age (year)		73.06 ± 5.53	73.33 ± 5.55	72.78 ± 5.52	0.342
Age	65–69	110 (30.6%)	53 (29.4%)	57 (31.6%)	0.316
	70–74	101 (28.0%)	46 (25.6%)	55 (30.6%)	
	75–79	108 (30.0%)	62 (34.4%)	46 (25.6%)	
	≥80	41 (11.4%)	19 (10.6%)	22 (12.2%)	
Educational level	illiteracy	43 (11.9%)	25 (13.9%)	18 (10.0%)	0.070
	<6 years	77 (21.4%)	30 (16.7%)	47 (26.1%)	
	≥6 years	240 (66.7%)	125 (69.4%)	115 (63.9%)	
BMI (kg/m^2)		24.80 ± 4.86	24.34 ± 4.41	25.27 ± 5.25	0.068
BMI	<18.5	17 (4.7%)	12 (6.7%)	5 (2.8%)	0.131
	18.5–23.9	144 (40.0%)	77 (42.8%)	67 (37.2%)	
	24.0–27.9	141 (39.2%)	62 (34.4%)	79 (43.9%)	
	≥28.0	58 (16.1%)	29 (16.1%)	29 (16.1%)	
Physical activity	Yes	267 (74.2%)	134 (74.4%)	133 (73.9%)	0.904
	No	93 (25.8%)	46 (25.6%)	47 (26.1%)	
Smoking habit	Yes	102 (28.3%)	58 (32.2%)	44 (24.4%)	0.102
	No	258 (71.7%)	122 (67.8%)	136 (75.6%)	
Alcohol intake	Yes	68 (18.9%)	38 (21.1%)	30 (16.7%)	0.281
	No	292 (81.1%)	142 (78.9%)	150 (83.3%)	
Hypertension	Yes	164 (45.6%)	87 (48.3%)	77 (42.8%)	0.290
	No	196 (54.4%)	93 (51.7%)	103 (57.2%)	
Diabetes	Yes	54 (15.0%)	33 (18.3%)	21 (11.7%)	0.077
	No	306 (85.0%)	147 (81.7%)	159 (88.3%)	
Hyperlipemia	Yes	97 (26.9%)	51 (28.3%)	46 (25.6%)	0.553
	No	263 (73.1%)	129 (71.7%)	134 (74.4%)	
MoCA (scores)	Total score	21.21 ± 5.60	18.02 ± 5.05	25.65 ± 3.04	<0.001
	Executive	2.63 ± 1.66	1.91 ± 1.49	3.19 ± 1.02	<0.001
	Naming	2.67 ± 0.71	2.41 ± 0.90	2.89 ± 0.39	<0.001
	Memory	4.84 ± 1.56	4.29 ± 1.72	5.65 ± 0.70	<0.001
	Attention	2.03 ± 0.95	1.64 ± 1.02	2.54 ± 0.66	<0.001
	Language	1.31 ± 0.87	0.81 ± 0.87	1.61 ± 0.67	<0.001
	Abstraction	2.31 ± 1.37	1.26 ± 1.47	3.37 ± 1.38	<0.001
	Orientation	5.77 ± 0.61	5.53 ± 0.98	5.96 ± 0.22	<0.001
25(OH)D_3	Severe deficiency	16 (4.4%)	15 (8.3%)	1 (0.6%)	<0.001
	Deficiency	271 (75.3%)	139 (77.2%)	132 (73.3%)	
	Insufficient	39 (10.9%)	24 (13.4%)	15 (8.3%)	
	Sufficient	34 (9.4%)	2 (1.1%)	32 (17.8%)	

**Results were shown as frequency percentages n (%) or mean ± SD. #Independent sample t-test or Mann–Whitney U test was used for continuous variables. Pearson's chi-squared was used for categorical variables.*

crude (unadjusted) and adjusted ORs for the MCI according to the quartile concentrations of plasma 25(OH)D_3, IL-1β, and IL-18 are shown in **Table 3**. In Model 1, compared with highest quartile of VD, lower quartiles of VD (Q_2: OR_1 = 9.289, 95% CI_1: 3.180–27.134; Q_3: OR_1 = 9.672, 95% CI_1: 3.063–30.544; Q_4: OR_1 = 15.725, 95% CI_1: 4.880–50.673) were associated with MCI; compared with the lowest quartile, the higher quartiles of IL-1β (Q_2: OR_1 = 3.200, 95% CI_1: 1.649–6.210; Q_3: OR_1 = 6.909, 95% CI_1: 3.531–13.519; Q_4: OR_1 = 10.400, 95% CI_1: 5.203–20.786) were associated with MCI, and the highest quartile of IL-18 (Q_4: OR_1 = 3.131, 95% CI_1: 1.704–5.752) was associated with MCI. The significant association among VD, inflammatory factors, and MCI persisted after further adjusting for economic status, BMI, smoking, drinking, exercising, hypertension, diabetes, and hyperlipoidemia, with p for trend <0.05.

The Relationship Between Vitamin D and Inflammatory Factors in Elderly Patients With Mild Cognitive Impairment

We further analyzed the association between the level of VD and inflammatory factors in the MCI group. **Table 4** illustrates that the plasma 25(OH)D_3 level was negatively correlated with the levels of IL-1β (r = −0.168, p = 0.025) and IL-18 (r = −0.257,

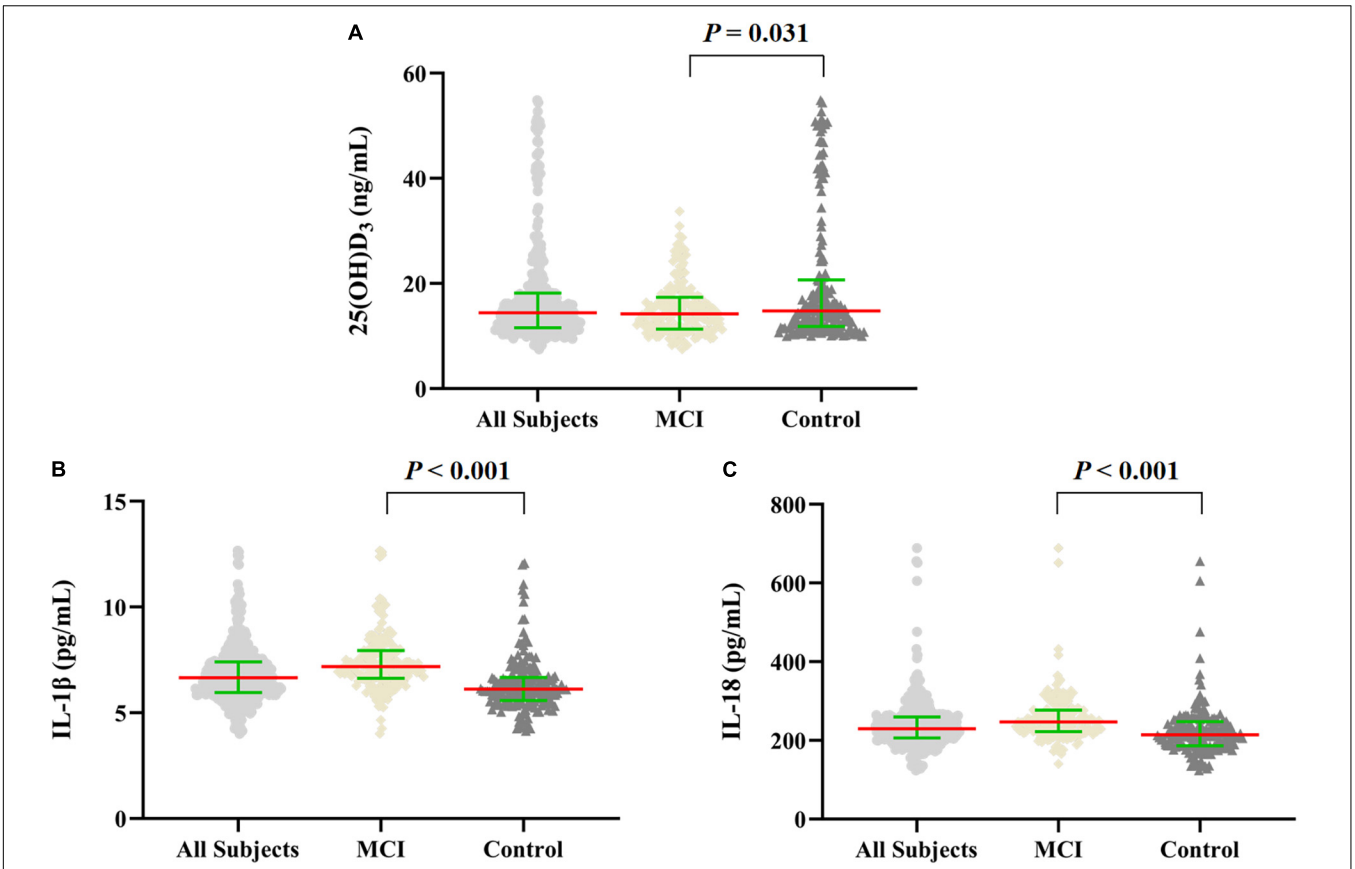

FIGURE 2 | Scatter dot plot of the levels of plasma 25(OH)D_3 and inflammatory factors in MCI and control groups. **(A–C)** The levels of plasma 25(OH)D_3 and IL-1β, IL-18 in all subjects (n = 360), and in MCI and control groups (n = 180, respectively). The data were shown by the median with interquartile range, the red line represents the median, and the green line represents the interquartile range. Mann–Whitney U test was used for between-group comparisons.

$p < 0.001$). In multivariate logistic regression, the levels of IL-1β and IL-18 (quartiles) were used as dependent variables, and VD status (deficiency or not) was used as the independent variable to analyze, and it was found that either in the crude model or adjusted model, there was no correlation between VD and IL-1β (**Table 5**). In Model 1, there was no correlation between VD and IL-18 levels. After adjusting for age, gender, educational level, economic status, BMI, smoking, drinking, exercising, hypertension, diabetes, and hyperlipoidemia, the analysis found that compared with subjects with sufficient VD, the subjects with VD deficiency were associated with the highest quartile of IL-18 (Q_4: OR_2 = 4.066, 95% CI_2: 1.654–9.995; **Table 5**).

TABLE 2 | Spearman's correlation of the association among cognitive function, 25(OH)D_3, and inflammatory factors.

Variables	25(OH)D_3 (ng/mL)	IL-1β (pg/mL)	IL-18 (pg/mL)
MoCA scores	0.257*	−0.440*	−0.434*
Executive	0.032	−0.303*	−0.341*
Naming	0.049	−0.246*	−0.277*
Memory	0.085	−0.239*	−0.206*
Attention	0.099	−0.274*	−0.288*
Language	0.121*	−0.282*	−0.296*
Abstraction	0.131*	−0.325*	−0.253*
Orientation	0.099	−0.239*	−0.230*

Spearman's correlation was used for the relationship between groups, $p < 0.05$.

Mediate Effects of Inflammatory Factors on the Association Between 25(OH)D3 and Cognition

We performed mediation analysis of inflammatory factors in the association between 25(OH)D_3 and cognitive status. We observed significant mediation effects of IL-1β and IL-18 in the association between 25(OH)D_3 and cognition in **Table 6** (p = 0.013, 0.004, respectively). The mediation analysis showed a mediation proportion of 25.4% (95% CI: 9.2–53.2%) in IL-1β and a mediation proportion of 17.5% (95% CI: 7.2–36.7%) in IL-18. These results suggested that IL-1β and IL-18 may be the potential mediators of 25(OH)D3 deficiency effect on the risk of cognitive impairment.

After further analysis of the various domains of cognition, the results found that IL-1β had significant mediation effects in the association between 25(OH)D_3 and memory, attention, language, and abstraction ($p < 0.05$), and IL-18 had significant

TABLE 3 | Logistic regression of the association among different cognitive status, 25(OH)D_3, and inflammatory factors.

Independent variables	Groups	Model 1			Model 2		
		OR_1	95%CI_1	P_1	OR_2	95%CI_2	P_2
25(OH)D_3*	Q_1	1.000	–	–	1.000	–	–
	Q_2	9.289	3.180 ~ 27.134	0.001	7.538	2.456 ~ 23.136	0.000
	Q_3	9.672	3.063 ~ 30.544	0.000	9.111	2.729 ~ 30.415	0.000
	Q_4	15.725	4.880 ~ 50.673	0.000	14.146	4.187 ~ 47.795	0.000
	P for trend	0.004			0.016		
IL-1β#	Q_1	1.000	–	–	1.000	–	–
	Q_2	3.200	1.649 ~ 6.210	0.001	4.221	2.031 ~ 8.773	0.000
	Q_3	6.909	3.531 ~ 13.519	0.000	7.848	3.740 ~ 16.465	0.000
	Q_4	10.400	5.203 ~ 20.786	0.000	12.619	5.889 ~ 27.040	0.000
	P for trend	0.000			0.000		
IL-18#	Q_1	1.000	–	–	1.000	–	–
	Q_2	1.727	0.952 ~ 3.133	0.072	1.532	0.804 ~ 2.921	0.195
	Q_3	1.652	0.911~ 2.997	0.098	1.764	0.925 ~ 3.361	0.085
	Q_4	3.131	1.704 ~ 5.752	0.000	3.373	1.736 ~ 6.553	0.000
	P for trend	0.001			0.000		

Model 1: Crude model.

Model 2: Adjusted for age, gender, education, economic status, BMI, smoking status (yes/no), drinking status (yes/no), exercising status (yes/no), hypertension (yes/no), diabetes (yes/no), and hyperlipoidemia (yes/no).

**The groups of 25(OH)D_3 status Q_1 for sufficient; Q_2 for insufficient; Q_3 for deficiency; Q_4 for severe deficiency.*

#The groups of IL-1β were divided by quartiles: Q_1 for <25% (~5.96 pg/mL); Q_2 for 25–50% (5.98–6.65 pg/mL); Q_3 for 50–75% (6.66–7.41 pg/mL); Q_4 for >75% (~7.44); the groups of IL-18 were divided by quartiles: Q_1 for <25% (~206.60 pg/mL); Q_2 for 25–50% (206.71–229.88 pg/mL); Q_3 for 50–75% (229.90–259.23 pg/mL); Q_4 for >75% (~259.49).

mediation effects in the association between 25(OH)D_3 and naming, attention, language, and abstraction ($p < 0.05$).

DISCUSSION

In this study, we observed the lower plasma 25(OH)D_3 concentration and higher IL-1β and IL-18 levels in the MCI group, and there were significant associations among 25(OH)D_3, inflammatory factors, and MCI. Significantly, after the mediation analysis, we also found that the 25(OH)D_3 deficiency could increase the risk of cognitive impairment by a mechanism partly involving inflammation, which could explain 25.4 (IL-1β) and 17.5% (IL-18) of effect of the risk of cognitive impairment related to 25(OH)D_3 deficiency, and be also applicable to different domains of cognition.

Plasma 25(OH)D_3 is determined by endogenous vitamin D synthesis and/or dietary intake, conversion into 25(OH)D_3, and, finally, distribution and usage (metabolism and excretion). The concentration of 25(OH)D_3 in plasma is largely unregulated, and it has a relatively long half-life of 2–3 weeks (Cui et al., 2017).

TABLE 4 | Spearman's correlation of the association between 25(OH)D_3 and inflammatory factors in mild cognitive impairment (MCI).

Inflammatory factors	25(OH)D_3 (ng/mL)	
	r	*P*
IL-1β (pg/mL)	−0.168	0.025
IL-18 (pg/mL)	−0.257	<0.001

Therefore, in this study, we assessed the VD nutritional status by detecting plasma 25(OH)D_3, which is the most commonly used marker of vitamin D status. In this study, we found that the median value of plasma 25(OH)D_3 concentration of all subjects was 14.43 ng/mL, and 16 subjects (4.4%) were severe deficiency, 271 subjects (75.3%) were deficiency, 39 subjects (10.9%) were insufficient, and 34 subjects (9.4%) were sufficient about the level of plasma 25(OH)D_3. The constituent ratio of 25(OH)D_3 deficiency in this study was higher than that of 25(OH)D_3 deficiency (39.2%) in CNNHS (Chen et al., 2017). We further explored the differences in age composition, seasons, and residences of the two surveys. It turned out that CNNHS survey results showed that 25(OH)D_3 deficiency was positively correlated with the spring season, low ambient UVB levels, and living in large cities (Chen et al., 2017). The Taiyuan city in Shanxi Province that we surveyed belongs to low ambient UVB levels and large cities, and the time when we did our survey and the collection time of blood samples were both in spring. In addition, the age among our subjects was older than CNNHS, which may be the reason why the constituent ratio of 25(OH)D_3 deficiency in our results was higher.

As a neurosteroid hormone, VD also has a certain neuroprotective effect on the brain. When VD deficiency occurred, it may be brought about cognitive dysfunction, cognitive decline, or neurodegenerative diseases, and so on (Brouwer-Brolsma and de Groot, 2015; Jones et al., 2015; Keeney and Butterfield, 2015). Several previous observational studies had reported an association between low levels of serum vitamin D and MCI or dementia in the elderly (Goodwill and Szoeke, 2017; Overman et al., 2017). In our research, focusing on the

TABLE 5 | Logistic regression of the association between vitamin D (VD) deficiency and inflammatory factors in MCI group.

Dependent variables	Groups	Model 1			Model 2		
		OR_1	95%CI_1	P_1	OR_2	95%CI_2	P_2
IL-1β*	Q_1	1.000	–	–	1.000	–	–
	Q_2	0.773	0.285–2.096	0.163	0.508	0.159–1.629	0.255
	Q_3	3.132	0.713–12.073	0.069	2.424	0.511–11.502	0.265
	Q_4	3.500	0.880–13.918	0.075	2.790	0.637–12.216	0.173
IL-18*	Q_1	1.000	–	–	1.000	–	–
	Q_2	1.444	0.468–3.732	0.599	0.995	0.509–1.943	0.987
	Q_3	2.235	0.612–5.637	0.274	1.681	0.831–3.398	0.148
	Q_4	5.090	0.777–13.403	0.094	4.066	1.654–9.995	0.002

Model 1: Crude model.
Model 2: Adjusted for age, gender, education, economic status, BMI, smoking status (yes/no), drinking status (yes/no), exercising status (yes/no), hypertension (yes/no), diabetes (yes/no), and hyperlipoidemia (yes/no).
**The groups of IL-1β were divided by quartiles: Q_1 for <25% (∼5.96 pg/mL); Q_2 for 25–50% (5.98–6.65 pg/mL); Q_3 for 50–75% (6.66–7.41 pg/mL); Q_4 for >75% (∼7.44); the groups of IL-18 were divided by quartiles: Q_1 for <25% (∼206.60 pg/mL); Q_2 for 25–50% (206.71–229.88 pg/mL); Q_3 for 50–75% (229.90–259.23 pg/mL); Q_4 for >75% (259.49∼).*

TABLE 6 | Mediate effects of inflammatory factors in the association between 25(OH)D_3 and cognitive status.

Variables	IL-1β		IL-18	
	Proportion mediated*	*p*-Value	Proportion mediated*	*p*-Value
MoCA scores	25.4% (9.2–53.2%)	0.013	17.5% (7.2–36.7%)	0.004
Executive	–	–	–	–
Naming	–	–	34.1% (7.4–76.9%)	0.012
Memory	16.6% (5.4–40.7%)	0.016	–	–
Attention	34.6% (8.7–74.6%)	0.012	22.2% (6.2–55.4%)	0.007
Language	33.4% (8.1–74.0%)	0.010	22.0% (4.8–61.4%)	0.029
Abstraction	21.1% (5.6–54.5%)	0.026	14.7% (3.9–42.1%)	0.030
Orientation	–	–	–	–

**Covariates in the SAS macro include age, gender, education, economic status, BMI, smoking status (yes/no), drinking status (yes/no), exercising status (yes/no), hypertension (yes/no), diabetes (yes/no), and hyperlipoidemia (yes/no).*

MCI population, we also found that plasma 25(OH)D_3 level was significantly decreased in MCI compared with the control group. Furthermore, plasma 25(OH)D_3 concentration was positively correlated with MoCA scores.

The underlying mechanisms of the association between 25(OH)D_3 deficiency and cognitive impairment remain an open question, and it may be related to the role of VD in the brain. 25(OH)D_3 and 1,25(OH)$_2D_3$ could regulate the survival, development, and function of neural cells (Annweiler et al., 2013). VD also could reduce amyloid-induced cytotoxicity and apoptosis in primary cortical neurons (Mizwicki et al., 2012). Additionally, vitamin D supplementation ameliorates age-related decline in learning and memory in aged rats and this may be one of the measures to prevent or delay cognitive impairment (Briones and Darwish, 2012). The preventive effect of VD may be exerted through an antiinflammatory mechanism (Laird et al., 2014). The level of IL-1β was increased in AD during 25(OH)D_3 was deficient and the effect of VD supplementation on ameliorating cognitive function through the antiinflammatory mechanism *in vivo* (Medhat et al., 2020).

Inflammation has been confirmed to be involved in the pathogenesis and progression of AD. Indeed, inflammatory processes play at least some roles in the pathology of AD and MCI. Particularly, intriguing are peripheral inflammatory cytokines, studies had found that the level of peripheral inflammatory cytokines in AD has reached its peak in the early stage of the disease, which may precede the clinical symptoms of AD (Motta et al., 2007). Moreover, the researchers found a third of patients with MCI remained as they were, a third reversed diagnosis to cognitively normal, thereby providing a large time "critical window" to the prevention (Manly et al., 2008). In this study, we may pay more attention to how much of the risk of cognitive impairment is caused by inflammation in this "critical window" to prevention. We selected IL-1β and IL-18 in the peripheral blood of patients with MCI as the inflammatory factors, which were not only related to the inflammation, but also related to the loss of neurons which as the typical pathological characteristics of MCI and AD. The high levels of inflammatory factors in the periphery and brain would further invade the brain and neurons, which make the condition worse. Our research found that compared with the control group, the levels of plasma IL-1β and IL-18 in MCI were significantly higher, and IL-1β and IL-18 were negatively correlated with MoCA scores and scores of different domains. After further regression analysis, changes

in two inflammatory factors were found, so it was speculated that systemic inflammation might be a risk factor for MCI. These higher levels of IL-1β and IL-18 may further remind us that, in the "critical window period," the detection of the levels of peripheral inflammatory factors in patients with MCI may play a certain role in the prognosis of disease.

As mentioned above, the previous studies reported that in patients with AD, the serum levels of IL-1β and $25(OH)D_3$ showed a strong negative correlation (Briones and Darwish, 2012). In this study, we found that the plasma $25(OH)D_3$ level was negatively correlated with the levels of IL-1β and IL-18, and VD deficiency may be a risk factor for high levels of inflammatory factors in MCI. Interestingly, however, we only found the role for IL-18, not both. This may be related to the production of IL-18. IL-18 can be produced by chondrocytes, osteoblasts, and macrophages in joints and also present in keratinocytes and nearly all epithelial cells (Dinarello et al., 2013). Additionally, these are also the main ways that VD plays the physiological functions *in vivo*. Furthermore, we further analyzed whether and how much the risk of increased cognitive impairment related to VD deficiency was explained by inflammation. The mediation analysis showed that IL-1β and IL-18 could explain 25.4 and 17.5% of effect of the risk of cognitive impairment related to $25(OH)D_3$ deficiency. In the analysis of different cognitive domains, it was found that IL-1β and IL-18 may have some relationships in attention, language, and abstraction. IL-1β was significantly related to memory, while IL-18 was significantly related to naming. But both have nothing to do with executive and orientation. Although our study found these interesting results, an interleukin is responsible for specific cognitive alteration that is seemly unrealistic. Therefore, we still need to conduct a lot of research to further explore or confirm them.

The following limitations of this study should be considered. Whether low vitamin D concentrations play a causal role in the pathogenesis of a cognitive disease or are the consequences of an inadequate intake secondary to the illness remains an open issue. Thus, further longitudinal studies and randomized controlled trials are needed to examine the temporal sequence of this association. Although we matched the confounding factors, the results of this case–control study may still be misinterpreted because of the influence of random and systematic recall errors, and selection bias. However, our research answers the mediation effects of inflammation on the association between VD and MCI partially and provides certain population data support for the follow-up study of the antiinflammatory mechanism of VD. At the same time, it also plays a certain value in the prevention and delay of cognitive impairment.

CONCLUSION

In summary, this study not only demonstrated that the elderly individuals with MCI presented decreased plasma vitamin D levels and increased IL-1β and IL-18 concentrations, and there were significant associations among $25(OH)D_3$, inflammatory factors, and MCI. Furthermore, it was revealed that the $25(OH)D_3$ deficiency could increase the risk of cognitive impairment by a mechanism partly involving inflammation. Although further prospective larger studies and rigorous animal or cell experiments should be conducted to examine the association between vitamin D and the risk of cognitive decline and to clarify and verify whether this association may be caused by systemic inflammation. This study still provides some nutritional intervention strategies for preventing cognitive decline in the elderly and hopes to lay a certain research foundation for the realization of "healthy aging."

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Shanxi Medical University, China (protocol code 2014030 and date of approval 7th March, 2014). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LC contributed to the execution of the experiment. RD, CS, XL, and LZ contributed to the acquisition, analysis, and interpretation of data. MS contributed to the creation of new software used in the work. CL, LW, JK, HX, and WF contributed to the evaluation, analysis, and wrote the data. HZ contributed to the guidance and substantive revision of the drafting work. LC and HZ take full responsibility for the contents of the manuscript, agree to be personally accountable for the author's own contributions, for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature. All authors performed revisions of the manuscript, contributed to the article, and approved the submitted version.

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EDITED BY
Zhigang Liu,
Northwest A&F University, China

REVIEWED BY
Anubhuti Dixit,
Amity University, India
Hao Wu,
Shandong University, China

*CORRESPONDENCE
Min Wen
woshiwenmin@163.com

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Ketogenic diet prevents chronic sleep deprivation-induced Alzheimer's disease by inhibiting iron dyshomeostasis and promoting repair *via* Sirt1/Nrf2 pathway

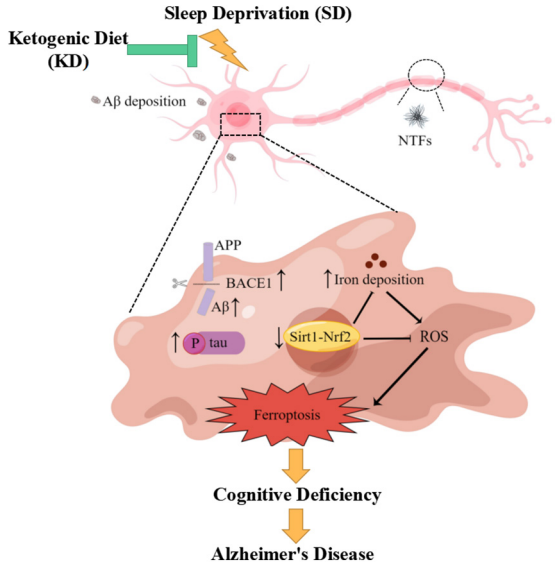
Yueqi Yang, Xueyan Wang, Aiai Xiao, Jun Han, Zhengping Wang and Min Wen*

Institute of Biopharmaceutical Research, Liaocheng University, Liaocheng, China

Sleep deprivation (SD) is one of the main risk factors for Alzheimer's disease (AD), but the underlying mechanism is still unclear. Ketogenic diet (KD) has been shown widely neuroprotective effects but less known about its effect on SD-induced AD. In the present study, a continuous 21 days SD mouse model with or without KD was established. The changes of cognitive function, pathological hallmarks of AD, ferroptosis, and intracellular signal pathways in mice were detected by Morris water maze, ThS staining, diaminobenzidine (DAB)-enhanced Perls' stain, antioxidant assay, immunohistochemistry, and western blot. The results showed that KD can prevent the cognitive deficiency, amyloid deposition and hyperphosphorylated tau induced by chronic SD. Analysis of ferroptosis revealed that KD can inhibit iron dyshomeostasis by down-regulating the expression of TfR1 and DMT1 and up-regulating the expression of FTH1, FPN1. Meanwhile, KD alleviated oxidative stress with elevated xCT/GPX4 axis, FSP1 and reduced MDA. In addition, KD could promote neuronal repair by enhancing BDNF and DCX. Further studies demonstrated that KD activated Sirt1/Nrf2 signaling pathway in the hippocampus in SD-exposed mice. Our finding firstly suggested that KD could prevent chronic SD-induced AD by inhibiting ferroptosis and improving the neuronal repair ability *via* Sirt1/Nrf2 signaling pathway.

KEYWORDS

chronic sleep deprivation, Alzheimer's disease, ketogenic diet (KD), ferroptosis, SIRT1, Nrf2



GRAPHICAL ABSTRACT
KD could prevent chronic SD-induced AD by inhibiting ferroptosis *via* Sirt1/Nrf2 signaling pathway.

Introduction

Alzheimer's disease (AD) is a common neurodegenerative disease and characterized by β-amyloid (Aβ) deposition, neurofibrillary tangles resulted from hyperphosphorylated tau, and neuronal loss (Ashraf and So, 2020). At present, although the amount of AD prevalence is growing year by year, there is no effective drug for AD, so it is vital to prevent AD. AD can be divided into familial and sporadic AD which accounts for more than 95% of AD cases and is caused by the combination of aging and environmental factors, including sleep disorder (Abulafia et al., 2017). With the change of lifestyle, sleep deprivation (SD) has become a common phenomenon in modern society and is recognized as one of the main risk factors for AD (Van Erum et al., 2018). There is a bidirectional link between SD and AD. AD patients are normally accompanied by different sleep problems, such as trouble falling asleep, and sleep interruptions. At the same time, long-term SD can lead to Aβ deposition, abnormally phosphorylated tau, which causes hippocampal damage and cognitive impairment, and thereby promoting the occurrence of AD (Han et al., 2021). Nevertheless, the underlying molecular mechanism of SD on AD remains largely unclear. Investigating the mechanism of SD-induced AD and executing targeted interventions may have significant implications for early prevention of AD.

Iron is the most abundant metal in the brain and is involved in multiple physiological processes to maintain normal brain function. Generally, the level of iron in the brain is slightly increased with aging, but iron deposition is more significant in the brains of AD patients (Yan and Zhang, 2019). The iron concentration in the hippocampus correlates positively with the Aβ deposition, but inversely with cognitive performance of AD patients (Yan and Zhang, 2019). Using iron chelator (deferoxamine) could reduce the level of iron in the brain and suppress the progression of AD, indicating an important role of iron dyshomeostasis in AD (Crowe and Morgan, 1994; Gassen and Youdim, 1997). The excessive iron can increase ROS and induce the production of lipids peroxide, ultimately leading to ferroptosis. Ferroptosis is an iron-dependent form of programed non-apoptotic cell death, characterized by intracellular iron deposition and accumulation of lipid peroxide products. Current research indicates that ferroptosis is linked to a variety of neurodegenerative diseases, including AD (Reichert et al., 2020). In addition, emerging evidence has reported that SD-caused memory impairment is related to hippocampal ferroptosis and can be reversed by ferrostatin-1, a specific inhibitor of ferroptosis (Wang X. et al., 2021, 2022). The above studies show that ferroptosis can act as a bridge between SD and AD, and targeting ferroptosis may be an effective means of preventing AD.

The ketogenic diet (KD) is characterized by high-fat, low-carbohydrate, which result in elevated levels of ketones, mainly β-hydroxybutyrate (BHB) in the blood (Ricci et al., 2020). Generally, the ratio of total energy from fat to carbohydrate and protein combined of KD is from 1:1 to 4:1, which reflects the ketogenic level and strictness of KD (Ricci et al., 2020). KD has aroused wide public concern owing to its therapeutic application in epilepsy successfully. Studies have uncovered that KD exerts neuroprotective effects against neurodegenerative diseases including AD, Parkinson's diseases (PD) and cognitive impairment (Pavón et al., 2021). Currently, studies have shown that the neuroprotective effects of KD involve antioxidant, anti-inflammatory and energy metabolism (Hernandez et al., 2018). Our latest research suggests that KD could ameliorate chronic SD-induced cognitive impairment by inhibiting hippocampus neuron ferroptosis (Wang X. et al., 2022), this indicates to us that KD could be used as a potential dietary treatment for the prevention of AD caused by SD. However, it is unclear whether and how KD treatment could suppress the occurrence of AD induced by chronic SD.

Accordingly, the present study intended to assess (1) the prophylactic effect of KD on the development of AD induced by chronic SD, (2) the effect of KD on SD-induced hippocampal damage, and (3) the molecular mechanisms by which KD mediates in preventing SD-induced AD.

Materials and methods

Reagents and antibodies

Bicinchoninic acid (BCA) protein assay kit was purchased from Beyotime Biotechnology (Shanghai, China).

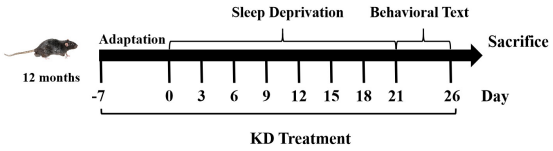


FIGURE 1
Flow schematic depiction of study design.

Malondialdehyde (MDA) and glutathione (GSH) assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). Beta-actin (β-actin), beta-myloid precursor protein cleavage enzyme-1 (BACE1), amyloid precursor protein (APP), amyloid β-protein (Aβ), microtubule-associated protein tau (tau), phosphorylated-tau (*p*-tau), brain-derived neurotrophic factor (BDNF), doublecortin (DCX), iron regulatory proteins 1 (IRP1), iron regulatory proteins 2 (IRP2), transferrin receptor 1 (TfR1), divalent metal-ion transporter-1 (DMT1), ferritin heavy chain 1 (FTH1), ferroportin 1 (FPN1), glutathione peroxidase 4 (GPX4), system xc-cystine-glutamate antiporter (xCT), ferroptosis suppressor protein 1 (FSP1), sirtunin 1 (Sirt1), nuclear factor E2 related factor 2 (Nrf2), phosphorylated-Nrf2 (*p*-Nrf2), and secondary HRP-conjugated antibodies were purchased from Abcam (Cambridge, MA, United States).

Animals and treatments

Consider that the risk of AD is higher in women than in men (Smith et al., 2020), a total of thirty female C57BL/6 mice (12-month-old) were used in this study. Animals were kept on a 12-h light/dark cycle at room temperature of 22 ± 2°C and received water and food *ad libitum*. All animal procedures were approved by the Experimental Animal Ethics Committee of Liaocheng University, and were done in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications No. 80-23). Mice were randomly allocated to control group (Con) or sleep deprivation group fed with AIN93M diet (SD) or KD. The composition of the experimental diets is listed in **Supplementary Table 1**. Conduct behavioral tests after sleep deprivation and after all behavioral tests, the mice were sacrificed for further analysis (As shown in **Figure 1**).

Chronic sleep-deprivation protocol

The model of chronic sleep deprivation was induced as we described previously (Wang X. et al., 2022). Briefly, an Automated Sleep Deprivation System (Shanghai XinRuan Information Technology CO., Ltd. Shanghai, China) was used for the chronic SD in mice. The system stops mice from sleeping by randomly rotating bars. Five mice from the same group were placed in the system and got food and water *ad libitum*. Mice were acclimated to the SD system for 7 days to minimize stress before starting the study as previously described (Zhao et al., 2019). SD was achieved with rotation (5 rev/min) from 14:00 to 10:00 + 1 day for 20 h. After sleep deprivation, mice were permitted to sleep at 10:00 and maintain for 4 h. The Con group mice stay in the same system, but are allowed normal sleep at the corresponding time.

Morris water maze test

The Morris water maze test was executed in a circular dark pool (height 50 cm, diameter 180 cm) filled with white water (25 ± 1°C). Mice were required to find a hidden platform which was placed about 1 cm under water surface in the center of one quadrant of the pool. The mice from each group experienced four consecutive daily training trials. A probe trial was then repeated in the absence of the platform. Behaviors of the mice were tracked using Any-maze software (Stoelting Co.), the escape latency during the training period, the time in the target quadrant, and the across numbers of the target area during the probe trial were analyzed.

Biochemical analysis

After behavioral experiments, mice were killed by inhalation of 2% isoflurane. Eyeball blood from mice in each group was collected for blood glucose and BHB and measured with handheld glucometer and ketone meter respectively (Abbott Labs, Abbott Park, IL, United States). Hippocampus were quickly isolated from the brain and frozen in liquid nitrogen immediately. The levels of total iron, MDA, and GSH were tested using kits according to the manufacturer's protocols.

Histopathological examination

Diaminobenzidine-enhanced Perls' staining

The brain tissue was embedded in paraffin and blocks were sectioned at 5 mm thickness. Slides were immersed in 4% ferrocyanide/4% hydrochloric acid in the dark for 30 min, then incubated with diaminobenzidine (DAB) for enhanced iron staining for 30 min, and counterstained with hematoxylin subsequently. After routine dehydration and transparent treatment, the neutral gum was sealed and recorded under a bright-field microscope (Olympus, Tokyo, Japan).

H&E staining

Incubate the slides with hematoxylin solution in a staining jar for 5–10 min to stain the nuclei, then wash with distilled

water and stained with eosin solution for 1–3 min. The sections of brain stained with H&E were observed and recorded under a bright-field microscope (Olympus, Tokyo, Japan).

Thioflavin-S staining

For thioflavin-S (ThS) staining, ethanol gradient-treated sections were stained with 1% ThS for 5 min and then destained with 70% ethanol. All of the sections were observed using a fluorescence microscope (Olympus, Tokyo, Japan).

Immunohistochemical assay

Immunohistochemical studies were executed as the previous method (Wang X. et al., 2022). Sections treated with primary antibodies against Sirt1 and DCX respectively overnight at 4°C. Next, we were treated with horseradish peroxidase-conjugated secondary antibodies for 3 h at 4°C. All of the sections were observed using a fluorescence microscope (Olympus, Tokyo, Japan).

Western blot assay

Total proteins of hippocampus were prepared for western blotting as described in our preceding study (Wen et al., 2021). The antibodies used in this study were β-actin, BACE1, APP, Aβ, tau, P-tau, BDNF, DCX, IRP1, IRP2, TfR1, DMT1, FTH1, FPN1, GPX4, xCT, FSP1, Sirt1, Nrf2, p-Nrf2, and specific peroxidase (HRP)-conjugated secondary antibodies. These proteins were visualized using the enhanced chemiluminescence substrate and analyzed by UVP Auto Chemi Image system (Tanon 4600SF, Tanon, Shanghai, China).

Statistical analysis

Data were expressed as mean ± standard error of the mean (mean ± SEM). The significance of mean values among different groups was analyzed by a one-way ANOVA with a Tukey's *post hoc* test. The level of significance was considered at $p < 0.05$.

Results

Effects of ketogenic diet on blood β-hydroxybutyrate and blood glucose in the chronic sleep deprivation exposed mice

As expected, blood BHB levels were obviously higher in the KD group mice than in the Con group and SD group ($p < 0.01$, **Supplementary Figure 1A**). While, there was no significant difference in the blood glucose among these groups (**Supplementary Figure 1B**).

Effects of ketogenic diet on chronic sleep deprivation-induced cognitive deficiency

We can see from **Figure 2** that the escape latency was obviously increased in the SD group compared with the Con group on the third and fourth day ($p < 0.05$). While KD treatment significantly shortened the escape latency in SD-exposed mice. Next, we evaluated the spatial memory ability of mice by a spatial probe test after finishing the navigation test. A longer periods of time finding the platform and reduced time spent in the target quadrant with less number of platform crossings were exhibited in the SD group compared to the Con group (**Figures 2B–D**, $p < 0.05$). Nonetheless, KD treatment could largely improve this condition in SD-exposed mice. **Figure 2E** shows the typical track of three groups in the probe trials. These results reflected the improved effect of KD on chronic SD-induced cognitive deficiency in mice.

Effects of ketogenic diet on amyloid deposition and tau phosphorylation in the chronic sleep deprivation exposed mice

Amyloid deposition and neurofibrillary tangles are two typical pathological features of AD. Therefore, we first assessed the effect of KD on them to investigate the preventive effect of KD on SD-induced AD. As shown in **Figure 3A**, sleep deprivation for 21 days obviously induced the increase in the protein expression of BACE1, Aβ (**Figures 3C,D**, $P < 0.05$), and amyloid deposits (ThS Staining) (**Figure 3F**). No changes were noticeable in the expression of APP (**Figures 3A,B**). Meanwhile, SD elevated the levels of p-tau and the ratio of p-tau/tau in the hippocampus (**Figures 3A,E**, $P < 0.05$). Nevertheless, their levels were suppressed by KD supplementation.

Effects of ketogenic diet on hippocampal damage in the chronic sleep deprivation exposed mice

H&E staining showed altered neurons characterized by obviously neuronal loss and increased shrinking neurons with nuclei shrinkage in the SD group as compared with the Con group (**Figure 4A**). In the KD group, more clear nuclei and complete morphology of neurons were exhibited. Then we investigated the effect of KD on the repair ability of the hippocampus. The expression of neurotrophic factors BDNF and new neuron marker DCX was evaluated by western blot (**Figures 4B–D**). We found that both DCX and BDNF were declined in the SD group as compared with to Con group, which

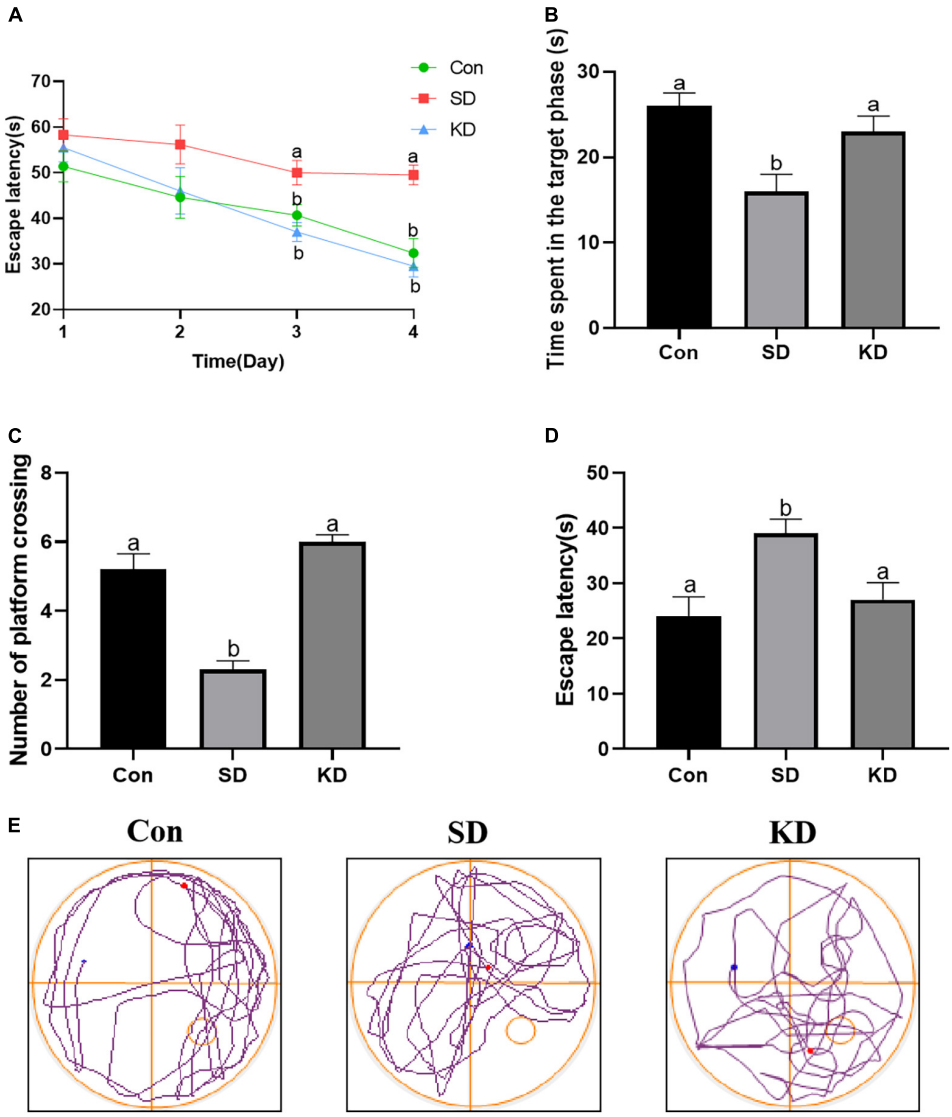


FIGURE 2
Effects of ketogenic diet (KD) on chronic sleep deprivation (SD)-induced cognitive deficiency. **(A)** The escape latency on training phase, **(B)** Time spent in the target quadrant, **(C)** Numbers of crossing platform, **(D)** escape latency, **(E)** Representative Morris water maze movement track from all groups. Data are presented as mean ± SEM (n = 10). Different letter indicates significantly different between each group ($p < 0.05$).

can be reversed by KD treatment (Figures 4B–D, $p < 0.05$). Simultaneously, the immunohistochemical results of DCX were comparable to the western blot results (Figure 4E).

Effects of ketogenic diet on hippocampal iron dyshomeostasis in the chronic sleep deprivation exposed mice

Iron accumulation is one of the main characters of ferroptosis. To evaluate the effect of KD on SD-induced iron dyshomeostasis in the hippocampus, total iron, iron accumulation and the expression of iron transport proteins were measured in the present study. We found that obvious iron deposition (Figure 5A) and increased total iron content (Figure 5B, $P < 0.05$) were presented in the SD group compared to the Con group. Meanwhile, significantly increased IRP1 (Figures 5C,D, $P < 0.05$), TfR1 (Figure 5F, $P < 0.01$) and DMT1 (Figure 5G, $P < 0.05$) accompanied with decreased FTH1 and FPN1 were shown in the SD group (Figures 5H,I, $P < 0.05$). No significant alteration was observed in IRP2 among three groups (Figure 5E). However, KD treatment reversed the expression of these iron proteins and alleviated iron deposition. These results revealed that KD supplementation could improve

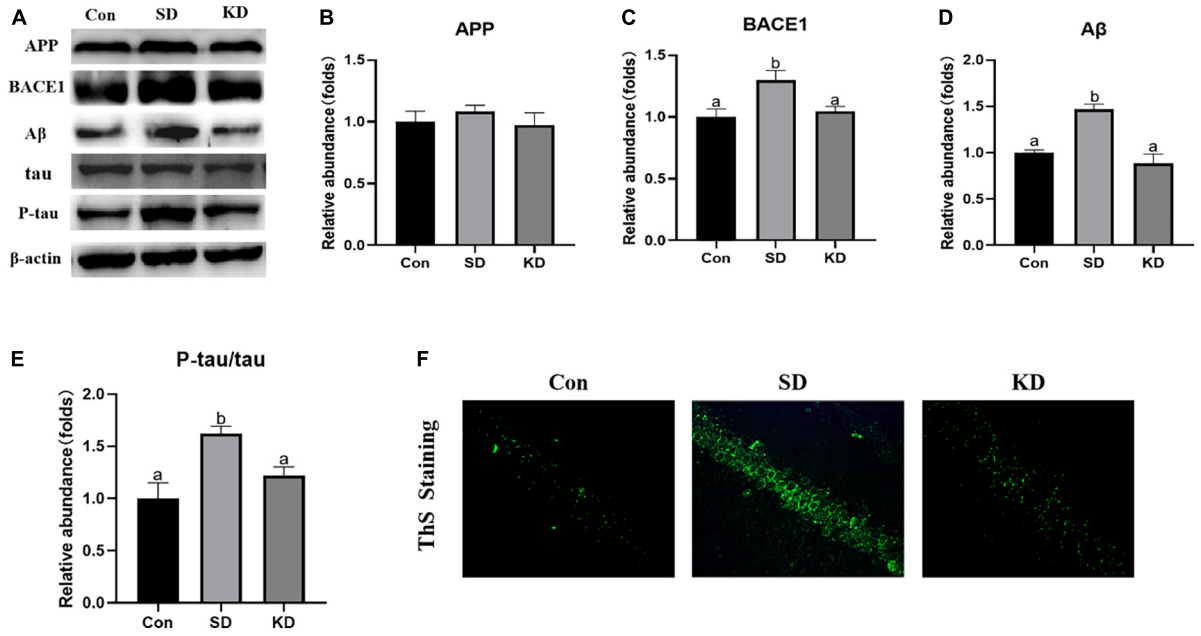


FIGURE 3
Effects of ketogenic diet (KD) on Aβ deposition and tau phosphorylation in the chronic sleep deprivation (SD) exposed mice. **(A)** Western-blots and **(B–E)** densitometry of APP, BACE1, Aβ, and p-tau/tau. Protein levels are normalized to β-actin which served as loading control and reproduced with Sham group. Values are indicated as the mean ± SEM (n = 7). Different letter indicates significantly different between each group ($p < 0.05$). **(F)** ThS staining in the hippocampus, scale bars = 50 μm (n = 3).

iron deposition by modulating the balance of iron transporter proteins.

Effects of ketogenic diet on lipid peroxidation in the hippocampus induced by chronic sleep deprivation

Lipid peroxides accumulation is another characteristic of ferroptosis. Therefore, we next to investigate the effect of KD on the lipid peroxidation in the hippocampus, we determined the levels of GSH, GPX4, xCT, FSP1, and MDA. In the SD group, an elevation in MDA was noted as compared to the Con group (**Figure 6A**, $P < 0.05$). On the contrary, as for antioxidant enzymes, significant decrease in GSH, GPX4, xCT, and FSP1 were presented (**Figures 6B–F**, $p < 0.05$). However, all above alterations can be reversed by KD intervention.

Effects of ketogenic diet on hippocampal Sirt1/Nrf2 signaling pathway in chronic sleep deprivation exposed mice

To evaluate the molecular mechanism by which KD exerted preventive effects on SD-induced AD, we examined the expression of Sirt1 and Nrf2 in the hippocampus by western blot (**Figure 7A**). Compared with the Con group, a down-regulation of Sirt1 (**Figure 7B**, $P < 0.05$), and p-Nrf2/Nrf2 (**Figure 7C**, $P < 0.01$) was exhibited in the SD groups. While KD treatment counteracted this SD-induced reduction. The results of Sirt1 immunohistochemistry in the hippocampus were consistent with the western blot of Sirt1 (**Figure 7D**).

Discussion

Our present study demonstrated that, following a 21-day sleep restriction, the wild-type 12-month-old C57BL/6 female mice developed AD accompanied by cognitive deficiency, Aβ deposition and tau hyper-phosphorylation in the hippocampus, but these phenomenons can be reversed when treated with KD, indicating the prophylactic effect of KD on SD induced AD. Further mechanism revealed that this protective effect of KD is related with the inhibition of ferroptosis and the promotion of neuron repair *via* Sirt1/Nrf2 pathway in SD mice. In our knowledge, this is the first report on the prevention of SD-induced AD just by adjusting the diet to a mild KD.

Growing evidence has demonstrated that SD induces cognitive impairment which also is the clinical characteristic of AD (Zhao et al., 2017; Wang X. et al., 2021). The parameters of the MWM test showed that the escape

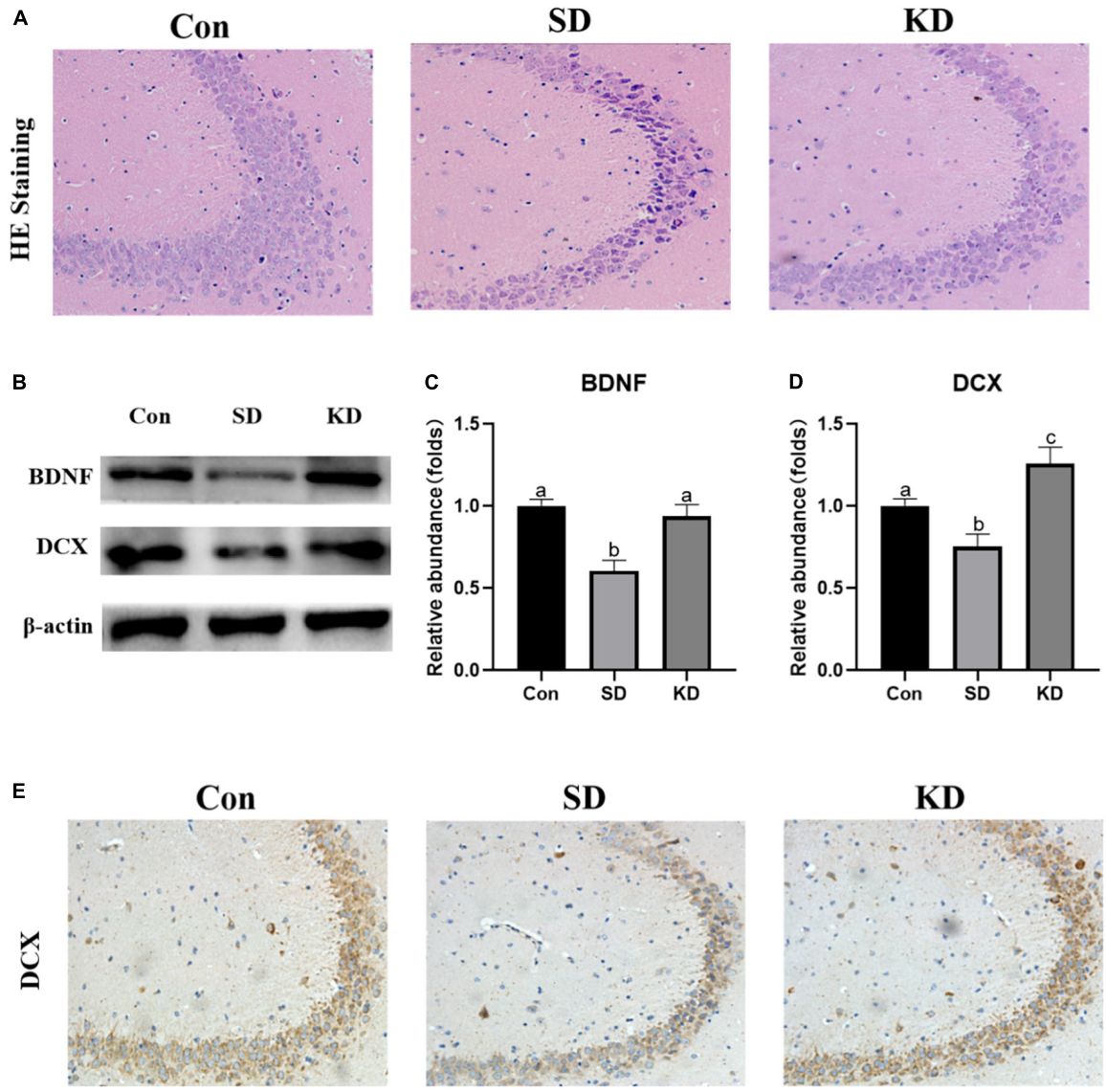


FIGURE 4
Effects of ketogenic diet (KD) on the hippocampal damage in the chronic sleep deprivation (SD) exposed mice. **(A)** H&E staining images of the hippocampus, scale bars = 50 μm (n = 3). **(B)** Western-blots and **(C,D)** densitometry of BDNF and DCX. Protein levels are normalized to β-actin which served as loading control and reproduced with Sham group. Values are indicated as the mean ± SEM (n = 7). Different letter indicates significantly different between each group ($p < 0.05$). **(E)** Immunohistochemistry analysis of DCX, scale bars = 50 μm (n = 3).

latency was markedly increased, whereas the numbers of platform crossing and the times spent in the target phase in probe trials were obviously reduced in SD group mice. While KD improved chronic SD-induced cognitive deficiency, which is consistent with our previous study that KD prevented the cognitive deficiency induced by 21 days SD in younger mice (7-week-old) (Wang X. et al., 2022).

It has been reported that the sleep-wake cycle regulates the levels of Aβ (Holth et al., 2019). Aβ increases significantly following acute sleep deprivation and decreases following sleep recovery (Shokri-Kojori et al., 2018). Studies in AD transgenic mice have demonstrated that Aβ plaque in the hippocampus after a chronic sleep restriction (Rothman et al., 2013). What's more, chronic SD promotes tau pathology spreading, which is also associated with the overproduction of Aβ (Holth et al., 2019). BACE1 is the major β-secretase and mediates the production of Aβ (Zhang and Song, 2013). In this work, the 21-day SD induced more Aβ deposition *via* up-regulating BACE1 and Aβ production, accompanied with increased p-tau in the hippocampus in the 12-month-old wild-type C57 BL/6 mice. The results were supported by

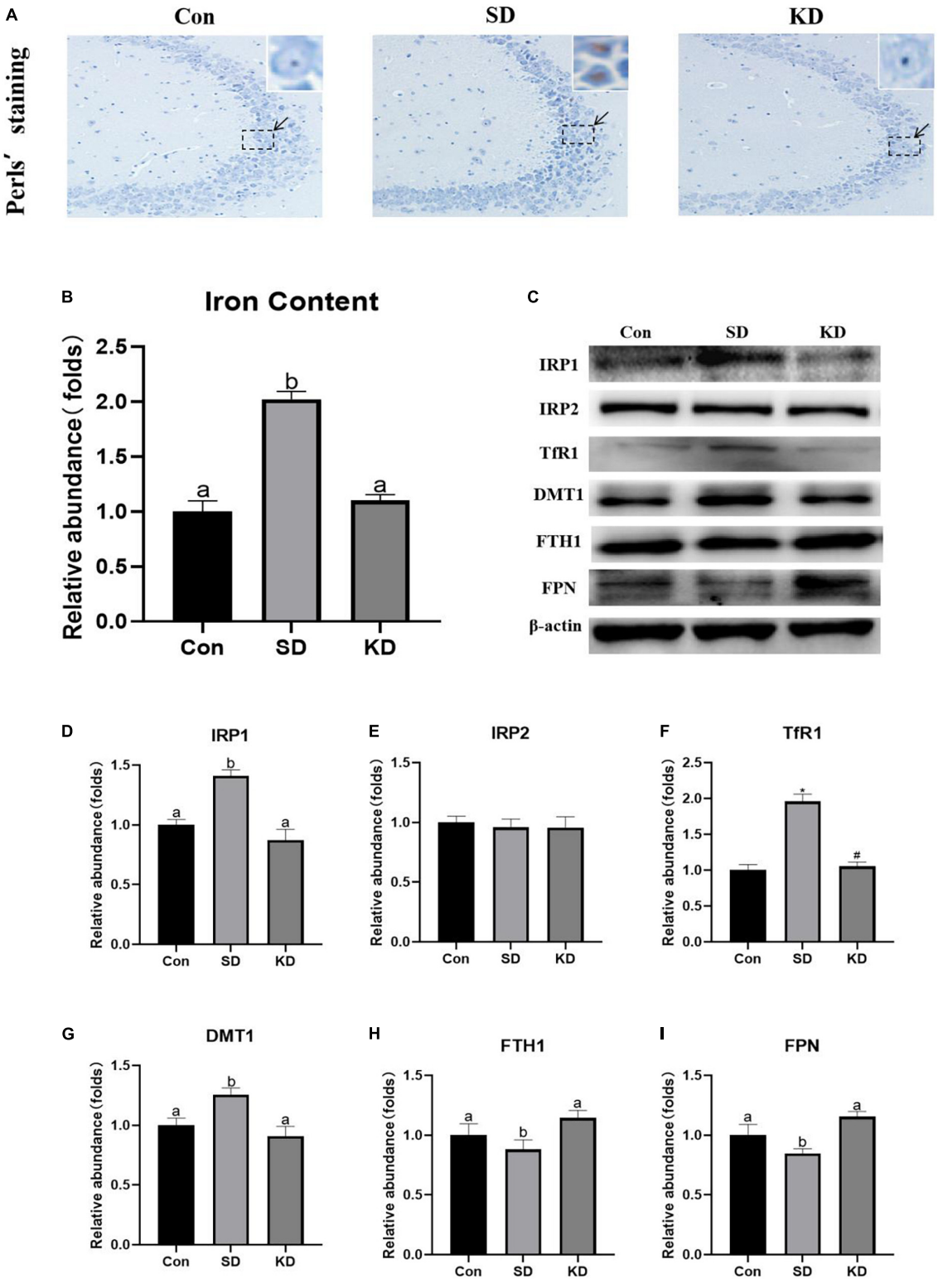


FIGURE 5
Effects of ketogenic diet (KD) on iron homeostasis dysregulation in hippocampus induced by chronic sleep deprivation (SD). **(A)** DAB staining in the hippocampus, scale bars = 50 μm (n = 3). **(B)** Total iron content in the brain and **(C)** Western-blots and **(D–I)** densitometry of IRP1, IRP2, TfR1, DMT1, FTH1, and FPN1. Protein levels are normalized to β-actin which served as loading control and reproduced with Sham group. Values are indicated as the mean ± SEM (n = 7). Different letter indicates significantly different between each group ($p < 0.05$); *$p < 0.01$, compared with Con; $^{\#}p < 0.01$, compared with SD.

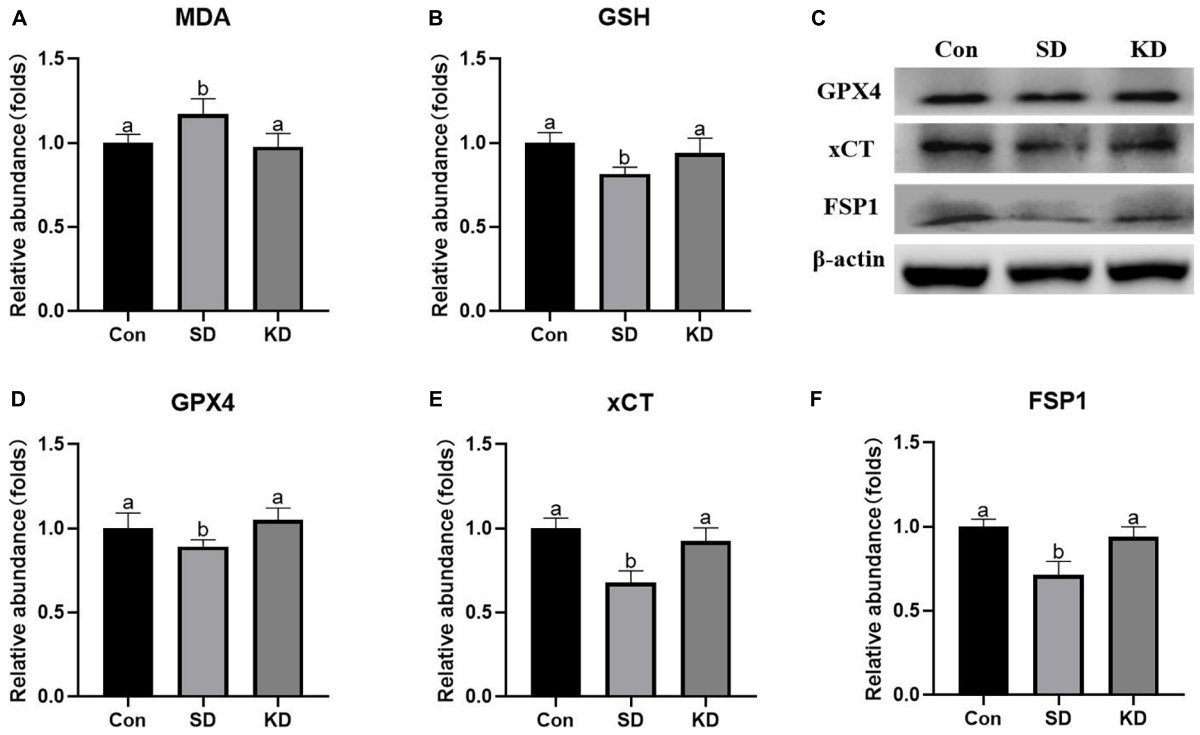


FIGURE 6
Effects of ketogenic diet (KD) on lipid peroxidation mediated oxidative stress in hippocampus induced by chronic sleep deprivation (SD). **(A)** Levels of malondialdehyde (MDA) and **(B)** glutathione (GSH). **(C)** Western-blots and **(D–F)** densitometry of GPX4, xCT, and FSP1. Protein levels are normalized to β-actin which served as loading control and reproduced with Sham group. Values are indicated as the mean ± SEM (n = 7). Different letter indicates significantly different between each group ($p < 0.05$).

the previous study demonstrating that Aβ plaque generated *via* up-regulating BACE1 in 9-month-old, adult and wild-type C57BL/6 mice after 60 days SD (Zhao et al., 2017). This phenomenon was reversed by KD treatment. In addition, it has been reported that sleep disturbances can impair Aβ clearance (Spinedi and Cardinali, 2019; Versele et al., 2020) found that ketone bodies (such as BHB) could promote Aβ clearance in a human *in vitro* blood-brain barrier model. Consideration of the elevated blood BHB in the present study (**Supplementary Figure 1**), it is reasonable to speculate that KD-induced the reduction of Aβ deposition and p-tau can be partially attributed to the promotion of Aβ clearance. These findings reinforced the causal link between SD and late-onset sporadic AD and proved a prophylactic intervention of moderate KD.

Growing evidence demonstrates that hippocampal damage is the main factor of SD-induced cognitive dysfunction (Zuo et al., 2020; Wang X. et al., 2021). Our present results showed that hippocampal neurons are characterized by pyknotic nuclei and vacuolated cytoplasm in SD group mice (**Figure 4A**). However, KD treatment alleviated this degeneration in SD-exposed mice. The effects were supported by our previous study showing that KD can attenuate chronic SD-induced hippocampal damage in young mice (7-weeks old) (Wang X. et al., 2022). DCX is a typical marker for adult neurogenesis and is close to cognitive abilities. The DCX diminished with aging, but is lower in AD patients than their peers (Moreno-Jiménez et al., 2019). BDNF is the most important neurotrophin involved in the growth, maintenance and survival of neurons, and a lower level of BDNF is related to a poorer cognition (Xue et al., 2022). Studies have shown that BDNF can induce DCX expression, and promote neurogenesis and reparation of neurons (Vitaliano et al., 2022; Xue et al., 2022). We found that 21-day SD induced a reduction in BDNF expression, as well as in DCX from the evidence of western blot and immunohistochemistry, which is in agreement with a previous study (Looti Bashiyan et al., 2021). These above indicate that SD suppressed neuronal repair and then enhanced hippocampal damage and cognitive deficiency. Notably, we found that KD upregulated the expression of BDNF and DCX, which suggested that the protective effect of KD on SD-induced hippocampal damage can be partly attributed to the promotion of repair in SD-exposed mice.

Emerging evidence indicated that SD-induced cognitive deficiency can be improved by Fer-1, a specific inhibitor of ferroptosis in mice, indicating ferroptosis involved in the

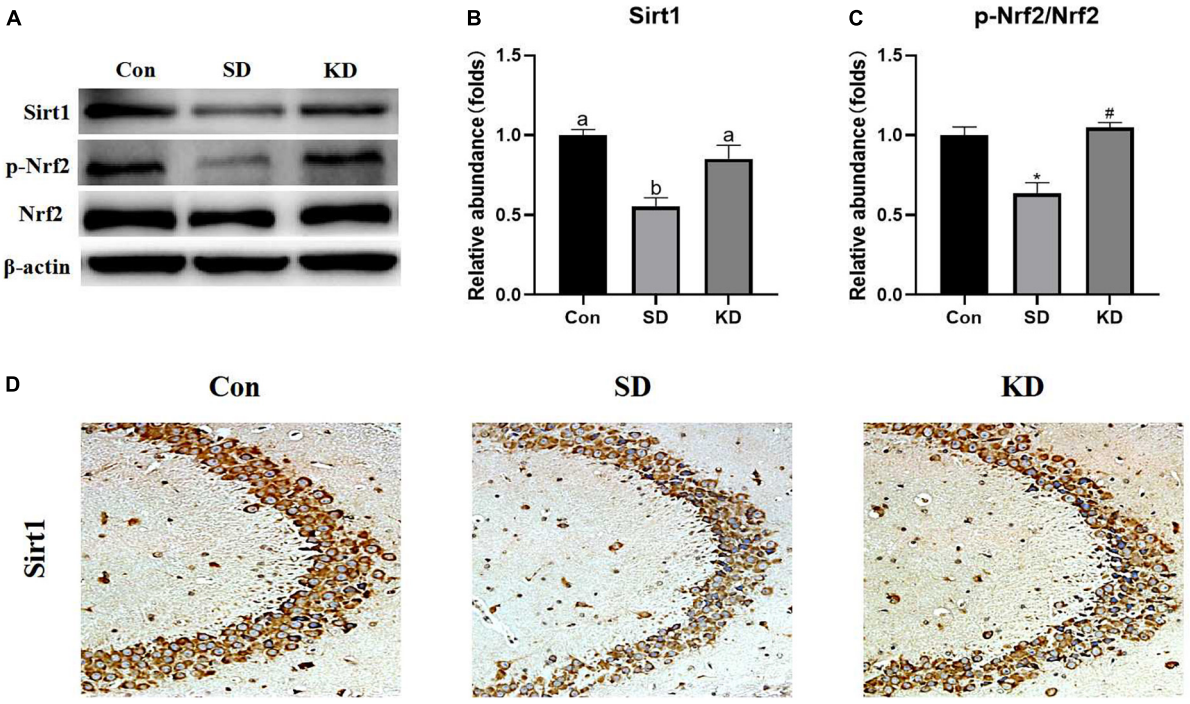


FIGURE 7
Effects of ketogenic diet (KD) on Sirt1/Nrf2 signaling pathway in hippocampus induced by chronic sleep deprivation (SD). **(A)** Western-blots and **(B,C)** densitometry of Sirt1 and p-Nrf2/Nrf2. Protein levels are normalized toβ-actin which served as loading control and reproduced with Sham group. Values are indicated as the mean ± SEM (n = 7). Different letter indicates significantly different between each group ($p < 0.05$); *$p < 0.01$, compared with Con; $^{\#}p < 0.01$, compared with SD. **(D)** Immunohistochemistry analysis of Sirt1, scale bars = 50 μm (n = 3).

mechanism of SD-induced cognitive deficiency (Wang X. et al., 2021, 2022). In addition, our previous study found that KD could improve chronic SD-induced hippocampal damage by inhibiting ferroptosis in younger mice (7-week-old). Accordingly, we investigated the effect of KD on ferroptosis-related indicators, including iron deposition, and lipid peroxides accumulation (Li et al., 2020). Improved Perls staining revealed that obviously intracellular iron accumulation in the hippocampus in the SD group mice in our present study. The results were supported by previous studies showing that an increase in intracellular iron in SD exposed mice (Wang X. et al., 2021, 2022). Similar to our previous study, the up-regulation of intracellular iron was reversed by KD treatment (Wang X. et al., 2022). What's more, it has been demonstrated that iron accumulation can worsen senile plaque deposition and promote tau phosphorylation (Wang F. et al., 2022). More β-secretase is activated in the presence of excessive iron and then accelerating the Aβ production and amyloid deposition (Ayton et al., 2020; Gleason and Bush, 2021). In addition, Ayton and colleagues conducted a research demonstrating an association between iron accumulation and steeper rate of cognitive decline in subjects displaying significant amyloid plaques and tau tangles (Spotorno et al., 2020). Hence, the prophylactic effects of KD on SD-induced AD can be partially attributed to its inhibition of iron overload in the hippocampus.

Iron is uptake by TFR1 (Fe^{3+}) or DMT1 (Fe^{2+}) and can be temporarily stored in the ferritin in the cytoplasm after being oxidated by FTH1 to prevent its toxicity or exported by the membrane protein FPN1 to maintain intracellular iron homeostasis. Neuron maintains intracellular iron homeostasis by modulating the expression of above iron transporter proteins *via* the IRP/IRE (iron-responsive element) system (Jing et al., 2021). IRPs regulate the iron homeostasis by binding to IRE of aforementioned iron transporter proteins' mRNAs dependent on cellular iron status (Vashisht et al., 2009). IRPs increase intracellular active iron *via* upregulating TfR1, downregulating FTH1 and FPN1. However, abnormal increase of IRPs can lead to the intracellular iron accumulation and thereby promoting ferroptosis. Animals with targeted deletions of IRP1 and IRP2 have demonstrated that IRP2 serves as the primary physiologic iron sensor, while IRP1 competes with IRP2 in regulating cellular iron homeostasis in response to ROS (Meyron-Holtz et al., 2004). We found SD elevated the expression of IRP1 rather than IRP2, thereby increasing TfR1 and DMT1, decreasing FTH1 and FPN1, leading to intracellular iron increase and

deposition in the hippocampus. Nevertheless, KD successfully suppressed the up-regulation of IRP1, subsequently modified SD-induced abnormality of iron transporters with diminished iron aggregation. These results revealed that KD prevented chronic SD-induced AD by inhibiting hippocampal iron dyshomeostasis.

Excess active iron promotes ROS production *via* the Fenton reaction, leading to lipid peroxides accumulation and thereby triggering ferroptosis. It has been well-believed that lipid peroxide MDA accumulation could present the development of ferroptosis (Yuan et al., 2020). GPX4/xCT system is the main lipid peroxide removal system by reducing lipid peroxides to non-toxic lipid alcohols (Yan et al., 2021). Inhibition of GPX4 in neurons causes cognitive deficiency and neurodegeneration (Hambright et al., 2017). FSP1 is in parallel with GPX4/xCT system to suppress ferroptosis by reducing lipid peroxidation by producing a reduced form of CoQ10, a well-known potent antioxidant (Doll et al., 2019). Consistent with the elevated iron, an increase in MDA levels, a reduction in GSH level, as well as in FSP1, xCT and GPX4 expression were presented in the hippocampus of SD group mice. These effects were suppressed by KD treatment, suggesting that KD can promote lipid peroxide removal by elevating GPX4/xCT system and FSP1 to prevent ferroptosis. These above results verified our previous speculation, KD could prevent the occurrence of chronic SD-induced AD partly by inhibiting ferroptosis.

Based on the results aforementioned, we further explored the signaling pathways by which KD was exerting its effect on neuroprotection. As a class III histone deacetylase, Sirt1 plays a role in neuroprotection and longevity (Wang C. et al., 2021). Up-regulation of the expression of Sirt1 can improve cognitive function, and prevent the onset of AD (Cao et al., 2018). Zuo et al. (2020) demonstrated that H_2S can prevent SD-induced hippocampal damage by up-regulating Sirt1 expression in the hippocampus, but this effect can be reversed by the Sirt1 inhibitor in rats. What's more, it has been reported that melatonin alleviates short-term SD-induced memory loss in mice by suppressing hippocampal ferroptosis (Wang X. et al., 2021) and increasing hippocampal Sirt1 (Hinojosa-Godinez et al., 2019). These results indicate that Sirt1 may participate in the regulation of ferroptosis but less has been reported about the mechanism of Sirt1 on ferroptosis. Nrf2, a key regulator of the cellular antioxidant response, is a downstream target of Sirt1 and has been linked to neurodegenerative disease treatment and ferroptosis regulation. Activation of Nrf2 could against chronic SD-induced memory impairment in rats (Tang et al., 2020). Moreover, a recent study using the EX527 (an inhibitor of SIRT1) proved the anti-ferroptosis effect of Sirt1/Nrf2 pathway in a sepsis mice model (Qiongyue et al., 2022). A recent study demonstrated that Nrf2 induction could prevent ferroptosis in Friedreich's Ataxia (La Rosa et al., 2021). Currently, it has been proved that Nrf2 not only contributes to mitigation of lipid peroxidation by activating GPX4/xCT axis (Yuan et al., 2021) but also modulates celluar iron homeostasis by increasing the expression of iron transport proteins FTH1 and FPN1 (Kerins and Ooi, 2018). In agreement with our previous study (Wang X. et al., 2022), we found that KD up-regulated the expression of Sirt1 and Nrf2 in SD exposed mice. In addition, Sirt1-Nrf2 is also associated with the neurogenesis by up-regulate the expression of BDNF and DCX (Namgyal et al., 2020; Suwannakot et al., 2021). These above results prompted us to conclude that KD prevents chronic SD-induced AD *via* Sirt1/Nrf2 signaling pathway.

In summary, our present study first demonstrated the prophylactic effect of KD on SD-induced AD in wild-type mice models *via* activating the Sirt1-Nrf2 pathway in the hippocampus. Our findings may provide new perspectives on the dietary treatment of AD.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

Our experimental protocol was approved by the Research and Ethics Committee of Liaocheng University.

Author contributions

MW and ZW: conception and design of research. YY, XW, and AX: performed the experiments. YY, XW, and MW: analyzed the data. MW, YY, JH, and ZW: interpretation of the results of experiments. YY and XW: prepared the figures. YY and MW: drafted the manuscript. All authors read and approved the final manuscript.

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Center for Nanomedicine and Drug Delivery Systems, and Shandong Province Engineering Laboratory of Anti-viral Drugs.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2022.998292/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Effects of ketogenic diet (KD) on blood β-hydroxybutyrate (BHB) **(A)** and blood glucose **(B)** in sleep deprivation (SD) exposed mice (mean ± SEM, $n = 10$). Different letter indicates significantly different between each group ($p < 0.05$). $^{*}p < 0.01$, compared with Con; $^{\#}p < 0.01$, compared with SD.

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EDITED BY
Yashi Mi,
University of Arizona, United States

REVIEWED BY
Farzad Salehpour,
University of Texas at Austin, United States
Rong Fan,
University of Massachusetts Amherst, United States

*CORRESPONDENCE
Saadia Zahid
saadia.zahid@asab.nust.edu.pk

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A quantitative meta-analysis of vitamin C in the pathophysiology of Alzheimer's disease

Maryam Hamid, Sumaiya Mansoor, Sanila Amber and Saadia Zahid*

Neurobiology Research Laboratory, Department of Healthcare Biotechnology, Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, Islamabad, Pakistan

Purpose: Alzheimer's disease (AD) is a multifaceted neurodegenerative disorder with many complex pathways feeding into its pathogenesis and progression. Vitamin C, an essential dietary antioxidant, is vital for proper neurological development and maintenance. This meta-analysis and systematic review attempted to define the relationship between vitamin C plasma levels and AD while highlighting the importance and involvement of vitamin C in the pathogenesis of AD.

Materials and methods: PRISMA guidelines were used to obtain studies quantifying the plasma levels of vitamin C in AD and control subjects. The literature was searched in the online databases PubMed, Google Scholar, and Web of Science. A total of 12 studies were included ($n = 1{,}100$) and analyzed using Comprehensive Meta-Analysis 3.0.

Results: The results show that there is a significant decrease in the plasma vitamin C levels of AD patients as compared to healthy controls (pooled SMD with random-effect model: −1.164, with 95%CI: −1.720 to −0.608, $Z = -4.102$, $p = 0.00$) with significant heterogeneity ($I^2 = 93.218$). The sensitivity analysis showed directionally similar results. Egger's regression test ($p = 0.11$) and visual inspection of the funnel plot showed no publication bias.

Conclusion: Based on these studies, it can be deduced that the deficiency of vitamin C is involved in disease progression and supplementation is a plausible preventive and treatment strategy. However, clinical studies are warranted to elucidate its exact mechanistic role in AD pathophysiology and prevention.

KEYWORDS

vitamin C, Alzheimer's disease, ascorbic acid, amyloid-β, oxidative stress

Abbreviations: CNS, Central nervous system; BDNF, brain-derived neurotrophic factor; CSF, cerebrospinal fluid; AD, Alzheimer's disease; DβH, dopamine β-hydroxylase; Aβ, amyloid-β; APP, amyloid precursor protein; AA, ascorbic acid; AGEs, advanced glycation end products; RAGE, receptors for AGE; BBB, blood brain barrier; LRP-1, low-density lipoprotein receptor-1; GSH, glutathione; SSRIs, selective serotonin reuptake inhibitors; 4-HNE, 4-hydroxynonenal; MCI, mild cognitive impairment; ROS, reactive oxygen species; CDR, Clinical Dementia Rating; HPA, hypothalamic–pituitary–adrenal; MMSE, Mini-Mental State Examination; CI, confidence interval; IQR, inter-quartile range; SMD, standard mean deviation.

Introduction

Alzheimer's disease (AD) is an irreversible neurodegenerative disorder associated with memory loss, functional impairment, and a host of psychiatric symptoms such as apathy, agitation, psychosis, and depression (Deardorff and Grossberg, 2019). AD contributes heavily to the global disease burden and is the largest cause of mortality in adults ≥ 65 years of age with over 50°million people living with AD worldwide in 2020 (Zhang et al., 2021). Almost 90% of AD cases are linked to sporadic occurrence, and the remaining cases of AD are inherited. It is a complex multifactorial disease attributed to factors including senescence, amyloid-β (Aβ) plaque formation, oxidative stress, and neuroinflammation (Bekris et al., 2010).

Vitamin C or ascorbic acid (AA; $C_6H_8O_6$) is a water-soluble organic compound (Padayatty and Levine, 2016). Unlike most mammals, humans and primates lack the essential enzyme L-gulono-γ-lactone oxidase required for the final stage of vitamin C biosynthesis because the mutations in the L-gulono-γ-lactone oxidase (GLO) gene, therefore, are unable to synthesize vitamin C within the body (Lachapelle and Drouin, 2011). Plant sources such as tomatoes, strawberries, citrus fruits, green, and red bell pepper along with other green leafy vegetables are high in vitamin C content (up to 5,000°mg/100°g) (Chambial et al., 2013), while the recommended dietary allowance is 75 mg/day and 90 mg/day for females and males, respectively (Padayatty and Levine, 2016).

Vitamin C has an imperative involvement in the prevention and treatment of a host of diseases and ailments like the common cold, tissue healing, cancer, diabetes, infertility, etc., (Padayatty et al., 2006; Jagetia et al., 2007; Hemilä and Chalker, 2013; Sadeghzadeh et al., 2019; Plevin and Galletly, 2020). In the human brain, the highest concentration of vitamin C is found in the cerebral cortex, hippocampus, and amygdala, while CSF concentrations are higher as compared to plasma (Travica et al., 2017). An increase in the generation of free radicals in various neurodegenerative disorders may indicate that vitamin C could have a prospective role in the therapeutic of such disorders including AD (Kocot et al., 2017). In the current work, we conducted a comprehensive meta-analysis along with systemic review of existing studies examining vitamin C in AD.

Vitamin C and brain

The intense physiochemical activity of the brain requires a high metabolic rate and consumption of copious amounts of oxygen and glucose. The high metabolic rate and enzymatic processes lead to the generation and accumulation of free radicals, most importantly reactive oxygen species (ROS) (Watts et al., 2018; Jelinek et al., 2021). Maintaining the delicate oxidative balance depends on antioxidants in the brain of which vitamin C has the highest concentration (Kaźmierczak-Barańska et al., 2020). The strong reducing property of AA can be attributed to the hydroxyl groups present in the lactone ring that serve to either donate electrons or protons. The hydroxyl groups react with hydroxyl radicals, peroxide radicals, hydrogen peroxide, etc.; as a result, AA is oxidized to dehydroascorbate, a bicyclic hemiketal which may be reduced back to AA by the enzyme dehydroascorbate reductase (Englard and Seifter, 1986; Njus et al., 2020).

Vitamin C is essential for neurodevelopment, neurotransmitter regulation, and glutamate-mediated neurotransmission and to maintain oxidative balance. It is a vital nutrient for brain function particularly due to its antioxidant properties. It regulates the biosynthesis of catecholamine's dopamine and norepinephrine and mediates the dopamine neuron differentiation through TET1- and JMJD3-dependent mechanism in the fetal midbrain indicating an epigenetic role (He et al., 2015). It is subsequently involved in the conversion of dopamine to norepinephrine by acting as an electron donor for dopamine β-hydroxylase (DβH) and prevents dopamine-mediated superoxide formation (May et al., 2013). Vitamin C also promotes DNA demethylation of pro-myelinating genes that result in the regulation of Schwann cell myelination (Huff et al., 2021).

The major excitatory neurotransmitter responsible for relaying messages in the CNS is glutamate. It binds to metabotropic NMDA receptors, activating them, and inducing calcium influx into neuronal cells. Dysregulation of glutamate concentration leads to a disturbance in neuronal calcium homeostasis that leads to neuronal damage and cell death in several neurological disorders (Lewerenz and Maher, 2015; Shah et al., 2015). Persistent elevation in intracellular calcium levels induces apoptosis by either activating apoptotic enzymes such as calpain, inhibiting normal cellular protein synthesis due to depletion of endoplasmic reticulum calcium levels, or by destroying the mitochondrial membrane potential (Demaurex and Distelhorst, 2003). Vitamin C is effective against glutamate-mediated cytotoxicity by reversible inhibition of NMDA activity and reduces the glutamate-induced phosphorylation of AMPK (Majewska et al., 1990; Shah et al., 2015). It is evident that vitamin C ameliorates the glutamate-induced neurotoxicity *via* the ascorbate–glutamate heteroexchange that keeps the excitatory overload of glutamate in check. Glutamate uptake results in the release of vitamin C in the extracellular fluid that later removes the glutamate-generated ROS. However, deficiency of ascorbate dysregulates glutamate clearance leading to its accumulation and ultimately excitotoxicity of neurons that contributes to cognitive impairment (Mi et al., 2018).

Vitamin C and Alzheimer's disease

The formation and deposition of neurofibrillary tangles and Aβ plaques are characteristics of an AD patient's brain. These

depositions are highly insoluble and are composed of densely packed filaments (Bloom, 2014). The proteolytic cleavage of amyloid precursor protein (APP) directs the formation of Aβ plaques, while the hyperphosphorylation of tau protein tends to generate neurofibrillary tangles. These cellular events can be affiliated with oxidative stress, although it is still ambiguous whether oxidative stress acts as an early or late event in the pathogenesis of AD (Apelt et al., 2004). The facilitation of amyloidogenic pathway of APP processing by oxidative stress leads to enhanced production and deposition of Aβ. Followed by oxidative stress, the increased activity of β-secretase is conceivably facilitated through phosphorylation of p42/44 MAPK (Muche et al., 2017).

Ascorbic acid (AA) plays a major role in AD pathophysiology due to its antioxidant and neuroprotective property particularly against ROS (Covarrubias-Pinto et al., 2015; **Figure 1**). It is released by the glial cells into the synaptic clefts of neurons in CNS (Röhl et al., 2010) and is responsible for encouraging the regeneration of antioxidant enzymes including glutathione (GSH) and catalase (Dringen et al., 1999). Several studies showed the potential use of AA as a therapeutic agent to stall the progression of AD. A study in APP/PSEN 1 transgenic mice served as evidence for nootropic abilities of AA. Mice injected parenterally with AA showed enhanced cognitive abilities despite no altering of oxidative stress and plaque deposition (Harrison et al., 2009). Another study suggested that the oral intake of AA reduced the Aβ fibrils-mediated oxidative stress (Rosales-Corral et al., 2003). Since the Aβ plaques contain metal-binding sites for zinc, iron, and copper (Bush et al., 2003), these bound metals can stabilize the Aβ plaques and increase the rate of deposition and cytotoxicity (Jomova et al., 2010). The metal redox activity of active iron and copper leads to the production of peroxynitrites, hydroxyl ions, carbonyls, and advanced glycation end products (AGEs). The hydroxyl ions generated by metal redox reactions increase the lipid peroxidation, DNA, and protein oxidation. The production and interaction of AGE with its cell membrane-bound receptors further lead to proinflammatory responses (Yao et al., 2004). However, the soluble forms of receptor for AGEs (RAGE) inhibit the binding of ligands to the cell membrane-bound RAGE, thus inhibiting the neurotoxic or proinflammatory responses (Lue et al., 2009).

The post-mitotic nature of neurons and their higher levels of oxygen requirement make the brain highly vulnerable to oxidative stress (Mecocci et al., 2002). Likewise, oxidative stress may activate microglia and astrocytes at the site of higher oxidative stress (García-Krauss et al., 2016) where the interaction of glial cells with neurons produces inflammatory cytokines, nitric oxide, and chemokines which drives the inflammation in the nervous system (Sultana et al., 2011). A few studies have emphasized the role of AA as a pro-oxidant due to the interaction of toxic forms of Aβ with AA resulting in the formation of −OH radicals (Valko et al., 2005). However, a recent finding demonstrates ascorbic acid-mediated destabilization of preformed amyloid fibrils and protection against amyloid-induced cytotoxicity (Alam et al., 2017).

Oxidative stress as a major epigenetic factor induces mitochondrial dysfunction that extensively contributes in AD as alterations in the energy metabolic pathways are witnessed (Perez Ortiz and Swerdlow, 2019). Salient observations include glucose hypometabolism due to reduced glycolysis and compensation of energy deficit by using fats and amino acids as an alternate energy source (Toledo et al., 2017). Several glycolytic and mitochondrial proteins exhibit altered levels due to oxidative modifications that perturbed the glucose metabolism in AD. These modified proteins also contribute toward dysregulation of various other biochemical and metabolic pathways altered in AD (Butterfield and Boyd-Kimball, 2018).

Similarly, ROS-mediated mitochondrial dysfunction may further damage mitochondrial membrane permeability and respiratory chain and induce mitochondrial DNA mutations (Guo et al., 2013). AA prevents mitochondrial membrane depolarization and ultimately oxidative injury by accessing the mitochondria in its oxidized form through glucose transporter 1 (Glut1) (Kc et al., 2005). Nonetheless, the complex mechanism and regulation of AA uptake by the organelles are attributed to its diversified antioxidant property that may alleviate the mitochondrial dysfunction by quenching the mitochondrial ROS (Fiorani et al., 2021). Moreover, various genes associated with cellular mechanisms of oxidative damage repair may serve as important candidates for AD. For instance, silent information regulator type-1 (SIRT1) which is involved in various cellular processes including cell survival, control of apoptosis, and modulation of ROS levels showed a positive association with the AD and is linked to pathways that may impair oxidative stress (Camporez et al., 2021). The upregulation of SIRT1 antagonizes AD through improvement in the oxidative balance (Xu et al., 2020). Similarly, the regulation of uncoupling protein 2 (UCP2) by stanniocalcin-1 (STC-1) alleviates oxidative stress and Aβ level as observed in the N2a/APP695s we cells treated with oleanolic acid (Guo et al., 2020). In addition, recent evidence on the involvement of NF-E2-related factor 2 (Nrf2), a stress-responsive transcription factor, ameliorates cognitive impairment by suppressing oxidative stress, and neuroinflammation observed in *App* knock-in AD mouse model (Uruno et al., 2020). Likewise, microRNA-23b attenuates tau pathology and inhibits oxidative stress by targeting N-acetylglucosaminyltransferase II (GnT-II) in AD (Pan et al., 2021). It is also noteworthy that NADPH oxidase 4 (NOX4) mediates the ferroptosis of astrocytes by oxidative stress-induced lipid peroxidation through the impairment of mitochondrial metabolism in AD (Park et al., 2021). Collectively, these modulating factors are crucial candidates

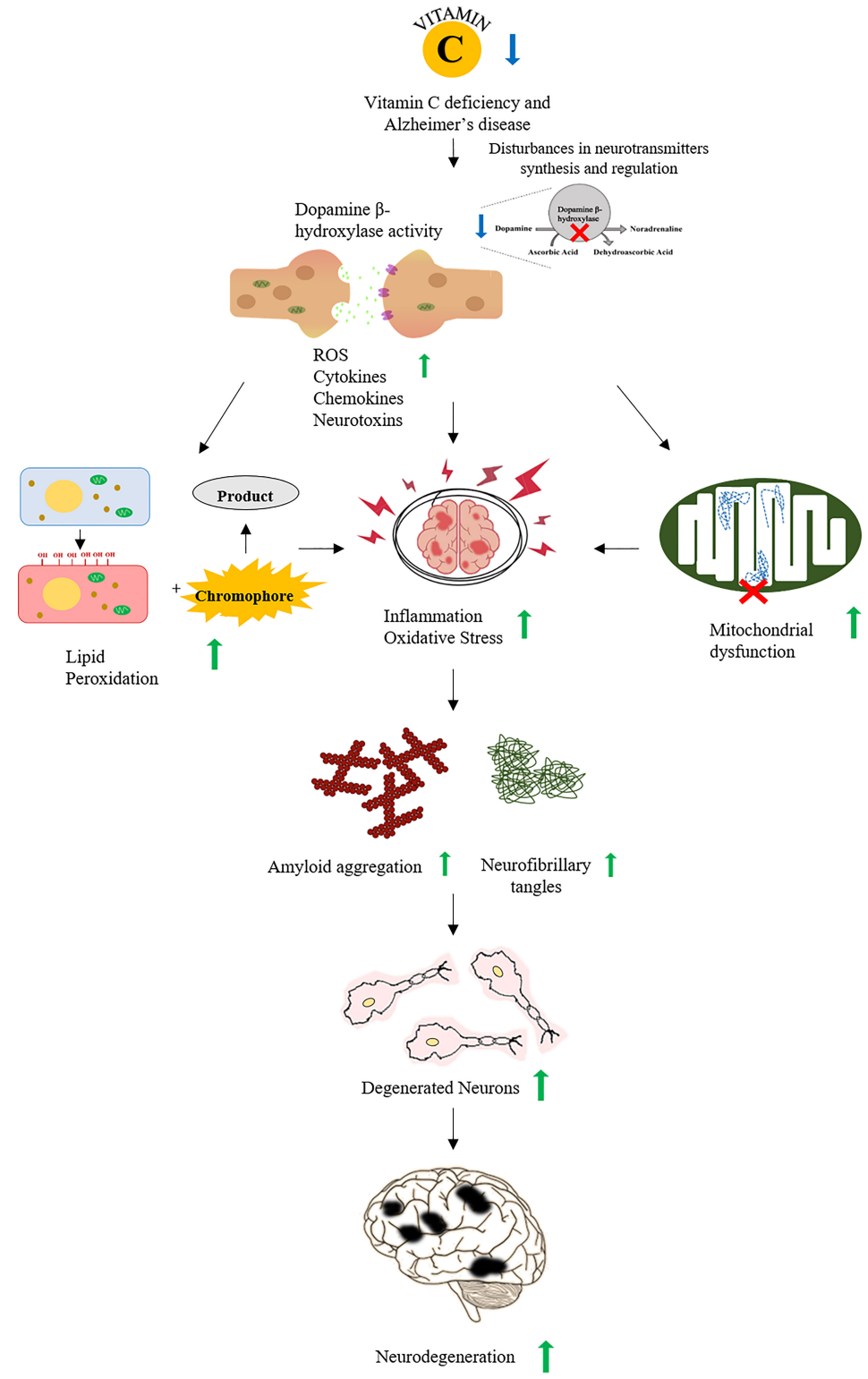

FIGURE 1
A schematic representation of association between vitamin C and Alzheimer's disease (AD). Vitamin C deficiency interrupts the synthesis and regulation of neurotransmitters increasing ROS, cytokines, and neurotoxins that lead to increased oxidative stress and inflammation. Oxidative stress also increases the accumulation of Aβ plaques and the formation of neurofibrillary tangles. All of these events promote the neurodegeneration observed in AD.

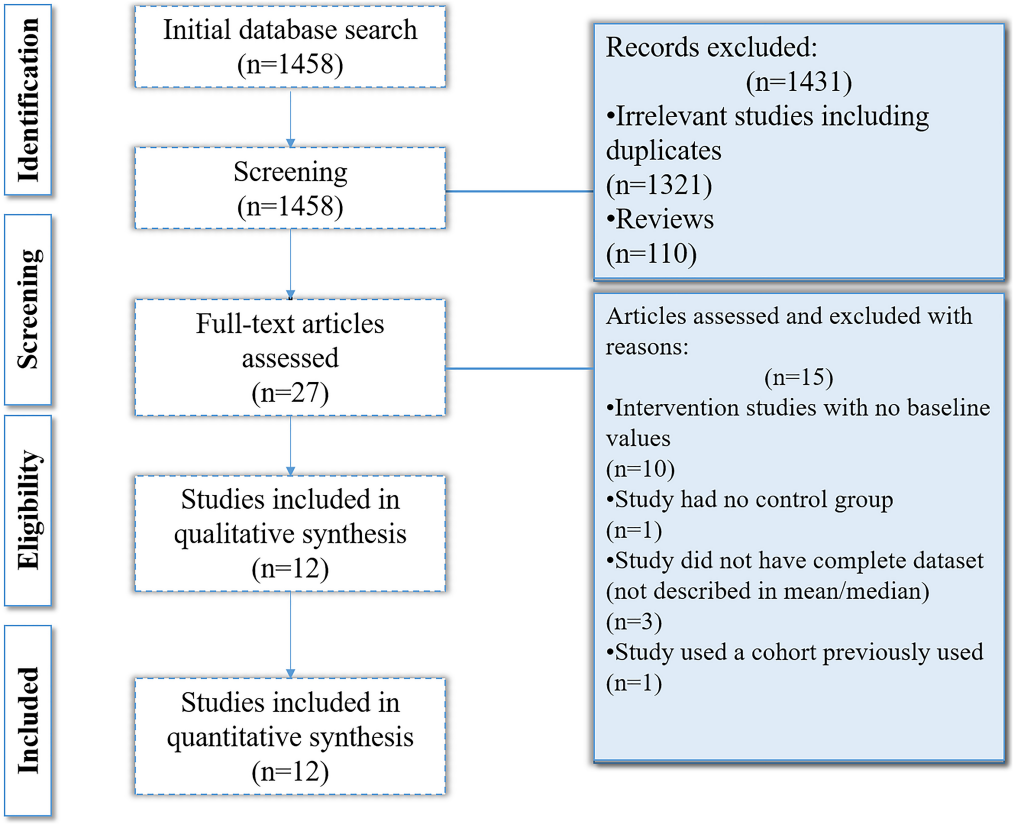

FIGURE 2
Flowchart of the selection process.

for AD and highlight a strong association of oxidative stress with AD pathology.

Vitamin C is vital for antioxidative mechanism and is extremely important for homeostasis and the proper functioning of the CNS. Besides general free radical trapping, the suppression of proinflammatory genes, neuroinflammation, and Aβ fibrillogenesis is also emerging roles of vitamin C (Monacelli et al., 2017; Kaźmierczak-Barańska et al., 2020). Although the concentration of vitamin C as ascorbate is higher in the CNS, however, the plasma levels are indicative of various key processes associated with CNS. For instance, the age-associated differences in plasma and brain vitamin C are linked to age-associated cognitive differences, alongside compromised vitamin C brain regulation (Travica et al., 2020). Interestingly, the high plasma vitamin C status also elevates overall mood in young adult males (Pullar et al., 2018). Taking into consideration the vital role of vitamin C, this meta-analysis was conducted to establish whether there was a statistically significant difference in the plasma levels of vitamin C/AA in AD patients and healthy controls. This information can be useful to determine if vitamin C supplementation interventions are a valuable avenue to look into as it may corroborate the role of AA in AD pathology.

Materials and methods

Search strategies and selection of studies

The purpose of this study was to describe the importance of vitamin C with relevance to the neurological disorder AD and elucidate the association of AD with vitamin C levels. The studies included were obtained according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2009).

The literature was searched in the online databases PubMed, Google Scholar, and Web of Science using the keywords "Alzheimer's disease (AD)," "vitamin C," and "ascorbic acid (AA)." The timeline of the searched literature was set for the last 21 years ranging from 2000 to January 2022.

Inclusion criteria

Those studies were identified that contained "Alzheimer's disease" (AD), "vitamin C," and "ascorbic acid" (AA) in the

title, abstract, or key descriptors. The selection criteria included: human subjects, non-randomized observational studies (case-control and cross-sectional), neuropsychological testing of subjects to determine AD, mean or median plasma levels of vitamin C as one of the main variables of interest, and the study should compare two groups of interest (AD patients vs. controls). Certain articles contained information on several antioxidants and micronutrients; in such cases, only the information about mean vitamin C levels was retrieved. The English language was used as a restriction during data collection.

Exclusion criteria

Studies were excluded if they were unrelated to plasma vitamin C levels or AD and if they were animal studies or reviews. Studies that reported plasma vitamin C levels without comparison to a healthy control group were excluded. Studies that did not report the median or mean values were also excluded. For those studies which used the same cohort to publish several articles, only the article having the maximum number of participants was included. In the end, six studies were included according to the inclusion and exclusion criteria.

Statistical analysis

Comprehensive Meta-Analysis Version 3.0 was used to perform the statistical analysis (Borenstein et al., 2013). To account for heterogeneity, the random-effects model was used. Standardized mean differences (SMDs) were calculated for differences in means between AD patients and healthy controls. Forest plots were generated, and 95%CI (confidence interval) was also included. The I^2 value was used as a measure of heterogeneity. For $I^2 \geq 75\%$, which suggests high heterogeneity, meta-regression was performed to identify the sources contributing to heterogeneity. Publication bias was determined using Egger's regression tests (Egger et al., 1997). Sensitivity analysis was also conducted using the one-study removal method in the Comprehensive Meta-Analysis Version 3.0. The Cochrane Collaboration's tool for Risk Of Bias in Non-randomized Studies–of Exposure (ROBINS-E) was used for each observational study included in the analysis (Bero et al., 2018; Robins-E Development Group, Higgins et al., 2022). The evaluation includes confounding factors, selection of participants, misclassification of variables, bias due to missing data, and reverse causation. These were evaluated as either high or low risk of bias; if the studies were unclear regarding the data, risk of bias was indicated as unclear.

Results

Selection of studies

The online databases search yielded 1,458 results out of which 1,331 were removed on the basis of irrelevancy and not meeting inclusion criteria. On the basis of title and abstract relevancy, 27 studies were selected which were further scrutinized. Fifteen of the 27 studies were removed as they either had no control groups, were intervention studies that lacked baseline values, did not have a complete data set, or the cohort was used in a previously published article. Ultimately, 12 studies were selected and included in the analysis (**Figure 2**).

Data collection was performed in a hierarchical manner. The articles were downloaded, reviewed, and filtered based on the inclusion and exclusion criteria. All such studies that were reporting the deficiency of plasma vitamin C levels in AD are summarized in **Table 1**.

Meta-analysis of vitamin C concentrations in plasma

The analyzed data from the selected studies showed that plasma vitamin C levels are significantly reduced in the AD patients as compared to the healthy controls (pooled SMD with random-effect model: -1.164, with 95%CI: -1.720 to -0.0608, $Z = -4.102$, $p = 0.00$). The heterogeneity in-between studies were significant ($Q = 162.191$, $df = 11$, $p = 0.00$, $I^2 = 93.218$). The p-value indicates that the dispersion is not due to random error, but in fact, it is due to real differences in the study effects (**Figure 3**). Sensitivity analysis showed that no single study greatly impacted the results of the analysis and there were no outliers.

Publication bias

Egger's regression test ($p = 0.11$) suggests no publication bias in the meta-analysis since $p > 0.05$ indicates no publication bias. Visual inspection of the funnel plot symmetry also corroborates this result (**Figure 4**).

Risk of bias

The risk of bias and the quality of the observational non-randomized studies were analyzed in the meta-analysis through Cochrane's Risk Of Bias tool in Non-randomized Studies–of Exposure (ROBINS-E). Overall, the risk of bias in

TABLE 1 Alzheimer's disease (AD) to control ratio for vitamin C.

Studies	Type	Disease	Number of participants		Mean age (years)		Vit C conc. in AD (μmol/L)	Vit C conc. in cont. (μmol/L)	Implications
			AD	Cont.	AD	Cont.			
Schippling et al. (2000)	Case-control study	AD	29	29	71.7 ± 10.1	55.1 ± 18.8	35.0 ± 18.6	48.8 ± 18.5	An increase in lipoprotein oxidizability and lower levels of AA in AD implicate oxidation in the pathogenesis of AD.
McGrath et al. (2001)	Case-control study	AD	29	46	74 ± 7.75	73 ± 5.75	9.9 ± 6.9	24.2 ± 8.6	The serum antioxidant levels of AD patients were considerably lower as compared to the control group. ($p < 0.05$)
Polidori and Mecocci (2002)	Case-control study	AD	35	40	85.9 ± 5.5	85.5 ± 4.4	18.1 ± 5.8	35.9 ± 6.3	AD patients exhibit statistically significant ($p < 0.001$) poorer levels of AA when compared to controls.
Mecocci et al. (2002)	Case-control study	AD	40	39	75.9 ± 5.4	74.8 ± 6.3	32.28 ± 10.8	56.74 ± 15.9	Biomarkers of oxidative damage are increased, and antioxidant levels are decreased in AD.
Rinaldi et al. (2003)	Case-control study	AD	63	56	76.8 ± 6.9	75.8 ± 7.2	25.9 ± 8.9	52.4 ± 16.5	Plasma levels and activity of AA were depleted in AD patients.
Quinn et al. (2003)	Case-control study	AD	10	10	65 ± 7	66 ± 6	58.1 ± 42	86.4 ± 39	The trend of lowered plasma vitamin C in AD patients was observed.
Glasø et al. (2004)	Case-control study	AD	20	18	75–85	75–85	46.2 ± 25	77.7 ± 28	Significant difference was observed between the AA levels of AD and control subjects.
Polidori et al. (2004)	Cross-sectional study	AD	63	55	76.8 ± 6.9	75.7 ± 7.3	25.9 ± 8.9	52.4 ± 16.4	Regardless of the nature of dementia, i.e., vascular or neurodegenerative, it is associated with a drastic decrease in the blood antioxidant level.
Giavarotti et al. (2013)	Case-control study	AD	23	42	82	82	52 ± 23.9	48 ± 19.4	Antioxidant levels are disturbed in AD along with an activation of the inflammatory pathways.
Mangialasche et al. (2015)	Cross-sectional study	AD	28	21	74.9 ± 6.9	79.1 ± 7.7	23.6 ± 3.5	25.9 ± 2.8	Mitochondrial dysfunction is observed in AD patients, and this dysfunction is correlated with plasma antioxidant levels.
Ulstein and Bøhmer (2017)	Cross-sectional study	AD	48	63	71 ± 8.2	72.7 ± 6.3	62.8 ± 28.9	62.8 ± 17.8	No significant difference was observed between the groups.
Lanyau-Domínguez et al. (2021)	Cross-sectional study	AD	43	250	78	82.8	61.6 ± 51.9	79.1 ± 64	Vitamin deficiency was observed in AD compared to controls.

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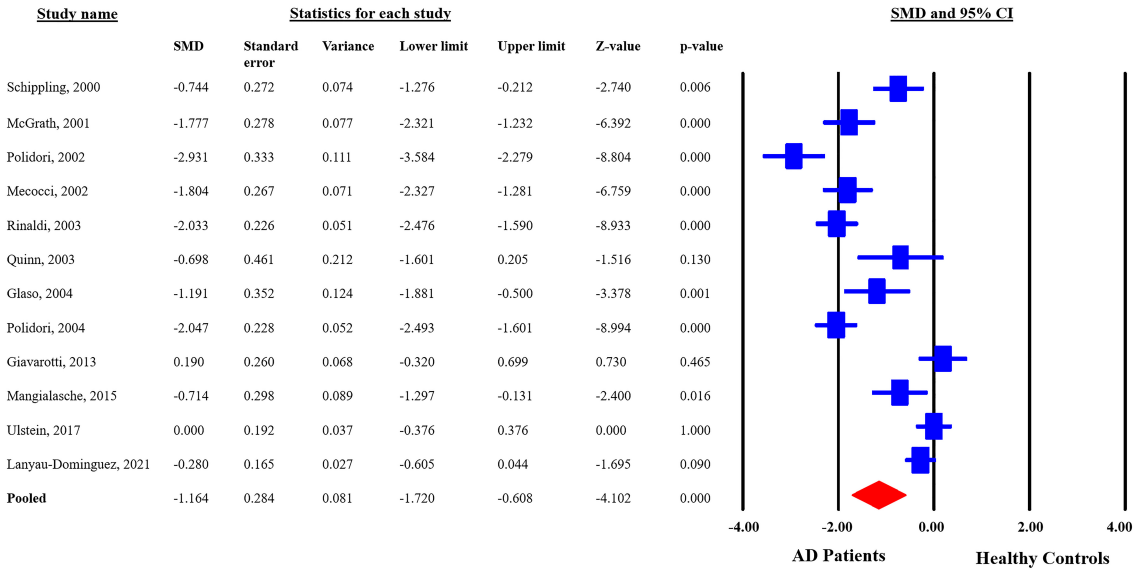

FIGURE 3
Forest plot for plasma concentrations of vitamin C in Alzheimer's disease (AD) patients and healthy controls. CI, confidence interval; SMD, standardized mean difference.

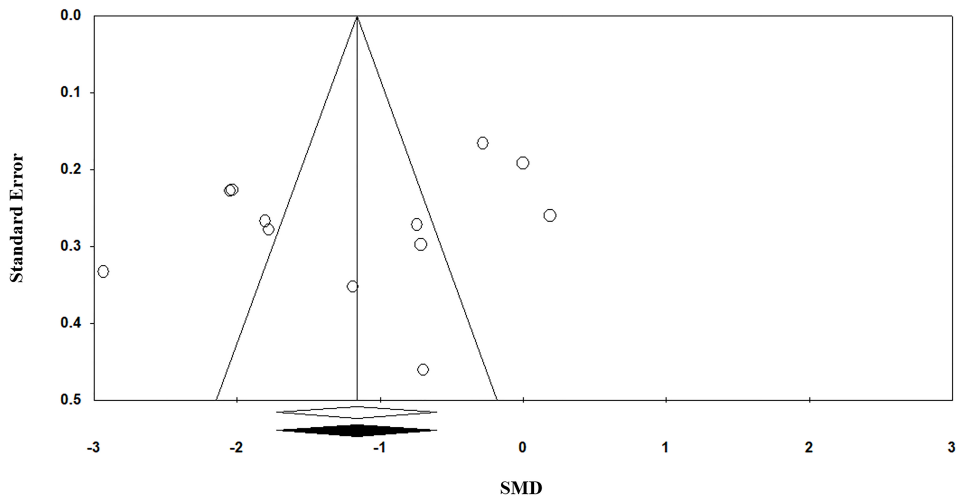

FIGURE 4
Funnel plot for the meta-analysis regarding the association between vitamin C deficiency and Alzheimer's disease (AD).

the analyzed studies is "low risk of bias except for concerns regarding residual confounding." The studies reported all possible variables that could influence the levels of vitamin C along with a single study having missing data for comorbidities of the patients. Most of the studies were unclear regarding the causation and concluded as decrease in plasma vitamin C levels was accompanied by or leading to AD along with a single study reporting a decrease in the antioxidant levels. The risk of bias for all 12 studies is shown in Figure 5.

Discussion

This study was designed to compare the plasma and serum levels of vitamin C in AD patients with healthy controls. Results of this meta-analysis show that the levels of vitamin C in AD patients were significantly decreased (12 studies, $n = 1{,}100$). A significant heterogeneity was also evident. To account for heterogeneity, three major moderators were identified which included latitude, age of patients, and percentage of females within the study. Latitude is an

FIGURE 5
Risk of bias analysis of studies evaluated using Cochrane's Risk Of Bias tool in Non-randomized Studies–of Exposures (ROBINS-E).

important moderator as dietary habits change according to the location of the test subjects and may have an effect on the study results. The age and sex of subjects were also taken into account. Due to missing data, comorbidities, and MMSE could not be applied, however, might be involved in the heterogeneity shown in the study. The R^2 value is 0.31 which indicates that 31% of the heterogeneity is accounted for when all three of the moderators are checked for heterogeneity.

Although when the moderators were individually checked for heterogeneity, latitude, and sex accounted for most of the heterogeneity in the model with R^2 values 0.10 and 0.22, respectively, indicating that 10 and 22% of the heterogeneity in the model being the result of variation in the latitude and sex of the patients. The heterogeneity shown by latitude is 10% which can be attributed to the fact that the conducted studies spanned a wide region; therefore, a considerable variation in the diet is anticipated (Podcasy and Epperson, 2022).

Age did not explain the heterogeneity in the model represented by a zero R^2 value (0.00) indicating the presence of 0% of the heterogeneity in the model. Age is not involved in the causation of AD as depicted by the zero contribution of age to heterogeneity in the analysis; however, previous studies have shown that the risk of AD increases with age (Guerreiro and Bras, 2015). One of the studies included patients with major comorbidities such as coronary heart disease, diabetes, and hypertension, while all other studies excluded patients that had any comorbidity or smoked. In addition, variation in MMSE scores of patients could be another potential factor as each study used a different range or threshold to include patients in the trial leading to diversity in the level of cognitive impairment (Mitchell, 2009; Nagaratnam et al., 2020).

There is no publication bias present in the meta-analysis. Most of the studies have taken the potential confounders such as age, gender, diet, MMSE, comorbidities, etc., under consideration with two studies that did not account for comorbidities. The sensitivity analysis was conducted to see whether an outlier significantly affected the results, and the analysis showed that there was no single study that greatly impacted the results of the meta-analysis. The combined SMD remained consistent even after the removal of studies separately.

The earliest study included in this review, published in 2000, assessed the cerebrospinal fluid (CSF) and plasma vitamin C level in AD patients versus healthy controls and the lipid oxidizability in AD patients, to establish a link between lipid oxidation, plasma antioxidants, and AD progression. The vitamin C levels were significantly decreased in CSF and plasma of AD patients. This implies that the increased lipid oxidizability could be involved in the pathogenesis of AD (Schippling et al., 2000). Vitamin C plasma levels are considered critical for the onset and the progression of aging and AD (Covarrubias-Pinto et al., 2015). Therefore, various studies tried to establish the link between peripheral levels of vitamin C

and disease progression as well as the result of antioxidant supplementation.

Oxidative stress has been strongly associated with the process of neurodegeneration and cognitive decline. Due to the increasing concentration of ROS and the decreasing neutralization activity of the antioxidants, oxidative stress causes irreversible neuronal damage and apoptosis (Guo et al., 2013). The crucial players involved in neutralizing ROS are enzymatic (SOD and GPx) and non-enzymatic antioxidants (vitamin E and vitamin C). Vitamin C is a free radical scavenger and donates two of its electrons that prevents the oxidation of other more harmful substances. A small quantity of the resulting compound dehydroascorbate is converted back to AA *via* reduction, and the rest is metabolized to oxalate by hydrolysis (Covarrubias-Pinto et al., 2015).

A case-control study assessed the increased oxidative stress in AD through quantitation of peripheral marker, a lipid peroxidation product 4-hydroxynonenal (4-HNE), and the plasma levels of AA and vitamin E along with sulfhydryls. There was a significant increase in levels of 4-HNE, while AA was substantially decreased in AD patients in comparison to healthy controls (McGrath et al., 2001). 4-HNE is considered to elevate γ-secretase activity, induce Aβ aggregation, and promote protofibril formation. The marked reduction in AA levels may indicate a possible failure to detoxify 4-HNE, exacerbating the oxidative stress and progression of AD (Siegel et al., 2007; Zhang et al., 2018).

Similarly, assessment of the levels of a wide range of antioxidants including vitamin C and the extent of lipid peroxidation revealed decreased plasma antioxidant levels in AD patients, while the difference in the level of vitamin C was also statistically significant indicating the susceptibility of AD patients to oxidative insult (Polidori and Mecocci, 2002). A case-control study revealed an increased consumption of antioxidants in the brain *via* the evaluation of CSF to plasma ratio of vitamin C (Quinn et al., 2003). Another study that evaluated the nutritional factors associated with late-onset dementia of the Alzheimer's type concluded that the decreased levels of several vitamins including vitamin C may contribute to the development of AD (Glasø et al., 2004).

Moreover, the biomarkers of oxidative damage to DNA such as 8-hydroxy-2'-deoxyguanosine on lymphocytes are also increased in AD along with a decline in plasma antioxidants and vitamin C, A, and E as well as carotenoids such as lutein, α-carotene, β-carotene, and lycopene (Mecocci et al., 2002). This further depicts that increased oxidative stress is related to a poor antioxidant status in AD (Fracassi et al., 2021). A decrease in the overall activity of enzymatic antioxidants along with reduced plasma levels of non-enzymatic antioxidants including vitamin C in AD and MCI patients was also reported. The study attributed the poor plasma concentrations of antioxidants as a result of rapid depletion through neutralizing free radicals produced due to oxidative stress (Rinaldi et al., 2003). A cross-sectional study evaluated the plasma antioxidant levels in patients with AD and vascular dementia. The study concluded that irrespective of the nature of dementia whether the cause is vascular or neurodegenerative in nature, both suffer from a drastic decrease in the blood antioxidant levels when compared with the controls (Polidori et al., 2004).

As AD is accompanied by increased inflammation and oxidative stress, another case-control study explicated a higher activation of circulation monocytes and inflammatory markers in AD along with a decrease in the circulating vitamin C and α-tocopherol levels (Giavarotti et al., 2013). Similarly, reduced mitochondrial aconitase activity is observed in AD patients which leads to mitochondrial dysfunction which was correlated with the decrease of plasma antioxidants (Mangialasche et al., 2015). Likewise, a cross-sectional study by Ulstein and Bøhmer (2017), explored the association between deficiencies of several vitamins and AD. Although their results do not support the notion that vitamin deficiencies are not involved in the causation of AD, perhaps it can be due to the limitations of the study as they did not account for whether the participants were taking vitamin supplements or not and the patients' age was comparatively younger than the general AD population (Ulstein and Bøhmer, 2017). Contrarily a recent cross-sectional study on Cuban older adults concluded that hyperhomocysteinemia and vitamin deficiencies are associated with AD (Lanyau-Domínguez et al., 2021).

Although the 12 studies explored relative quantities of different antioxidants and oxidative stress markers in plasma of AD patients when compared to healthy controls, a consistent decrease in the plasma vitamin C concentration of AD patients was observed across all studies. This marked reduction in plasma vitamin C levels may be attributed as a possible contributing factor for the causation of AD since the substantial decrease in the antioxidant level would lead to increased oxidative stress, neuroinflammation, Aβ fibrillogenesis, and aberrations in various other molecular processes regulated by vitamin C.

Intervention studies

As elevated levels of ROS are a characteristic of AD and accelerate disease progression, dietary antioxidants are vital to offset the detrimental effects of oxidative stress. Dietary antioxidants such as vitamin C can decrease ROS level thereby decelerating disease progression. Although the dosage is not optimized, a diet rich in vitamin C may benefit AD patients as evident through various observational studies and clinical trials (Polidori and Nelles, 2014).

A cohort study with 5,395 participants was examined for incident dementia over a period of 6°years. The data revealed that 197 of the participants developed dementia out of which 146 had AD. The study observed that even after adjusting for factors such as age, sex, body mass index, base pack years of smoking, etc., the high intake of vitamin E and C decreased the risk of AD (Engelhart et al., 2002). Another cross-sectional study showed that vitamin C, E, or a combination of both decreased the prevalence of AD (Zandi et al., 2004).

Although the mechanism through which vitamin C delays AD progression is not properly described, vitamin C supplementation is involved in the proteolytic processing of APP and leads to a decrease in the Aβ peptides along with a substantial reduction of lysosomal enzymes in sporadic AD as shown by the effects of supplementing 50°uM vitamin C to skin fibroblasts of AD patients (Costanzi et al., 2008). Similar results were obtained through animal model studies where increased supplementation of vitamin C of 3.3°g/l through drinking water reduced the Aβ plaque burden in the hippocampus and cortex of KO-Tg mice, alleviating the mitochondrial aberrations and BBB disruption (Kook et al., 2014). Whereas, APP + PSEN1 transgenic mice when fed a blueberry supplement diet for 8°months showed no memory and learning deficits although there was no change in the Aβ deposition (Joseph et al., 2003). Interestingly, even a mild deficiency of vitamin C may accelerate amyloid pathogenesis *via* modulation of oxidative stress mechanism contributing toward impaired cognition (Dixit et al., 2015). Treatment with solution of vitamin C for a period of 6°months demonstrated a significant decline in ROS, Aβ peptides, and synaptophysin in an AD mouse model (Murakami et al., 2011).

Evaluation of nutrient intake of elderly community-dwelling AD patients showed poor dietary intakes associated with significant differences in levels of macronutrients, energy, zinc, calcium, iron, etc., (Shatenstein et al., 2007). Another similar report including 8,085 participants over a 4-year period showed that consumption of fruit and vegetables decreased the risk of AD and dementia (Barberger-Gateau et al., 2007), while increased intake of vitamin C-rich food like strawberries and star fruit substantially curtails the risk of developing

TABLE 2 Interventional studies on the status of vitamin C and Alzheimer's disease (AD).

Study	Type	Participants	Vitamin C intake (diet or supplements)	Follow-up/ Treatment period	Effects	Result
Kontush et al. (2001)	Cohort study	20 AD patients were divided into two groups	Vitamin C supplementation 1,000°mg/d	1°month	Effect on antioxidants and lipoproteins.	Supplementation increases plasma and CSF antioxidants while decreasing plasma lipoproteins.
Engelhart et al. (2002)	Cohort study	5,395 participants	Vitamin C average measurement 121.6 mg/d (baseline)	6 years	197 participants developed dementia and 146 developed AD.	High intake of vitamin C through diet was associated with low incidence of AD.
Zandi et al. (2004)	Cross-sectional study	4,740 participants with 200 AD patients	Supplemental use of vitamin C 500°mg/d	2 years	104 more participants developed AD.	Vitamin C intake was lower in participants that developed AD.
Barberger-Gateau et al. (2007)	Cohort study	8,085 participants	Fruit consumption	4 years	183 developed AD and 281 developed dementia.	Frequent consumption of fruit and vegetables decreases the incidence of AD.
Cornelli (2010)	Cohort study	52 AD patients were divided into two groups	Multivitamin supplementation plus donepezil	6 months	Effect on oxidative stress.	Significant decrease in oxidative stress and homocysteine levels was observed.
Galasko et al. (2012)	Randomized controlled trial	78 AD subjects were divided into three groups	Vitamin C supplementation 500°mg/d	16 weeks of treatment period	Effect on oxidative stress.	Supplementation decreased oxidative stress in the brain of AD patients.
Leelarungrayub et al. (2016a)	Preliminary study	29 elderly participants	Starfruit consumption 100°g daily	4 weeks	Effect on inflammatory cytokines.	Consumption decreases proinflammatory cytokines.
Leelarungrayub et al. (2016b)	Preliminary study	27 elderly participants	Starfruit consumption 100°g daily	2 weeks	Effect on blood antioxidants.	Consumption increased antioxidant levels.
Agarwal et al. (2019)	Cohort study	925 participants	Strawberry consumption	6.7 years	245 participants developed AD.	Consumption decreased the incidence of AD.

AD by minimizing the oxidative stress and inflammation (Leelarungrayub et al., 2016a,b; Agarwal et al., 2019). A cohort study evaluated the effect of antioxidants when combined with a cholinesterase inhibitor, i.e., donepezil in AD. The group which was supplemented with antioxidants showed a significant reduction in homocysteine levels and number of sickle erythrocytes as compared to placebo. Increase in GSH levels was strongly correlated with improvement in MMSE II scores. The improvement corresponded to a decrease in oxidative stress in antioxidant combined with donepezil-treated group (Cornelli, 2010). Goschorska et al. (2018) observed a significant suppression in the antioxidant action of AChE inhibitors; however, it is postulated that supplementation of antioxidants like vitamin C may improve the clinical consequences associated with oxidative stress in AD. Lipids and lipoproteins are the main targets of oxidation in the brain. Combined supplementation of vitamin E and C decreased the susceptibility of lipoproteins for oxidation in AD patients, essentially inhibiting lipid peroxidation in the brain (Kontush et al., 2001; Arlt et al., 2002). The key observations of these interventional studies are presented in **Table 2**.

A randomized clinical trial was conducted between 2006 and 2008 that involved 78 participants with diagnosis of AD divided into three groups with 26 participants each. The treatment group was supplemented with a combination of vitamin C, vitamin E, and alpha-lipoic acid in capsule form for 16 weeks, while the other two were given coenzyme Q and placebo, respectively. The results revealed that although the vitamin C group did not show any change in CSF Aβ42 or tau levels, oxidative stress was substantially decreased and remained unchanged in the other groups, comparatively. Further, longer trials would be required to corroborate their results (Galasko et al., 2012). Another ongoing clinical trial evaluating the effect of lifestyle changes on AD patients recruited 100 AD patients that were randomly divided into two groups. The interventional group received changes in diet including vitamin C supplementation. Their primary purpose is to reduce or possibly stop the progression of AD *via* such changes (Cornelli, 2010; Ornish, 2020).

Limitations and future directions

Vitamin C as an essential antioxidant mitigates the damage caused by ROS. The reduced concentration of vitamin C in the plasma of AD patients reiterates the involvement of ROS in the incidence and progression of the disease. Treatment protocols involving supplementation and dietary enrichment of vitamin C reduced disease progression and alleviated symptoms of AD patients. The supplementation studies also demonstrated reduction in risk of disease in subjects consuming high doses of vitamin C supplements or high consumption of fruits and vegetables. Although one of the limitations of this meta-analysis is that the studies included were conducted in neighboring European countries with similar latitudes, it is recommended to conduct studies from other regions so as to diversify the findings. Diversification of studies will help to find the link between diet, genotype, and disease link.

Conclusion

In conclusion, this meta-analysis suggests that the consumption of vitamin C may be used as a public health measure to reduce the onset and progression of the disease. The present findings highlight the underlying pathophysiological association between vitamin C and AD. Clinical studies are warranted to elucidate its exact mechanistic role in AD pathophysiology and prevention.

Data availability statement

The original contributions presented in this study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

SZ was responsible for the conceptualization of the study, data review and analysis, and manuscript preparation and editing. MH and SM were responsible for data collection (literature review), data entry, statistical analysis, data interpretation, and manuscript drafting and editing. SA contributed to the content of the article and manuscript editing and write-up. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Zhigang Liu,
Northwest A&F University, China

REVIEWED BY
Qian Liu,
Northwest University, China
Ning Zhang,
Liaocheng University, China

*CORRESPONDENCE
Yu-Ming Wang
wangyuming@ouc.edu.cn

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Antarctic krill oil exhibited synergistic effects with nobiletin and theanine in ameliorating memory and cognitive deficiency in SAMP8 mice: Applying the perspective of the sea–land combination to retard brain aging

Cheng-Cheng Wang[1], Jing-Ya Kong[1], Xiao-Yue Li[1], Jin-Yue Yang[1], Chang-Hu Xue[1,2], Teruyoshi Yanagita[3] and Yu-Ming Wang[1,2]*

[1]College of Food Science and Engineering, Ocean University of China, Qingdao, China, [2]Laboratory for Marine Drugs and Bioproducts, Pilot National Laboratory for Marine Science and Technology, Qingdao, China, [3]Laboratory of Nutrition Biochemistry, Department of Applied Biochemistry and Food Science, Saga University, Saga, Japan

The complex pathogenesis of Alzheimer's disease (AD) leads to a limited therapeutic effect; therefore, the combination of multiple bioactive ingredients may be more effective in improving AD due to synergistic effects. Based on the perspective of the sea–land combination, the effects of sea-derived Antarctic krill oil (AKO) combined with land-derived nobiletin (Nob) and L-theanine (The) on memory loss and cognitive deficiency were studied in senescence-accelerated prone 8 mice (SAMP8). The results demonstrated that AKO combined with The significantly increased the number of platform crossings in the Morris water maze test by 1.6-fold, and AKO combined with Nob significantly increased the preference index in a novel object recognition test. AKO exhibited synergistic effects with Nob and The in ameliorating recognition memory and spatial memory deficiency in SAMP8 mice, respectively. Further research of the mechanism indicated that AKO exhibited synergistic effects with Nob in suppressing β-amyloid (Aβ) aggregation, neurofibrillary tangles, and apoptosis and neuroinflammation, while the synergistic effects of AKO and The involved in synaptic plasticity and anti-neuroinflammation, which revealed that the combination was complex, not a mechanical addition. These findings revealed that the sea–land combination may be an effective strategy to treat and alleviate AD.

KEYWORDS

sea–land combination, neurodegenerative diseases, Antarctic krill oil, nobiletin, theanine, n-3 PUFAs-enriched phospholipids, Alzheimer's disease, neuroinflammation

Introduction

Aging is the primary risk factor for the development of most neurodegenerative diseases, including Alzheimer's disease (AD). AD is characterized by memory loss and cognitive impairment. Due to hidden symptoms, most patients with AD are difficult to diagnose and treat in time at the early stage of onset. In addition, the complex pathogenesis of AD leads to limited therapeutic effects, leading to irreversible development of AD and large socioeconomic and personal costs (Hou et al., 2019). Therefore, it is of great significance to develop a multitarget diet or natural bioactive ingredient to prevent the occurrence and development of AD. The Yin–Yang doctrine is an important scientific concept in modern biomedicine and nutrition, which is derived from ancient Chinese Philosophy (Sun et al., 2020). The opposite but complementary relationship between Yin and Yang is the key concept of the Yin/Yang doctrine, which is like that of the land and the sea. Foods from land and sea jointly contribute to human well-being with the coordination of resources and nutritional balance due to the complementary nutritional composition. Based on the Yin–Yang doctrine, it is considered that the sea–land combination may be an effective strategy to treat and alleviate AD.

Antarctic krill (*Euphausia superba*) oil (AKO) has been reported for its multiple health benefits as a novel food ingredient, which is abundant in phospholipids associated with n-3 polyunsaturated fatty acids (n-3 PUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Xie et al., 2019). A growing number of studies revealed that AKO significantly ameliorated memory impairment by inhibiting β-amyloid (Aβ) aggregation and its damage to neurons in mice (Li et al., 2018). In addition, previous studies suggested that n-3 PUFA-enriched phospholipids exhibited stronger effects than n-3 PUFA-enriched ethyl esters and triglyceride, and recombination of ethyl esters with phospholipids from egg yolk improved the dysfunction of memory and cognition in mice (Wang et al., 2019b).

It has been reported that the neuroprotective and anti-aging effects of phytochemicals derived from land, such as nobiletin (Nob, a representative lipophilic polymethoxylated flavone) and L-theanine (The, a unique hydrophilic amino acid of tea). Some evidence revealed that Nob could cross the blood–brain barrier (BBB) and enter the brain to inhibit neuronal damage in various animal models and cultured cells and slices (Braidy et al., 2017; Nakajima and Ohizumi, 2019). However, the oral bioavailability of Nob is limited due to its special chemical structure and limited solubility, which restricts its neuroprotective activity. Therefore, it was hypothesized that AKO phospholipids with amphiphilic and emulsifying performance had synergistic effects in ameliorating memory and cognitive deficiency with Nob by improving Nob bioavailability and multitarget effects. In addition, a large number of studies have reported that The significantly improves memory loss and cognitive deficiency by modulating neurotransmitter levels and inhibiting neuron loss, which may be a result of its glutamate-like chemical structure (Türközü and Sanlier, 2017). However, it was unclear whether the hydrophilic The had synergistic effects with the amphiphilic AKO in improving cognitive function.

Therefore, based on the proposed sea–land combination for coordinated resource development and balanced nutritional intake, the effects of the combination of sea-derived AKO and the land-derived phytochemicals (Nob and The) on memory loss and cognitive deficiency were invested in the present study in senescence-accelerated prone 8 mice (SAMP8). Furthermore, possible underlying molecular mechanisms were explored.

Materials and methods

Materials

Antarctic krill oil (purity >97%) was obtained from Kangjing Marine Biotechnology Co., Ltd. (Qingdao, China), and Nob (purity >98%) and The (purity >99%) were purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China) and Jonk Biological Technology Co., Ltd. (Wuhan, China), respectively. The total DNA–RNA–Protein kit was purchased from Omega Bio-Tek, Inc. (San Francisco, USA). Primary antibodies were obtained from Cell Signaling Technology (Beverly, USA), ABclonal (Wuhan, China), or BIOSS (Beijing, China).

Animals and treatments

Approximately 6-month-old male SAMP8 mice, SPF grade, were provided by the Animal Center of Medical College, Peking University (Beijing, China, SCXK(Jing)2016-0010), which were kept in a room with standard conditions and provided with food and water *ad libitum*. After adaptation, mice were randomly divided into six groups, including the model SAMP8 group, the AKO group, the Nob group, the The group, the combination of AKO and Nob groups (AKO + Nob), and the combination of AKO and The groups (AKO + The). Mice were fed a slightly modified AIN-93G standard diet consisting of 1% (w/w) AKO, 0.075% (w/w) Nob and The, correspondingly, for 3 months. Mice were evaluated by the Morris water maze test and novel object recognition test, followed by CO_2 euthanasia with, and brains were rapidly separated and frozen with liquid nitrogen and then stored at −80°C until used or fixed in 4% buffered paraformaldehyde.

Morris water maze test

The Morris water maze test was performed according to the previous protocol. In brief, after 5-day place navigation test, the 6th-day spatial probe test was carried out. Mice were monitored by a video camera on the apparatus. The time spent finding the platform during the place navigation test, and the number, time, and distance in the target quadrant, as well as the number of platform crossings in the spatial probe test, were recorded and analyzed using the ANY-maze software (Stoelting Co., Wood Dale, IL, USA).

Novel object recognition test

In the familiarization phase of the novel object recognition test, mice were trained in a test chamber with two identical objects in a square open field. Briefly, mice were successively placed on the side of the wall away from the objects and back toward them. The time spent sniffing two objects was recorded. After 24 h, the test phase was performed. One of the objects in the test chamber was replaced with a novel one, and mice were monitored and recorded the time spent sniffing the familiar and the novel object. The preference index was calculated according to the following formula: preference index = time spent sniffing the novel object/(time spent sniffing the novel object + time spent sniffing the familiar).

Bielschowsky silver staining, Nissl staining, and immunofluorescence

Fixed tissues were dehydrated and embedded in paraffin, followed by cutting into thin sections. Bielschowsky silver staining was performed by a modified method. Briefly, slices were immersed in 10% silver nitrate solution for 15 min and ammonium silver nitrate solution at 40°C for 30 min, and then stopped in 1% ammonium hydroxide solution. For Nissl staining, slices were stained with 0.5% cresyl violet, followed by dehydration with ethanol and xylene. To detect the levels of ionized calcium binding adapter molecule 1 (IBA1) and glial fibrillary acidic protein (GFAP) in the brain, slices were incubated with primary antibodies against IBA1 (1:400) and GFAP (1:400) and fluorescent-labeled secondary antibodies, respectively. Slices were viewed under a microscope and analyzed by image J.

Protein extraction and western blotting assay

The total DNA–RNA–Protein kit was used to extract the total protein according to the manufacturer's instructions. The protein was separated by electrophoresis on 5–12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gels and transferred to polyvinylidene fluoride (PVDF) membrane. Blots were blocked in 5% bovine serum albumin (BSA) and then incubated with primary antibodies against amyloid precursor protein (APP, 1:2,000), β-site APP cleaving enzyme 1 (BACE1, 1:2,000), phosphorylated tau (p-Tau) (Ser396) (1:5,000), phosphorylated-glycogen synthase kinase-3β (Y216 + Y279) (p-GSK3β, 1:1,000), B-cell lymphoma 2 (Bcl-2, 1:1,000), Cleaved-Caspase-9 (1:1,000), Cleaved-Caspase-3 (1:2,000), synaptophysin (SYN, 1:5,000), postsynaptic density protein-95 (PSD-95, 1:1,000), brain-derived neurotrophic factor (BDNF, 1:2,000), Toll-like receptor 4 (TLR4, 1:2,000), tumor necrosis factor-α (TNF-α, 1:2,000), nuclear factor-κB (NF-κB, 1:2,000), NOD-like receptor family pyrin domain containing 3 (NLRP3, 1:2,000), and interleukin-6 (IL-6, 1:2,000) at 4°C overnight, respectively. After incubation with horse radish peroxidase-conjugated secondary antibodies (1:3,000) for 2 h, blots were visualized using an enhanced chemiluminescence (ECL, EpiZyme, China) substrate with a UVP Auto Chemi Image system (UVP, Inc., Upland, CA, USA). Protein load was evaluated using anti-β-actin antibodies (1:2,000, EpiZyme #LF201, China).

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM, indicated by error bars), and significant differences were assessed by one-way analysis of variance (ANOVA) followed by a *post-hoc* test and Student's *t*-test. Latency curves in behavioral tests were analyzed with a two-way repeated-measures ANOVA (group × day) followed by a *post-hoc* test. Different letters indicate significant differences when $p < 0.05$.

Results and discussion

The effects of memory and cognitive deficiency on SAMP8 mice

The complex pathogenesis of AD leads to limited therapeutic effects; therefore, the development of multitarget bioactive ingredients to improve AD may be an effective strategy. In the present study, based on the Yin–Yang doctrine, the sea–land combination therapy strategy, sea-derived AKO combined with land-derived Nob or The, was attempted to improve memory and cognitive deficiency in SAMP8 mice. The Morris water maze test is a typical behavioral test to evaluate spatial memory and cognition of mice, which was performed in the present study. The results during the place navigation test suggested that the latency of SAMP8 mice decreased only slightly and not significantly after 5 days of the place navigation

test, suggesting spatial memory and cognitive deficiency of SAMP8 mice. However, from the 2nd day until the 5th day, the latency of mice in the AKO+The group was significantly ($p < 0.01$) lower than that in the SAMP8 group and exhibited more superior effects than other diets. Dietary AKO+Nob significantly reduced latency from the 3rd day with no significant differences with AKO and Nob (Figure 1A). In the spatial probing test, the number of platform crossings, target quadrant entries, and target quadrant distance traveled were significantly increased with AKO+The though no significant difference was observed in AKO and The groups, compared with the SAMP8 group (Figures 1B–D). In addition, AKO+Nob exhibited significant effects by increasing the number of platform crossings (Figure 1B). The results from the Morris water maze test revealed that AKO exhibited synergistic effects with The rather than Nob in ameliorating spatial memory and cognitive deficiency in SAMP8 mice.

The novel object recognition test is a recognition memory test based on the innate preference of the rodent to explore the novel object rather than the familiar one. Mice remembered and preconized that the familiar object would spend more time exploring the novel object (Bevins and Besheer, 2006; Leger et al., 2013). The results suggested that no significant differences were observed between the time spent sniffing two objects in all groups during the familiarization phase. However, the time spent sniffing the novel object was significantly increased in the AKO, AKO+Nob, and AKO+The groups, compared with the familiar one during the test phase (Figures 1E,F). In addition, two-stage preference index enhancement was significantly increased with AKO+Nob, though no significant differences were observed in the AKO and Nob groups, compared with the SAMP8 group (Figure 1G), suggesting that AKO exhibited synergistic effects with Nob rather than The in ameliorating recognition memory in SAMP8 mice.

It has been reported that AKO significantly ameliorate memory impairment in mice and AKO-rich n-3 PUFA-enriched phospholipids exhibited more superior effects than n-3 PUFAs in other forms (Li et al., 2018; Wang et al., 2019b). In addition, Nob improved memory impairment and context-dependent fear memory impairment in SAMP8, and neuroprotective effects have been verified in other rodent models, such as Aβ-infused rats and 3XTg-AD mice (Onozuka et al., 2008; Nakajima et al., 2013; Ghasemi-Tarie et al., 2022b). Theanine significantly ameliorated D-galactose-induced brain damage in rats and Aβ-induced cognitive dysfunction and neurotoxicity in mice (Kim et al., 2009; Zeng et al., 2021). Consistently, memory and cognitive impairment of SAMP8 mice was ameliorated by AKO, Nob, and The only to some extent in the present study. Further, AKO exhibited synergistic effects with Nob and The in ameliorating recognition memory and spatial memory deficiency in SAMP8 mice, respectively. The combination of AKO and Nob and that of AKO and The showed different synergistic effects in the Morris water maze test and in the novel object recognition test, which might be due to the different processes underlying recognition memory and spatial memory (Bevins and Besheer, 2006).

The effects of neurofibrillary tangles and Aβ aggregation on the brain

β-amyloid aggregation and neurofibrillary tangles, the main pathological characteristics of AD, were determined by Bielschowsky silver staining in the present study. The results showed that obvious Aβ plaques and neurofibrillary tangles were found in the brain of SAMP8 mice, which were alleviated to some extent by AKO+The and AKO+Nob with stronger effects than AKO, Nob, and The alone (Figure 2A). Protein levels related to Aβ aggregation and neurofibrillary tangles were subsequently determined by western blotting, and the results suggested that protein levels were significantly reduced by AKO, Nob, and The alone and in combination (Figures 2B–G). Importantly, AKO+Nob had stronger effects than others in inhibiting the expressions of proteins related to Aβ aggregation and neurofibrillary tangles, which revealed the synergistic effects of AKO with Nob in suppressing the aggregation of Aβ and neurofibrillary tangles in SAMP8 mice (Figures 2B–G). Our previous study revealed that AKO significantly suppressed the level of Aβ in the brain, and the AKO-rich n-3 PUFA-enriched phospholipids significantly inhibited the generation of Aβ by reducing the level of APP and BACE1, which was consistent with the data in the present study (Li et al., 2018; Wang et al., 2019b). In addition, it has been reported that Nob reduces intracellular and extracellular Aβ in iPS cell-derived AD model neurons and hyperphosphorylation of tau in SAMP8 mouse (Nakajima et al., 2013), and The significantly decreases the generation of Aβ in D-galactose-induced rats, which was consistent with the present study (Kimura et al., 2018; Zeng et al., 2021). Furthermore, it has been suggested in the present study that the synergistic effects of AKO with Nob rather than The reduce the generation of Aβ and neurofibrillary tangles by inhibiting APP/BACE1 and p-Tau in SAMP8 mice.

The effects of neuronal cell loss on the brain

Neuronal cell loss is another important pathological characteristic of AD, which is accompanied by Aβ aggregation and neurofibrillary tangles. The number of neuronal cells was determined by Nissl staining in the present study, and the results suggested that dietary AKO+Nob significantly increased the number of neuronal cells in CA1 and CA3 of the hippocampus, though no significant differences were observed in AKO and Nob alone (Figures 3A,B). In addition, the number of

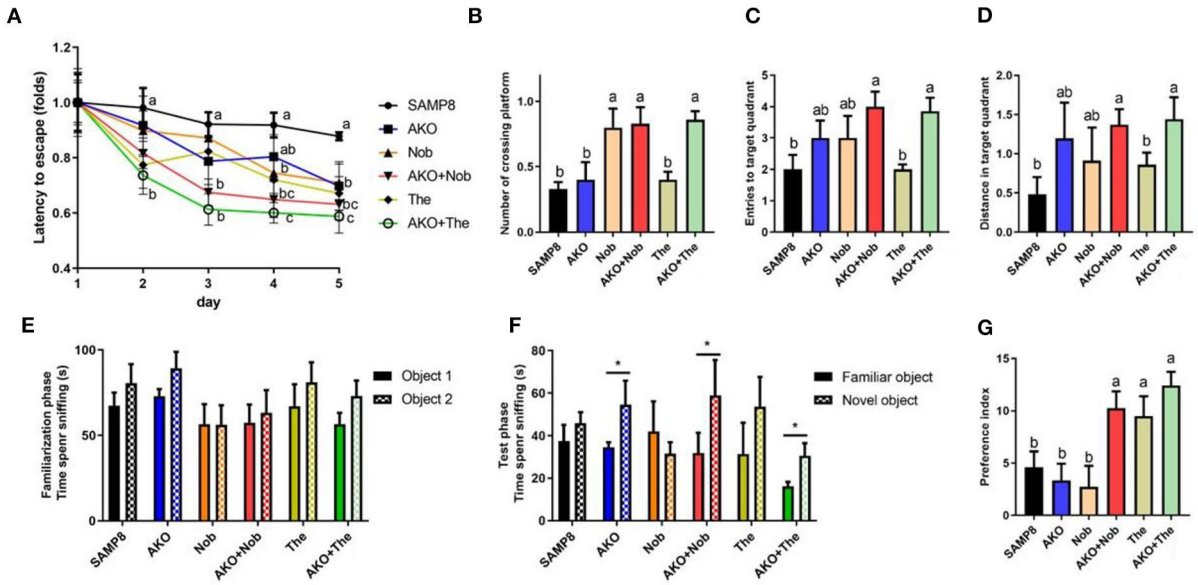

FIGURE 1
Effects on memory and cognitive deficiency indicated by performance in the Morris water maze test **(A–D)** and the novel object recognition test **(E–G)**. **(A)** Latency to escape to the platform during the place navigation test. **(B)** The number of platform crossings. **(C)** The entries to the target quadrant. **(D)** The distance traveled in the target quadrant. **(E)** The time spent sniffing two objects in the familiarization phase. **(F)** The time spent sniffing two objects in the test phase. **(G)** Increase of the preference index in two stages. All data were presented as mean ± standard error of the mean (SEM). Different letters indicate a significant difference at $p < 0.05$ by analysis of variance (ANOVA), and * indicates a significant difference at $p < 0.05$ by *t*-test.

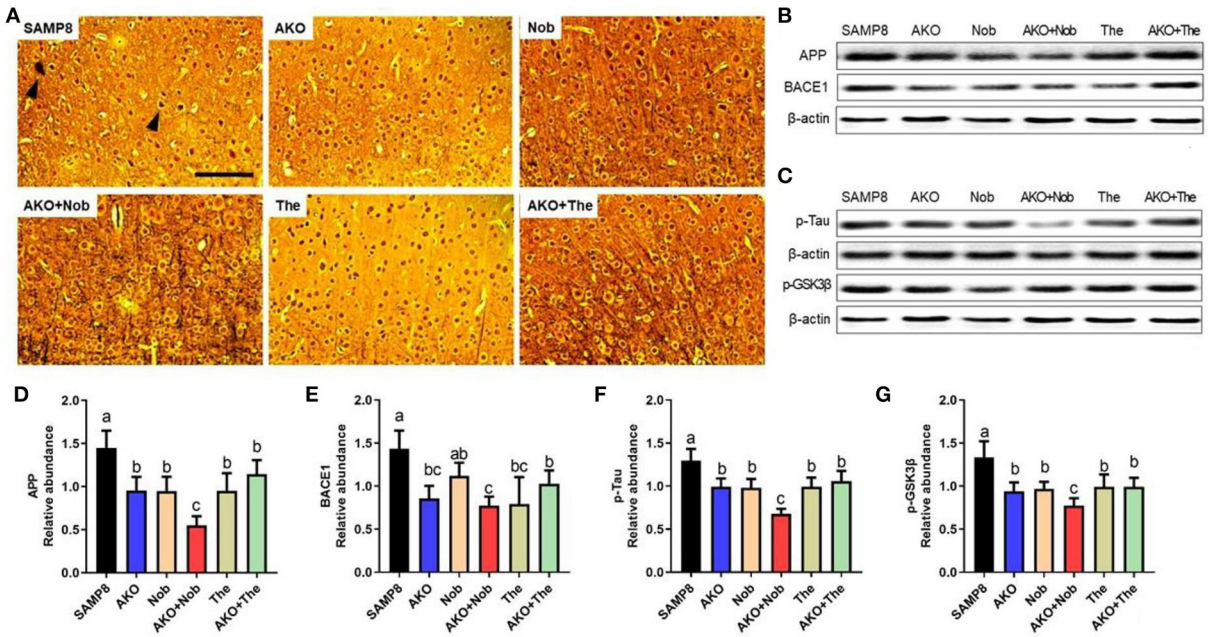

FIGURE 2
Effects of neurofibrillary tangles and β-amyloid (Aβ) aggregation. **(A)** The representative image in the brain stained by Bielschowsky silver staining, scale bar = 100 μm. Brain parenchyma contains lesions such as neurofibrillary tangles and Aβ aggregation (arrowheads). **(B)** The representative bands of amyloid precursor protein (APP) and β-site APP cleaving enzyme 1 (BACE1). **(C)** The representative bands of phosphorylated tau (p-Tau) and phosphorylated-glycogen synthase kinase-3β (p-GSK3β). **(D–G)** The relative expressions of APP **(D)**, BACE1 **(E)**, p-Tau **(F)**, and p-GSK3β **(G)**. All data were presented as mean ± SEM. Different letters indicate a significant difference at $p < 0.05$ by ANOVA.

neuronal cells in CA3 of the hippocampus was increased by AKO+The, which was significantly superior to AKO alone (Figures 3C,D). Our previous study suggested that n-3 PUFA-enriched phospholipids significantly suppressed neuronal cell loss in Aβ-induced rats and protected SH-SY5Y cells against hydrogen peroxide-induced damage (Che et al., 2018; Wen et al., 2019). In addition, it has been reported that Nob prevents CA1 neuronal loss in Aβ1-40-induced rats (Ghasemi-Tarie et al., 2022a) and The inhibits neuronal cell loss in SAMP8 mice (Cai et al., 2018). The results revealed that AKO exhibited synergistic effects with Nob rather than The in suppressing neuronal cell loss in the brain of SAMP8 mice.

The effects of apoptosis on the brain

Apoptosis plays an important role in Aβ-induced neurotoxicity in AD, which leads to neuronal cell loss (Wang et al., 2019b, 2020a). In the present study, the levels of apoptosis-related proteins were determined by western blotting, and the results suggested that the level of Cleaved-Caspase 3 was significantly inhibited by AKO+Nob with stronger effects than AKO and Nob alone, and AKO+The exhibited the effects similar to AKO alone (Figures 4A,C), while the level of Cleaved-Caspase 9 was significantly suppressed by AKO+Nob, AKO, Nob, and AKO+The to the same extent (Figures 4A,B). Consistently, the anti-apoptosis effects of AKO and its n-3 PUFA-enriched phospholipids were widely reported in a variety of neurodegenerative diseases models, such as AD and Parkinson's disease (Wang et al., 2018a,b, 2019b). In addition, Nob significantly ameliorated Aβ-induced apoptosis by inhibiting Bcl-2 and Cleaved-Caspase 3 in the brain of mice (Lee et al., 2019). The above data suggested that AKO exhibited synergistic effects with Nob rather than The in suppressing neuronal cell loss by inhibiting apoptosis in the brain of SAMP8 mice.

The effects of synaptic plasticity on the brain

It has been verified that synaptic plasticity is damaged by Aβ aggregation but protected by neurotrophins, such as BDNF. The results of the present study suggested that AKO+Nob significantly increased the level of PSD-95, a typical marker of synaptic function, to the same extent with AKO and Nob alone, while AKO+The exhibited a stronger effect than AKO and The alone (Figures 5A,B). The level of SYN was increased with AKO+The with a better effect than AKO and The alone (Figures 5A,C). In addition, the level of BDNF increased with AKO+Nob with a superior effect than AKO and Nob alone, meanwhile the effects of AKO+The were stronger than those of AKO and The alone (Figures 5A,D). It has been reported that the expression of BDNF is upregulated in the hippocampus of rats receiving 7 weeks of AKO supplementation, and n-3 PUFA-enriched phospholipids significantly increased the level of SYN, which was consistent with the present study (Wibrand et al., 2013). The previous study suggested that dietary treatment with n-3 PUFA-enriched phospholipids elevated notable expressions of PSD-95 in n-3 PUFA deficient mice, contributing to recovery from cognitive deficiency (Wen et al., 2021). In addition, the expression of BDNF was also increased by Nob in rats with cerebral ischemia and chronic unpredictable mild stress-induced rats (Li et al., 2013; Zhang et al., 2013), while The significantly increased the level of BNDF in the brain of rats and mice (Wakabayashi et al., 2012; Zeng et al., 2021). It has been reported that Nob upregulates synaptic transmission *via* postsynaptic α-amino-3-hydroxy-5-methyl-D-aspartate (AMPA) receptors to restore memory impairment (Matsuzaki et al., 2008). The results in the present study suggested the synergistic effects of AKO with The in enhancing synaptic plasticity, while the synergistic effects of AKO with Nob were only observed in increasing the level of BDNF in the brain of SAMP8 mice.

The effects of the activation of glial cells on the brain

The aggregation of Aβ can activate microglia and astrocyte, which lead to the development of neuroinflammation in the brain (Minter et al., 2016). In the present study, the activation of microglia and astrocytes in the brain was determined by their marker of activation IBA1 and GFAP, and the results showed that the levels of IBA1 and GFAP in CA3 of the hippocampus were significantly reduced by AKO+Nob and AKO+The with a stronger effect than AKO, Nob, and The alone, respectively (Figures 6A,B). In addition, IBA1 levels in dentate gyrus (DG) of the hippocampus were significantly reduced by AKO+Nob and AKO+The with a stronger effect than AKO, Nob, and The alone, respectively, while the level of GFAP was significantly reduced by AKO+Nob, AKO+The, and The alone (Figures 6C,D). It has been reported that n-3 PUFA-enriched phospholipids and Nob significantly suppress the activation of glial cells in the brain of mice, which is consistent with the present study (Wang et al., 2019b, 2021).

The effects of neuroinflammation on the brain

Further, the levels of neuroinflammation-related proteins were determined by western blotting. The results suggested that the level of TNF-α was significantly reduced by AKO+Nob and AKO+The, while AKO, Nob, and The alone exhibited no significant effects (Figures 7A,B). The level of IL-6 decreased

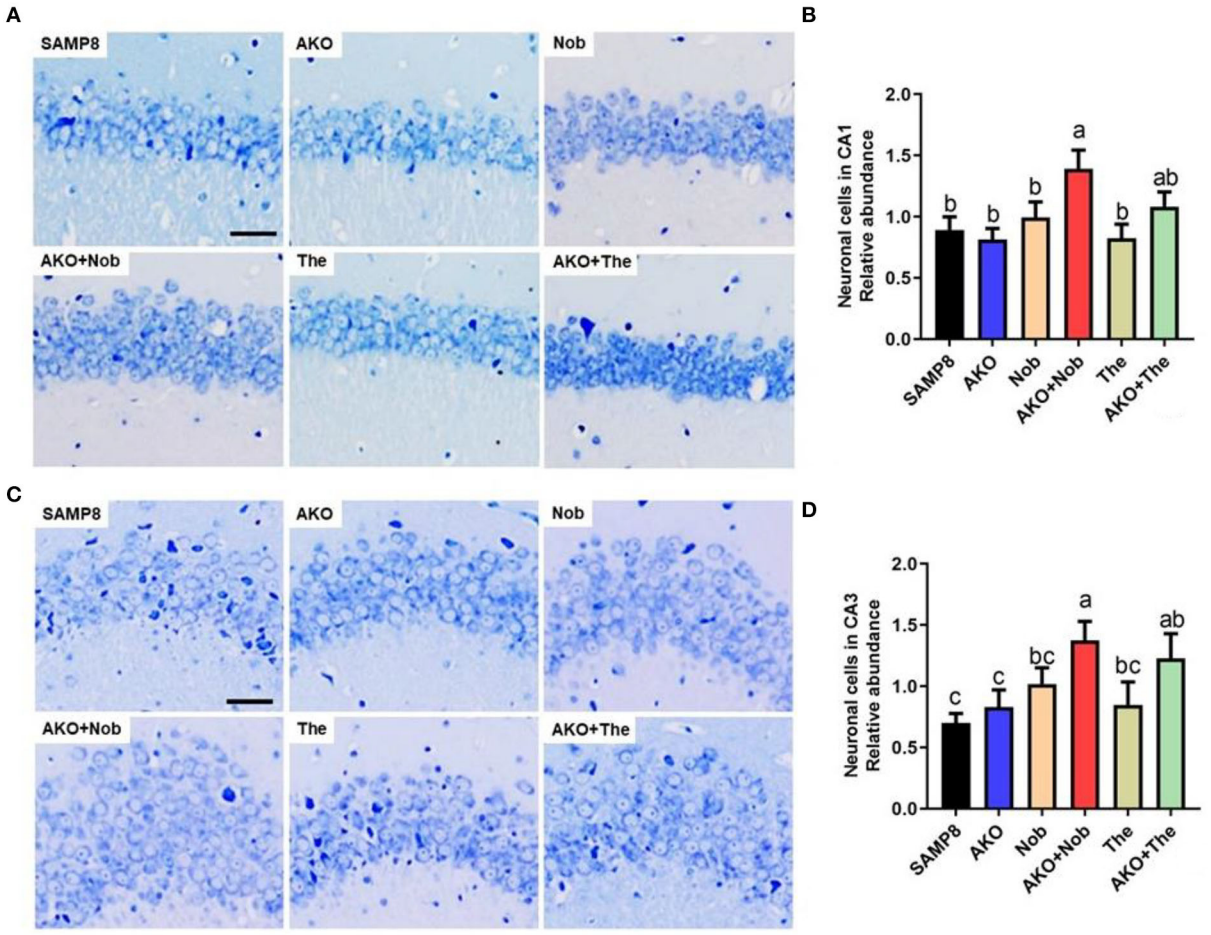

FIGURE 3
Effects of neuronal cell loss. **(A)** The representative image in CA1 of the hippocampus stained by Nissl staining, scale bar = 200 μm. **(B)** The relative number of neuronal cells in CA1 of the hippocampus. **(C)** The representative image in the CA3 of hippocampus stained by Nissl staining. **(D)** The relative number of neuronal cells in CA3 of the hippocampus. All data were presented as mean ± SEM. Different letters indicate a significant difference at $p < 0.05$ by ANOVA.

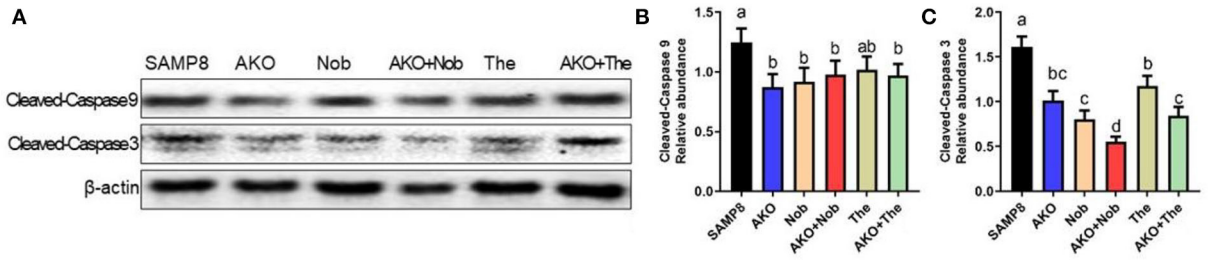

FIGURE 4
Effects of apoptosis on the brain. **(A)** The representative bands were determined by western blotting. **(B,C)** The relative expressions of Cleaved-Caspase 9 **(B)** and Cleaved-Caspase 3 **(C)**. All data were presented as mean ± SEM. Different letters indicate a significant difference at $p < 0.05$ by ANOVA.

significantly with AKO+The (Figures 7A,C). The expression and release of inflammatory factors are regulated by nuclear transcription factors NF-κB, which could be activated by TLR4. Therefore, the levels of NF-κB and TLR4 were determined and the results suggested that the level of TLR4 was significantly reduced by AKO+Nob and AKO+The, though no significant

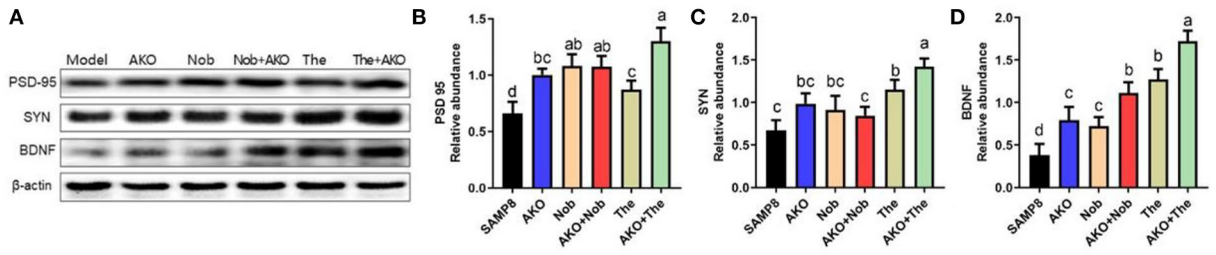

FIGURE 5
Effects of synaptic plasticity on the brain. **(A)** The representative bands were determined by western blotting. **(B–D)** The relative expressions of postsynaptic density protein-95 (PSD-95) **(B)**, synaptophysin (SYN) **(C)**, and brain-derived neurotrophic factor (BDNF) **(D)**. All data were presented as mean ± SEM. Different letters indicate a significant difference at $p < 0.05$ by ANOVA.

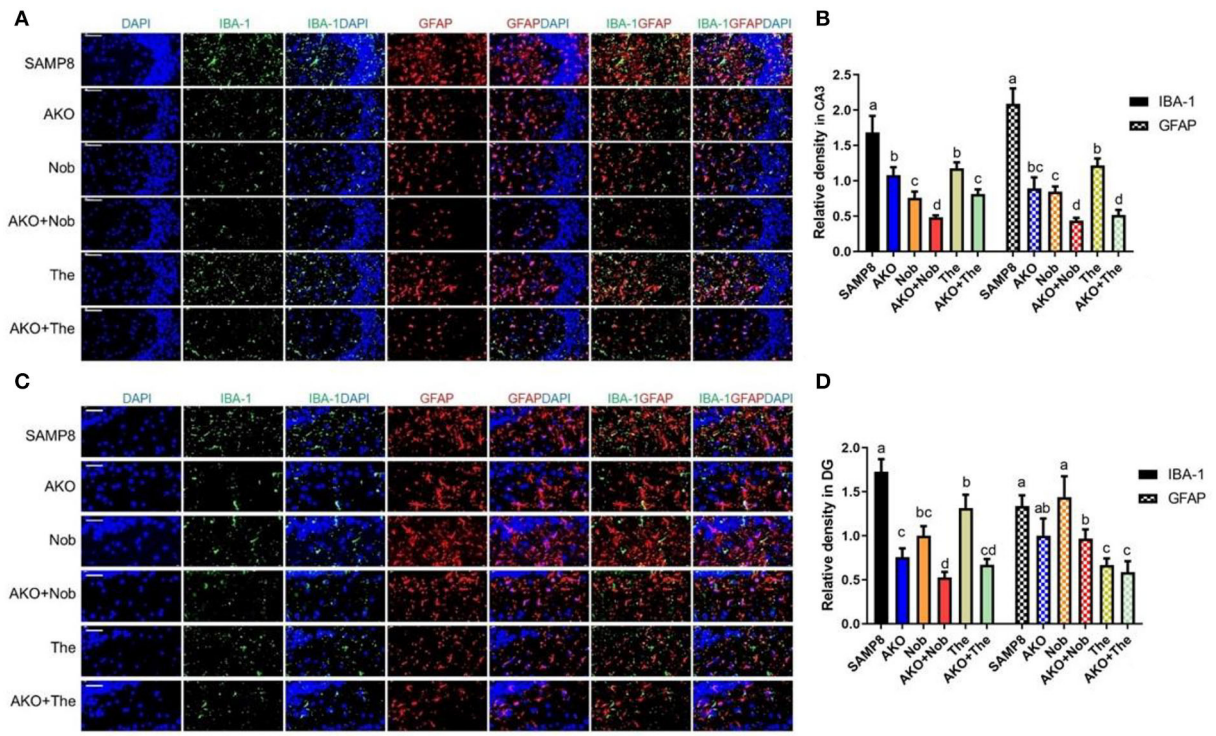

FIGURE 6
Effects on the activation of glial cells in the brain. **(A)** The representative image in CA3 of the hippocampus was determined by immunofluorescence, scale bar = 100 μm. **(B)** The relative density of ionized calcium binding adapter molecule 1 (IBA1) and glial fibrillary acidic protein (GFAP) in CA3. **(C)** The representative image in dentate gyrus (DG) of the hippocampus was determined by immunofluorescence, scale bar = 50 μm. **(D)** The relative density of IBA1 and GFAP in DG. All data were presented as mean ± SEM. Different letters indicate a significant difference at $p < 0.05$ by ANOVA.

effects were found in AKO, Nob, and The alone (Figures 7A,D). In addition, the expression of NF-κB was significantly reduced by AKO+Nob with stronger effects than AKO and Nob alone (Figures 7A,E), while no significant difference was found in AKO + The groups, which revealed that NF-κB might not be involved in the synergy of AKO with The in suppressing the expression of inflammatory factors.

It has been reported that AKO protects against lipopolysaccharide- (LPS-) induced neuroinflammation (Choi et al., 2017), and n-3 PUFA-enriched phospholipids exhibits the effects of suppressing neuroinflammation in a variety of neurodegenerative diseases (Wang et al., 2019b; Du et al., 2022). In addition, the antineuroinflammatory effects of Nob and The have also been demonstrated by inhibiting LPS-induced production and secretion of proinflammatory mediators, such as TNF-α and IL-6 *in vivo* and *in vitro* (Park et al., 2018; Qi et al., 2019; Wang et al., 2019c). The abovementioned results showed that AKO exhibited synergistic

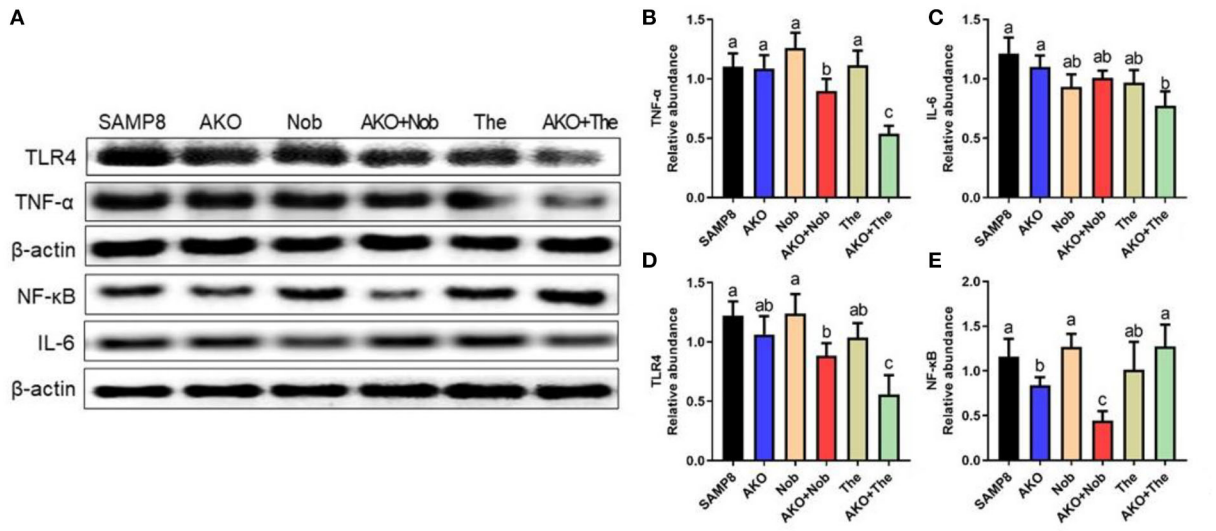

FIGURE 7
Effects of neuroinflammation in the brain. **(A)** The representative bands were determined by western blotting. **(B–E)** The relative expressions of tumor necrosis factor-α (TNF-α) **(B)**, interleukin-6 (IL-6) **(C)**, Toll-like receptor 4 (TLR4) **(D)**, and nuclear factor-κB (NF-κB) **(E)**. All data were presented as mean ± SEM. Different letters indicate a significant difference at $p < 0.05$ by ANOVA.

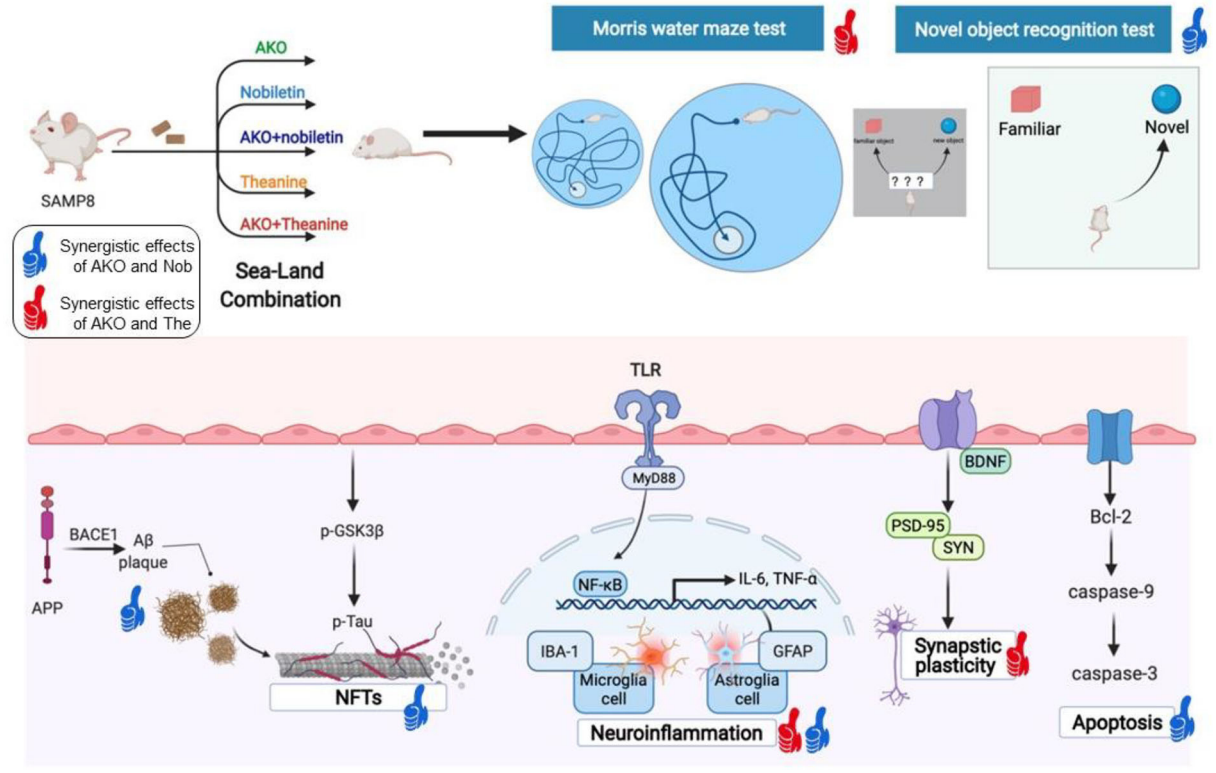

FIGURE 8
The synergistic effects of Antarctic krill oil (AKO) with nobiletin (Nob) and theanine (The) in ameliorating memory and cognitive deficiency in senescence-accelerated prone 8 mice (SAMP8) mice. The synergistic effects of AKO with Nob were signed by blue thumb, and the synergistic effects of AKO with The were signed by red thumb.

effects with Nob and The in suppressing neuroinflammation in the brain of SAMP8 mice. However, the expression level of NF-kB was inconsistent with that of IL-6 and TNF-α, which might be due to other pathways involved in the expression and secretion of inflammatory factors.

The study of mechanisms indicated the synergistic effects of AKO and Nob on Aβ aggregation, neurofibrillary tangles, apoptosis and neuroinflammation, and the synergistic effects of AKO and The on synaptic plasticity and neuroinflammation (Figure 8). The synergistic effects of AKO with Nob might be the result of a higher bioavailability of Nob. It has been reported that the soybean phospholipid increases the absorption of Nob in rats due to the increased hydrophilicity of Nob by interaction with soybean phospholipid (Lin et al., 2009). Multitarget interaction might be another important reason for the synergistic effects of AKO with Nob. It has been reported that Nob inhibits phosphodiesterase (PDE) activity and then increases the intracellular cAMP concentration to activate multiple protein kinase A (PKA) substrates (Nagase et al., 2005; Matsuzaki et al., 2008). In addition, the influx of Ca^{2+} was stimulated by activation of the N-methyl-D-aspartate (NMDA) receptors, which in turn stimulated cAMP/PKA signaling pathway and then regulated nuclear transcription factors, such as cAMP-response element binding (CREB) and NF-κB (Adams and Sweatt, 2002). In addition, AKO-rich n-3 PUFA-enriched phospholipids regulated the function of synaptic membrane-associated proteins, influencing membrane fluidity and protein–protein interactions in the brain, affecting signal transmission and synaptic function (Barceló-Coblijn et al., 2003). Another important reason for improving AD could be that n-3 PUFA-enriched phospholipids could improve brain energy metabolism and promote glucose utilization as glucose utilization was insufficient in the brain of patients with AD (An et al., 2018; Wang et al., 2019a, 2020b).

Theanine might play a complementary role with AKO through some unique mechanisms in ameliorating memory and cognitive deficiency. Due to its chemical structure similar to glutamate, The could improve neuronal function by regulating neurotransmitters, such as serotonin and dopamine (Unno et al., 1999). Furthermore, as a glutamine carrier, The inhibited the combination of extra cellular glutamine with neurons (Kakuda, 2011). The united role of sea-derived AKO and land-derived Nob and The in different aspects may be an important reason for their synergistic effects in ameliorating memory and cognitive deficiency. A broader mechanism should be involved, such as glutamatergic nerve function in the future.

Conclusion

In summary, our study demonstrated that AKO worked synergistically with Nob in ameliorating recognition memory deficiency in the novel object recognition test and with The on spatial memory deficiency in the Morris water maze test in SAMP8 mice. Further research of the mechanism indicated that the synergistic effects of AKO and Nob in ameliorating memory and cognitive deficiency mainly involved Aβ aggregation and neurofibrillary tangles, apoptosis, while the synergistic effects of AKO and The in improving memory and cognitive deficiency mainly involved synaptic plasticity. Neuroinflammation in the brain of SAMP8 mice was synergistically inhibited by both of Nob combined with AKO and The combined with AKO. The results revealed that the sea–land combination may be an effective strategy to treat and alleviate AD, providing a perspective to retard brain aging and neurodegenerative diseases.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Animal Ethics Committee of experimental animal care at College of Food Science and Engineering, Ocean University of China (Qingdao, China, Approval No. SPXY2020032501).

Author contributions

Y-MW had full access to all study data and took responsibility for the integrity of the data and accuracy of the data analysis. Y-MW and C-CW conceived the original idea for the study, supervised the conception, and revised and drafted the manuscript. J-YK, X-YL, and J-YY performed behavioral tests and molecular biological analysis and analyzed the data. TY and C-HX revised the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Tiantian Zhang,
Ocean University of China, China

REVIEWED BY
Lin Li,
Shanxi Medical University, China
Shingo Miyata,
Kindai University, Japan

*CORRESPONDENCE
Qingpeng Wang
lywqp@126.com
Ning Zhang
zhangning1111@126.com

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Effect of β-hydroxybutyrate on behavioral alterations, molecular and morphological changes in CNS of multiple sclerosis mouse model

Wei Sun[1], Min Wen[1], Min Liu[1], Qingpeng Wang[1]*, Quiqin Liu[2], Lanjie Li[2], Hans-Christian Siebert[3], Gabriele Loers[4], Ruiyan Zhang[1] and Ning Zhang[1,5]*

[1]Institute of Biopharmaceutical Research, Liaocheng University, Liaocheng, Shandong, China, [2]Shandong Donkey Industry, Technology Collaborative Innovation Center, Liaocheng University, Liaocheng, China, [3]Schauenburgerstr, RI-B-NT - Research Institute of Bioinformatics and Nanotechnology, Kiel University, Kiel, Germany, [4]Center for Molecular Neurobiology Hamburg, University Medical Center Hamburg-Eppendorf, University of Hamburg, Hamburg, Germany, [5]Laboratory of Food Science and Technology, Jiangnan University, Wuxi, China

Multiple sclerosis (MS) is a chronic inflammatory and degenerative disease of central nervous system (CNS). Aging is the most significant risk factor for the progression of MS. Dietary modulation (such as ketogenic diet) and caloric restriction, can increase ketone bodies, especially β-hydroxybutyrate (BHB). Increased BHB has been reported to prevent or improve age-related disease. The present studies were performed to understand the therapeutic effect and potential mechanisms of exogenous BHB in cuprizone (CPZ)-induced demyelinating model. In this study, a continuous 35 days CPZ mouse model with or without BHB was established. The changes of behavior function, pathological hallmarks of CPZ, and intracellular signal pathways in mice were detected by Open feld test, Morris water maze, RT-PCR, immuno-histochemistry, and western blot. The results showed that BHB treatment improved behavioral performance, prevented myelin loss, decreased the activation of astrocyte as well as microglia, and up-regulated the neurotrophin brain-derived neurotrophic factor in both the corpus callosum and hippocampus. Meanwhile, BHB treatment increased the number of MCT1$^+$ cells and APC$^+$ oligodendrocytes. Furthermore, the treatment decreased the expression of HDAC3, PARP1, AIF and TRPA1 which is related to oligodendrocyte (OL) apoptosis in the corpus callosum, accompanied by increased expression of TrkB. This leads to an increased density of doublecortin (DCX)$^+$ neuronal precursor cells and mature NeuN$^+$ neuronal cells in the hippocampus. As a result, BHB treatment effectively promotes the generation of PDGF-Ra$^+$ (oligodendrocyte precursor cells, OPCs), Sox2$^+$ cells and GFAP$^+$ (astrocytes), and decreased the production of GFAP$^+$ TRAP1$^+$ cells, and Oligo2$^+$ TRAP1$^+$ cells in the corpus callosum of mouse brain. Thus, our results demonstrate that BHB treatment efficiently supports OPC differentiation and decreases the OLs apoptosis in CPZ-intoxicated mice, partly by down-regulating the expression of TRPA1 and PARP, which is associated with the inhibition of the p38-MAPK/JNK/JUN pathway and the activation of ERK1/2, PI3K/AKT/mTOR signaling, supporting BHB treatment adjunctive nutritional therapy for the treatment of chronic demyelinating diseases, such as multiple sclerosis (MS).

KEYWORDS
multiple sclerosis, β-hydroxybutyrate, demyelination, TRPA1, PARP

Introduction

Aging, along with its associated ailments, including cancer, cardiovascular, diabetes, hypertension and neurodegenerative disorders, is a primary public health concern. Multiple sclerosis (MS) is a chronic degenerative neurological disease characterized by autoimmune inflammation, demyelination and axonal degeneration (Liu et al., 2020; Zhang et al., 2020) MS is the main cause of non-traumatic neurological disability in young adults (Klein et al., 2018). Although the disease patterns of MS are different, aging is a critical factor in the pathological progress of MS and increasing disability (Manouchehrinia et al., 2017; Klein et al., 2018).

Ketogenic diet (KD) has been widely used to treat neurodegenerative diseases, including Pelizaeus-Merzbacher disease, Alzheimer's disease (AD), Parkinson's disease (PD) and MS (Stumpf et al., 2019; Pavón et al., 2021). Studies have shown that a KD elevates the levels of BHB, improving many age-related neurological diseases, such as AD and PD (Han et al., 2020). BHB, has recently been considered not only as an energy support for cellular requirements, but also as signaling molecule that modulates oxidative stress, inflammatory responses, epigenetics (such as histone methylation and acetylation), RNA-binding proteins and G protein-coupled receptors (Stumpf et al., 2019; Wang et al., 2021). Exogenous BHB has been reported to suppress pathological microglial activation by inhibiting the NLRP3 inflammasome (Wang et al., 2021). In addition, exogenous BHB improved stem cell homeostasis by activating Notch signaling, which is a key signaling axis of tissue regeneration. Thus, BHB can be considered as an important mediator with regenerative potential of neural tissues.

The CPZ mouse model, mimicking the central event of MS pathology, exhibits oligodendrocyte apoptosis following the inflammatory response and demyelination (Zhang et al., 2020). These characteristics make the CPZ model a helpful tool to study primary oligodendrocyte loss and to identify the potential therapeutic targets for demyelination therapy in humans. Activated astrocytes and microglia produce the brain-derived neurotrophic factor (BDNF) during demyelination to promote OL differentiation and survival. BDNF/tropomyosin-related kinase B (TrkB) intracellular signaling is critical for neuronal growth, plasticity, and survival (Sha et al., 2018). Moreover, the BDNF/TrkB signaling pathway is known to stimulate myelin regeneration after demyelination, and is suggested to be used as a therapeutic agent for demyelinating diseases, including stroke and MS (Salehi et al., 2013; Fletcher et al., 2018). It has been reported that inhibition of HDAC3 can promote oligodendrocyte precursor cells (OPCs) to successfully transform into OLs, accelerate remyelination and delay demyelinating injury (Ding et al., 2020). RGFP966, a specific inhibitor of HDAC3, improves functional recovery after spinal cord injury (SCI) by dampening inflammatory cytokines (Kuboyama et al., 2017). Sox2 is expressed in OPCs, which can effectively promote remyelination, and seems to be necessary for the differentiation and development of hippocampal neurons (Peltier et al., 2011; Zhao et al., 2015). Platelet-derived growth factor receptor alpha (PDGF-Rα) is suggested to promote the proliferation and migration of OPCs (An et al., 2020). Moreover, monocarboxylate transporter 1 (MCT1) co-localizes with myelinating oligodendroglia, which are critical for the survival of axons in the central nervous system and support the transport of lactate from oligodendroglia to axons (Lee et al., 2012). In the first 3 weeks after CPZ induction OL apoptosis is strongly regulated by caspase-3. Then, reduction in the activity of caspase-3 was seen in late stages of CPZ intoxication, while the activity of poly ADP-ribose polymerase (PARP) increases, eventually leading to cell dysfunction and death *via* activating apoptosis-inducing factor (AIF; Vega-Riquer et al., 2019).

Another protein playing a role in MS and as cellular stress sensor is transient receptor potential ankyrin 1 (TRPA1), a non-selective cation channel. Recent studies have reported the expression of TRPA1 in glial cells, including astrocytes and oligodendrocytes (Bölcskei et al., 2018). There is considerable evidence suggesting that TRPA1 contributes to increased resting Ca^{2+} levels in astrocytes and long-term potentiation (Shigetomi et al., 2011, 2013). Some studies indicate that TRPA1 deficiency exerts neuro-protection in the CPZ model, which is characterized by preventing anxiety and depressive-like behavior, attenuating demyelination, reducing the apoptosis of mature oligodendrocytes, as well as inhibiting astrocyte and microglia activation (Shigetomi et al., 2013; Sághy et al., 2016). Another *in vivo* study on an Alzheimer's disease mouse model suggests that TRPA1 mediates in Aβ-induced inflammatory responses of astrocytes and contributes to AD development (Lee et al., 2016). A report by Bosson et al. shows that astrocyte Ca^{2+} hyperactivity contributes to early Aβ toxicity, which involves TRPA1 channels and is associated with CA1 neuron hyperactivity (Bosson et al., 2017), while blocking of TRPA1 channels can reduce myelin damage during the energy deprivation following ischaemia or hypoxia in MS (Davies et al., 2013; Hamilton et al., 2016).

The mitogen-activated protein kinase (MAPK) family includes p38 MAPK, extracellular signal regulated kinase (ERK1/2) and c-jun N-terminal kinase (JNK), which are responsible for signal transduction underlying regulation of cellular functions such as cell proliferation, survival and apoptosis (Ahmed et al., 2020). Previous studies demonstrate that CPZ

induced apoptosis of OLs was mediated *via* JNK and p38-MAPK pathways (Sághy et al., 2016), and ERK1/2 activation plays a crucial role in the survival of OLs (Domercq et al., 2011). Sághy et al. (2016) reported that TRPA1 deficiency plays a neuroprotective role in CPZ-induced demyelination by inhibiting the p38-MAPK/JUN pathway and by activating the ERK1/2 pathway, reducing OLs apoptosis and promoting the survival of OLs. Inhibition of poly (ADP-ribose) polymerase (PARP) reduces demyelination in the CPZ model by suppressing p38-MAPK and JUN activation and increasing the activation of the cytoprotective phosphatidylinositol-3 kinase-Akt pathway (Veto et al., 2010). In addition, PI3K/AKT/mTOR signaling plays important roles in OPCs proliferation, differentiation and development of healthy myelin (Mammana et al., 2018).

In this study, we observed the therapeutic potential of BHB treatment in ameliorating anxiety, as well as alleviating memory and learning deficits in CPZ-intoxicated mice. The mechanistic studies showed that BHB inhibited demyelination by up-regulating the expression of BDNF, CNTF and SOX2, down-regulating the expression of TRPA1 and PARP1, inhibiting the p38-MAPK/JNK/JUN pathways, and activating ERK1/2 and PI3K/AKT/mTOR signaling. Thus, our data show that BHB could act as a promising therapeutic agent for chronic demyelinating diseases, such as MS.

Methods

Animals

C57BL/6 male mice (8-week-old, weighing 18-20 g) were purchased from Jinan Pengyue Experimental Animal Company, Jinan, China. This study was approved by the laboratory animal ethics committee of Shangdong University. All animals received human care in compliance with the guidelines outlined in the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). Standard rodent pellets food and tap water were available *ad libitum*. The animals were housed under standard laboratory conditions (12:12 h light/dark cycle) for 1 week prior to experimental manipulation.

CPZ-induced demyelinating model and BHB treatment

C57BL/6 male mice were fed with standard rodent chow diet with 0.2% (w/w) CPZ (Sigma-Aldrich Inc., St. Louis, MO, USA) for 5 weeks (35 days). Animals were randomly divided into 4 groups ($n = 10$/group) as follows: (A) control group, mice were fed a standard rodent chow diet; (B) CPZ group, mice were fed with a standard rodent chow diet with CPZ and injected with normal saline; (C) BHB + CPZ group, which were intraperitoneally (i.p.) injected with BHB, starting 5 days before CPZ administration; (D) CPZ + BHB group, which were i.p. injected with BHB, starting at CPZ administration for consecutive 35 days until the end of the experiments. BHB (#298360, Sigma, USA, dissolved in normal saline) was administered to mice at a dose of 20 mmol/kg three times per day. Animals were euthanized, CC tissues were quickly dissected out from the mouse brains. Blood samples were collected from the tail, and blood ketone levels were measured using a handheld ketone meter (Blood ketone body tester, T-1, Beijing Yicheng bioelectronics Co., Ltd. Beijing, China). The study time-line with milestones is reported in Figure 1.

Behavioral assessment

Open field test

OFT was performed using the same protocol as previously described (Zhang et al., 2020). Anxiety level was measured in an open field square chamber (45 cm width × 45 cm length × 30 cm height). The travel paths of each animal were recorded for 10 min. Measurements included time spent in the central area and traveled distance (total and center) that were recorded by the video-tracking program (BW-OF302, Shanghai Biovill Co., Ltd., China).

Morris water maze (MWM)

The MWM test was used to evaluate spatial learning and memory (Zhang et al., 2021). This test consists of three parts: a conditioning test (1 day), a spatial acquisition test trial (5 days), and a probe trial test (1 day). MWM consists of a circular pool (120 cm diameter, 40 cm deep), filled with water at 22 ± 2°C. A black curtain surrounded the pool and contained distinctive marks as visual cues. The pool was divided into four equally sized quadrant: northeast (NE), southeast (SE), southwest (SW) and northwest (NW). The escape platform was placed in quadrant SE (2 cm below the surface of water). Each mouse was placed in the pool in the four different quadrants to find the escape platform. The mouse training time was set to 120 s and mice remained on the platform for 15 s. If a mouse could not find the platform within 120 s, it was gently guided to the platform for 30 s. The probe trial test was evaluated on

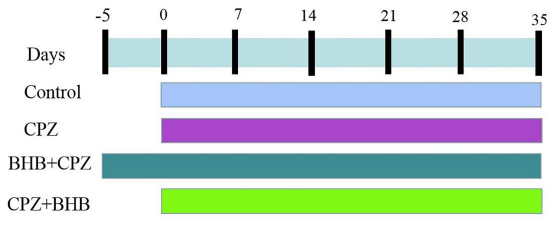

FIGURE 1
Study time-line of the present study. Mice in the BHB+CPZ group were injected intraperitoneally with BHB (three times per day) prior to initiation of CPZ-feeding. Mice in CPZ+BHB group were intraperitoneally treated with BHB (three times per day), when those animals were fed with CPZ. Body weight was recorded from the beginning of the study to the end, a total of seven time points.

the 7th day and the escape platform was removed from the pool. The escape latency, total distance swum, speed and number of times crossing the platform were recorded using ANY-maze software.

Cytokine ELISA assay

The levels of cytokines IL-1β, IL-6 and TNF-α in mouse brains were determined using ELISA assay kits (IL-1β: Beyotime Biotechnology, PI301; IL-6: Beyotime Biotechnology, PI326; TNF-α: Beyotime Biotechnology, PT512) following the manufacturer's instructions. The results were expressed as pg./mL.

Luxol fast blue/Cresyl violet (LFB/CV) staining

Brian tissues were incubated with 0.1% luxol fast blue solution (LFB, Sigma-Aldrich) for 24 h at 60°C. Afterwards, brain sections were counterstained with 0.1% cresyl violet solution (BDH, England) and rinsed in distilled water. Myelin fibres appeared blue, nuclei appeared stained in pink and violet. The images were obtained under a light microscope (Olympus, Tokyo, Japan) equipped with a digital camera. The selected fields were chosen from five independent animal slices. The demyelinated areas (LFB staining) were analyzed using ImageJ software (NIH, Bethesda, USA).

Ultrastructural analysis

C57/BL6 male mice ($n = 3$/group) were anesthetized by intraperitoneal injection of 4% chloral hydrate (400 mg/kg) and fixed by perfusion (4% paraformaldehyde). The CC of mice brains were dissected (tissue blocks of approximately 1 mm^3) and quickly immersed in 2% glutaraldehyde for 2 hours, then washed with 0.1 M sodium dimethyl arsenate solution for 2 hours, soaked by 1% osmium tetroxide for 2 hours, and sodium dimethyl arsenate solution for 15 min. Then the tissue samples were dehydrated and embedded in epoxy resin (Kim et al., 2018; Shao et al., 2020). The ultrastructure of corpus callosum myelin was examined *via* transmission electron microscope (TME, Hitachi H-7650).

Histological staining

Nissl staining

Neuronal survival was assessed with Nissl staining. The brain sections are stained with Nissl staining solution (0.1% cresyl violet) for 10 min, then rinsed with double distilled water for 1 min. Then dehydration of samples with various concentrations of alcohol was performed, sections were transparentized with xylene for 3 min and sealed with neutral resin for observation (Liu et al., 2020).

He staining

HE staining was performed using a HE Staining Kit (G1120, Solarbio). Briefly, staining of brain sections was carried out using Mayers hematoxylin, followed by eosin. Following eosin staining for 50 s, and dehydration by ethanol (95, 100%), the sections were cleared by xylene and mounted. These images were obtained using Nikon's confocal microscope (Nikon, Japan).

Immunohistochemistry staining

The immunohistochemistry was performed as previously described (Zhang et al., 2020). Paraffin-embedded sections of mice brains were stained using an anti-myelin basic protein antibody (MBP, 10458-1-AP, 1:500, Proteintech), GFAP (16825-1-AP, 1:800, Proteintech), Iba-1 (ab178846, 1:500, Abcam), Doublecortin (DCX, ab18723, 1:5,000, Abcam), NeuN (ab177487, 1:500, Abcam), secondary antibody was goat anti-rabbit IgG conjugated with alkaline phosphatase (ab6721, 1:500, Abcam). Vectastain® ABC Kit (Vector Laboratories) was used for immunohistochemical staining. Images were randomly selected from five independent animals. The positive MBP areas were analyzed using ImageJ software (NIH, Bethesda, USA).

Immunofluorescence staining

The paraffin-embedded brain tissues were processed for immunofluorescent staining as reported previously (Liu et al., 2020). The sections were incubated with MCT1 antibody (20139-1-AP, 1:800, Proteintech) and APC antibody (P25054, 1:200, Cusabio) at 4°C overnight, and secondary antibodies conjugated with Alexa Green 488 (1:1,000; cat. no. A-11001; Molecular Probes) or with Cy3-labelled secondary antibodies (1:1,000; cat. no. 111–136-144; Jackson ImmunoResearch Laboratories) were used as a secondary antibody for 1 h at room temperature. The slides were visualized by Nikon Eclipse Ti-E microscope.

Immunofluorescence double-labeling

Double immunofluorescence staining was performed as previously described (Liu et al., 2020).

Briefly, antigen retrieval was performed in sodium citrate buffer at pH 6, and sections were incubated in blocking solution containing 20% goat serum for 2 h. Then, the paraffin sections were incubated with primary antibodies overnight at 4°C. Primary antibodies were as follows: PDGF-Rα (rabbit, ab234965, 1:200, Abcam), GFAP (rabbit, 16,825-1-AP, 1:800, Proteintech), SOX2 (mouse, ab79351, 1:200, Abcam), TRPA1 (rabbit, 19,124-1-AP, 1:300, Proteintech), Oligo2 (mouse, 66,513-1-Ig, 1:300, Proteintech), and GFAP (mouse, ab4648, 1:50, Abcam). Secondary antibodies were goat anti–rabbit IgG-FITC, Texas red dye-conjugated goat anti-rabbit IgG, goat anti–mouse IgG-FITC, and Texas red dye-conjugated goat anti-mouse IgG (Jackson Immunology) and sections were incubated in the dark for 1.5 h at room temperature. Nuclei were counter-stained with

DAPI at 0.5 μg/ml. The images were captured by Nikon Eclipse Ti-E microscope.

Western blot analysis

The protein of the corpus callosum in mice brains were extracted using the MinuteTM Total Protein Extraction Kit (Invent) supplemented with phosphatase inhibitor and protease inhibitor cocktail (1:100; Sigma). The protein concentration was determined by using the BCA method (BCA Protein Assay kit, Sheng gong® Sangon Biotech). Equal amounts of sample proteins (25 μg) were separated *via* SDS-PAGE (10% or 12% polyacrylamide gels). Subsequently, the proteins were transferred onto polyvinylidene difluoride membranes (Millipore Co, Billerica, MA, USA). After washing, the membranes were blocked for 1.5 h at 37°C with a 5% skim milk solution in TBS-T followed by incubation with primary antibodies at 4°C overnight. Membranes were washed again and then incubated for 1.5 h with horse-radish peroxidase coupled secondary antibodies. Primary antibodies were as follows: PARP1 (ab191217, 1:1,000, Abcam), AIF (ab32516, 1:1,000, Abcam), NG2 (ab275024, 1:1,000, Abcam), SOX2 (11,064-1-AP, 1:1,000, Proteintech), TRPA1 (19124-1-AP, 1:1,000, Proteintech), BDNF (ab108319, 1:2,000, Abcam), TrkB (13129-1-AP, 1:1,000, Proteintech), CNTF (ab270992, 1:1,000, Abcam), p44/42 MAPK (Erk1/2; 9,102, 1:1000, Cell Signaling Technology), phospho-p44/42 MAPK (Erk1/2; 4370S, 1:2,000, Cell Signaling Technology), p38 MAPK (8,690, 1:1,000, Cell Signaling Technology), phospho-p38 MAPK (4511S, 1:1,000, Cell Signaling Technolog), JNK (9,258, 1:1,000, Cell Signaling Technolog), phospho-SAPK/JNK (81E11, 1:1,000, Cell Signaling Technolog), c-Jun (9,165, 1:1,000, Cell Signaling Technolog), Phospho-c-Jun (3,270, 1:1,000, Cell Signaling Technolog). ATK (60203-2-Ig, 1:5,000, Proteintech), phospho-ATK (4,060, 1:2000, Cell Signaling Technolog), PI3 kinase p110α (4,249, 1:1,000, Cell Signaling Technolog), phospho-PI3 Kinase p85 (4,228, 1:1,000, Cell Signaling Technolog), mTOR (2,983, 1:1000, Cell Signaling Technolog), phospho-mTOR (2,971, 1:1,000, Cell Signaling Technolog), HDAC3 (10255-1-AP, 1:2,000, Proteintech), and β-actin (20536-1-AP, 1:5,000, Proteintech). The secondary antibodies were as follows: goat anti-rabbit IgG conjugated to horseradish peroxidase (ab6721, 1:10,000, Abcam). After washing with PBST, the immunoreactive protein bands were visualized using chemiluminescence (ECL) substrate with the UVP digital image system (UVP, USA).

Statistical analysis

SPSS statistics 17.0 software was used for statistical analysis. One-way analysis of variance (ANOVA) determined differences between treatments, followed by the Bonferroni post-hoc test. The data is expressed as means ± SEM. $p < 0.05$ was considered statistically significant.

Results

BHB dose–response curve and body weights

The blood BHB concentration in BHB-treated mice was significant higher than that in control mice, which confirms that exogenous BHB supplementation could significantly elevate the concentration of β-hydroxybutyrate in the blood (Figure 2A) and brain (Supplementary data 1). In the present study, the blood BHB concentrations were 8.38 ± 0.28 mmol/l (***$p < 0.001$) at 0.5 h, 0.80 ± 0.28 mmol/l (***$p < 0.001$) at 1 h, 6.65 ± 0.30 mmol/l (***$p < 0.001$) at 2 h, 5.53 ± 0.30 mmol/l (***$p < 0.001$) at 4 h, 3.47 ± 0.36 mmol/l (***$p < 0.001$) at 6 h, and 2.65 ± 0.25 mmol/l (**$p < 0.01$) at 8 h after administration. The body weight of mice in all experimental groups was similar to average value, there was no statistical difference among the groups (control: 24.97 ± 0.32 g; CPZ: 24.97 ± 0.44 g; BHB + CPZ: 24.56 ± 0.54 g; CPZ + BHB: 24.9 ± 0.52 g). The body weight in the control group increased gradually over time, while it decreased in the CPZ group. At the end of the study, average body weight was 27.37 ± 0.57 g for control mice, 19.02 ± 0.50 g for CPZ-fed mice, 21.51 ± 0.51 g for BHB + CPZ-fed mice, and 21.25 ± 0.58 g for CPZ + BHB mice. Although body weights of mice tended to increase in the BHB + CPZ and CPZ + BHB groups compared to the CPZ group, no significant differences between groups were observed at the end of the experiment ($p = 0.056$, BHB + CPZ vs. CPZ; $p = 0.050$, BHB + CPZ vs. CPZ; Figure 2B).

Behavioral tests

OFT test

OFT test was performed on mice to evaluate the anxiety-like behavior (Wang et al., 2018). A remarkable decrease in the distance traveled (total distance and distance traveled in the central area, ***$p < 0.001$ vs. control group), and time spent in the central area (***$p < 0.001$ vs. control group) were observed in the CPZ group (Figures 3A–D). These results suggest that mice in the CPZ group have increased anxiety-like behaviors. Upon treatment with BHB, the distance moved (total distance and distance traveled in the central area, ***$p < 0.001$ vs. control group), and time spent in the central area (***$p < 0.001$ vs. control group) were both increased in BHB + CPZ and CPZ + BHB groups (Figures 3B–D), suggesting that BHB pre-intervention or simultaneous intervention can decrease the anxiety-like behavior of CPZ-fed mice.

MWM test

To investigate whether BHB can ameliorate the deficits in spatial learning and memory in CPZ-fed mice, the MWM test was carried out. Representative trajectory paths are shown in Figure 4A. Images show that the CPZ-fed mice needed mor time to find the platform than the mice in the control group and BHB treatment group. The mice in the CPZ group showed

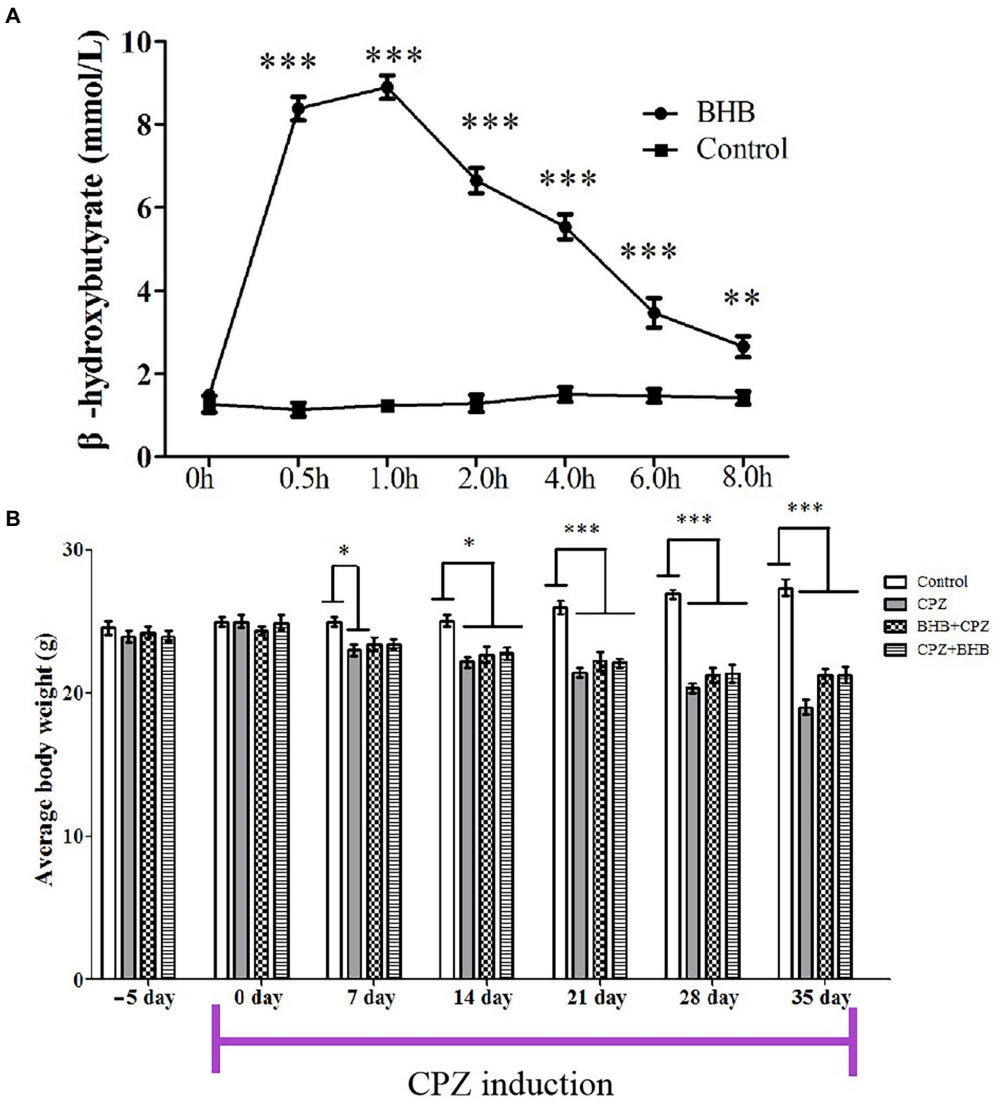

FIGURE 2
Dose–response curves of blood β-hydroxybutyrate concentrations after intraperitoneal injection of BHB **(A)** and average body weights of mice at each experimental time point **(B)**. *N*=10 mice/group. The data were presented as mean. *p<0.05, **p<0.01 and ***p<0.001 versus the control group.

a longer escape latency on the 3rd (30.63 ± 1.59 s), 4th (31.63 ± 4.64 s) and 5th (29.25 ± 2.17 s) days compared with the control group (the 3rd day, 23.65 ± 3.5 s; the 4th day, 21.85 ± 2.72 s; the 5th day, 20.03 ± 2.55 s; Figure 4B), while mice in the BHB + CPZ and CPZ + BHB showed a shorter escape latency on the 3rd (BHB + CPZ, 27.78 ± 5.85 s; CPZ + BHB, 24.73 ± 3.23 s), 4th (BHB + CPZ, 23.75 ± 3.02 s; CPZ + BHB) and 5th (BHB + CPZ, 21.12 ± 3.66 s; CPZ + BHB, 22.10 ± 3.07 s) days compared to the CPZ group (Figure 4B). The traveled total distance in all four groups of mice showed a decreased tendency (Figure 4C). Compared to the control group, mice in the CPZ group traveled longer distances from day 1 to day 5. On the 4th and 5th day, the total traveled distances in the BHB + CPZ and CPZ + BHB groups were shorter than those in the CPZ group (Figure 4C). No differences in average travel speeds were detected among the four groups ($p > 0.05$; Figure 4D). Moreover, the CPZ-fed mice took longer to cross the hidden platform for the first time ($p < 0.001$ vs. control group, Figure 4E) and the number of platform crossings was lower than that of the control group ($p < 0.001$ vs. control group, Figure 4F). However, mice spent a shorter time to cross the hidden platform in the BHB + CPZ group and CPZ + BHB group than that in the CPZ group for the first time ($p < 0.01$ BHB + CPZ vs. control group; $p < 0.01$ CPZ + BHB vs. control group, Figure 4E), and crossed the platform location more times in the BHB + CPZ group and CPZ + BHB group than that in the CPZ group ($p < 0.001$ BHB + CPZ vs. control group; $p < 0.05$ CPZ + BHB vs. control group, Figure 4F), suggesting that BHB treatment can improve the spatial learning and memory abilities in CPZ-fed mice.

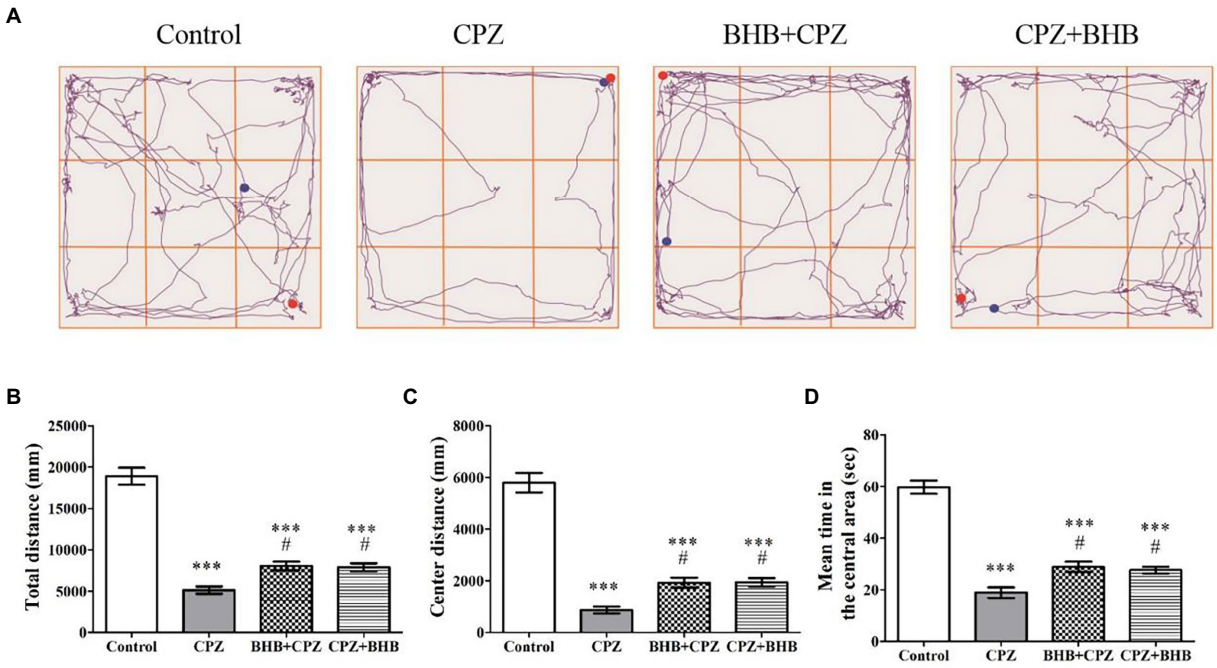

FIGURE 3
OFT behavioral assessment. **(A)** Representative images show typical examples of mice trajectories in the OFT at 35days after CPZ induction; blue dots represent the starting point and red dots represent the final point. Total distance traveled **(B)**, distance traveled in the central zone **(C)**, and time spent in the central area **(D)** were compared among control, CPZ, BHB+CPZ and CPZ+BHB groups. Data are presented as mean±SEM. *N*=10 mice/group. ***$p<0.001$ versus the control group; #$p<0.05$ versus the CPZ group.

BHB improves demyelinating lesion in CPZ-Fed mice

Histologically, demyelination lesions were revealed by the LFB/CV stain. CPZ-fed mice revealed significantly lower LFB/CV staining indensity in the corpus callosum (Figure 5A; $p<0.001$). The LFB/CV level was significantly increased in BHB + CPZ group and CPZ + BHB group, when compared with the CPZ group (BHB + CPZ group vs. CPZ group, $p<0.01$, Figure 5A; CPZ + BHB group vs. CPZ group, $p<0.05$, Figure 5A). Moreover, we used the MBP immunohistochemical staining to further confirm the extent of demyelination. MBP staining showed the loss of MBP-positive fibers in the corpus callosum of CPZ-fed mice (CPZ group vs. control group, $p<0.001$, Figure 5B), indicating severe demyelination. However, BHB + CPZ-fed mice and CPZ + BHB mice both show increased intensity of myelin staining by MBP in the corpus callosum, as compared to CPZ-fed mice (BHB + CPZ group vs. CPZ group, $p<0.01$, Figure 5B; CPZ + BHB group vs. CPZ group, $p<0.05$, Figure 5B). In the hippocampus, a similar phenomenon was observed. MBP immunostained areas of CA3 sub-regions were significantly increased in BHB + CPZ-fed mice and CPZ + BHB mice, when compared with the CPZ-fed mice (BHB + CPZ group vs. CPZ group, $p<0.05$, Figure 5B; CPZ + BHB group vs. CPZ group, $p<0.05$, Figure 5B). These results suggest demyelination lesions were significantly reduced by BHB treatment in CPZ-fed mice.

NG2, a marker of oligodendrocyte precursor cells, was dramatically increased in the CPZ-fed mice (Zhang et al., 2020). In the present study, Western Blot results showed that the NG2 expression was higher in the corpus callosum and hippocampus in CPZ-fed mice, while BHB treatment decreased the level of NG2, as compared to CPZ-fed mice (corpus callosum, BHB + CPZ group vs. CPZ group, $p<0.05$, Figure 5C; corpus callosum, CPZ + BHB group vs. CPZ group, $p<0.05$; hippocampus, BHB + CPZ group vs. CPZ group, $p<0.05$; hippocampus, CPZ + BHB group vs. CPZ group, $p<0.05$, Figure 5C), indicating that proliferation and survival of OPCs were regulated by BHB. In addition, quantitation of myelinated axons within lesion sites of the corpus callosum was conducted by TEM image analyses. It revealed that CPZ-fed mice exhibited abnormal morphology, including demyelination, de-compaction of myelin sheath, swelling of axons, myelin distortion and some loss of myelinated profiles (Figure 5D), while more myelinated axon were observed in the corpus callosum area of BHB + CPZ-fed mice and CPZ + BHB mice vs. CPZ-fed mice (Figure 5D). Both, the increased myelinated axon density and myelin thickness indicated that the structure and function of axons recovered with BHB administration.

Effects of BHB on microglia and astrocytes in the corpus callosum and hippocampus of CPZ-induced mice

Increasing evidences suggest that the activation of astrocytes and microglia can aggravate demyelinating lesions in the CPZ model. Astrocytosis and microgliosis were evaluated by GFAP and

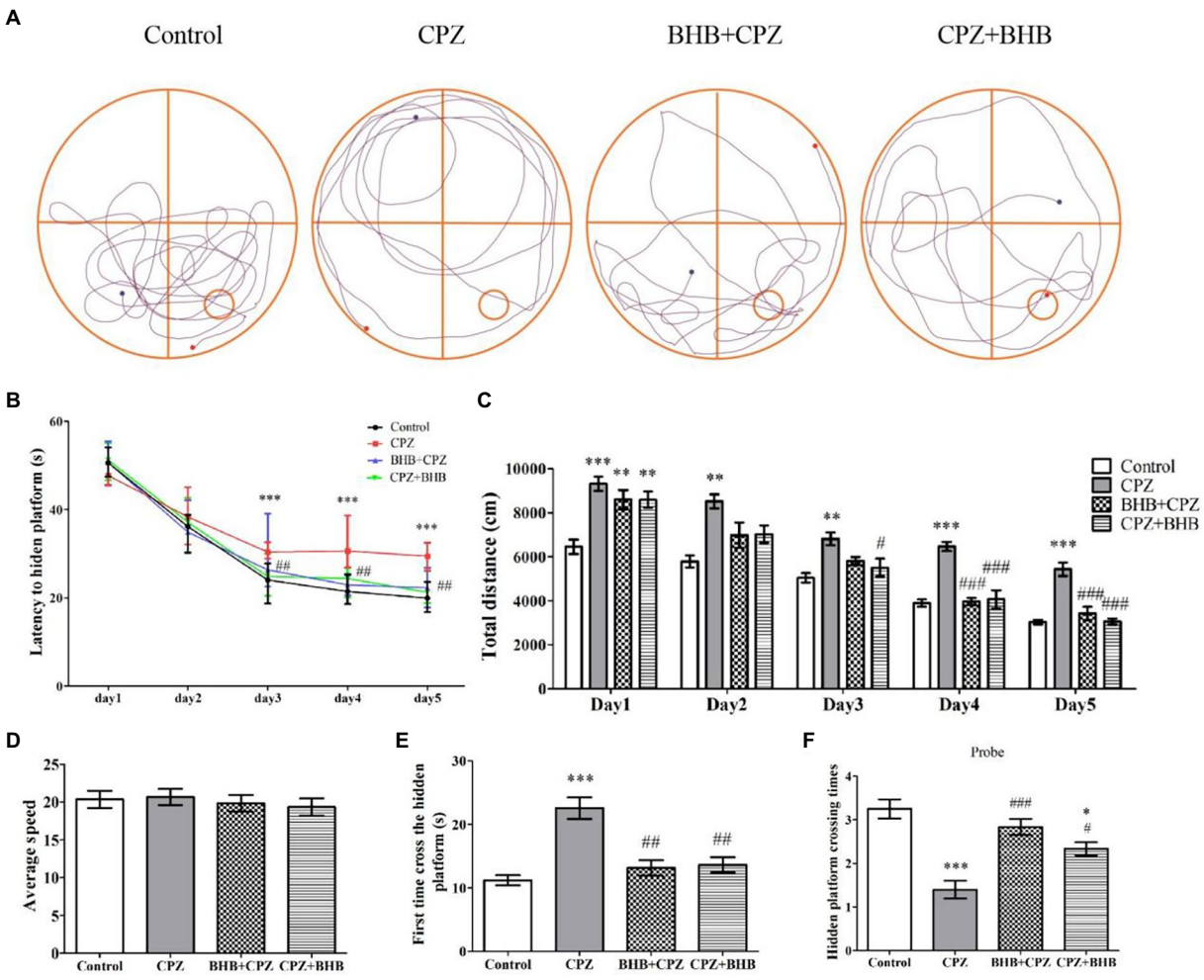

FIGURE 4
Effect of BHB on memory impairment in CPZ-fed mice. **(A)** Representative mouse search paths on the 6th day after starting with the MWM experiment, blue dots represent the starting point and red dots represent the final point. The red square indicates the platform location and the blue square indicates the start location. **(B)** Escape latency of the control, CPZ, BHB+CPZ and CPZ+BHB groups over five-day training. **(C)** Total distance traveled during training days and **(D)** average speed among the four groups were recorded (mm/s). **(E)** First time to cross the hidden platform and **(F)** the hidden platform crossing times among the four groups. Data are presented as mean±SEM. *N*=10 mice/group. **p*<0.05, ***p*<0.01, and ****p*<0.001 versus the control group; #*p*<0.05, ##*p*<0.01, and ###*p*<0.001 versus the CPZ group.

Iba-1 staining, respectively. In the present study, our results were similar to our previous study on the CPZ-fed mice, significantly elevated expression levels of GFAP-positive astrocytes were noted in the corpus callosum and hippocampus of CPZ-fed mice compared with the control group ($p < 0.001$), whereas a decrease was found in the BHB + CPZ treated mice and CPZ + BHB treated mice (as shown in Figure 6A). It has been reported that over-activation of astrocytes prevents myelin sheath regeneration and causes neuronal damage. Thus, reduction of the extent of astrogliosis might be beneficial for remyelination. In the study, we found that pretreatment or after-treatment with BHB significantly reduced GFAP expression and the morphology of GFAP return to normal conditions, suggesting that BHB treatment makes an environment conducive to normal neuronal growth and remyelination.

Microglia were visualized using Iba-1$^+$ immunohistochemical staining. Our previous study indicated that microglia were activated in mice after receiving CPZ, and hyperactivation of microglia could produce proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, which subsequently induced chronic inflammation and aggravate the process of demyelination (Zhang et al., 2020). In the present study, we investigated the Iba1$^+$ microglia in the corpus callosum and hippocampus of mice fed with CPZ. The number of Iba1$^+$ microglia in the corpus callosum and hippocampus was significantly increased (corpus callosum, CPZ group vs. control group, $p < 0.001$, Figure 6B; hippocampus, CPZ group vs. control group, $p < 0.001$, Figure 6B), while the number of Iba1$^+$ microglia was significantly decreased in corpus callosum and hippocampus sections of CPZ-fed mice after treating with BHB (corpus callosum,

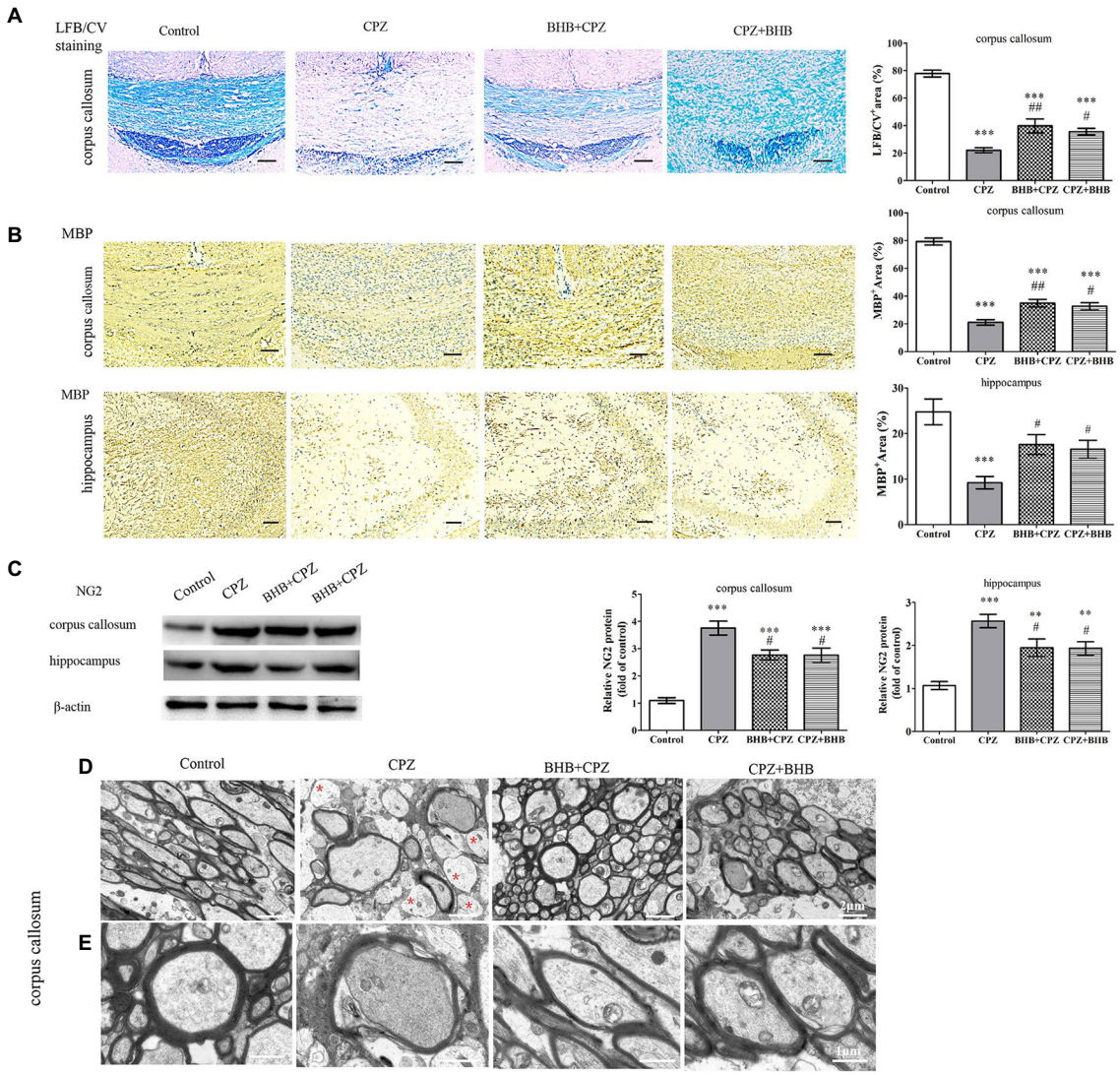

FIGURE 5
BHB treatment reduced demyelination in CPZ-fed mice. **(A)** Histological evaluation of demyelination by LFB/CV staining. Scale bars=50μm. **(B)** Representative MBP staining and intensity quantification in the corpus callosum and hippocampus of CPZ-fed mice among different groups. Scale bars=50μm. **(C)** Western blot analysis of NG2 expression in CPZ-fed mice. (D and E) Representative TEM images of demyelination in the corpus callosum of CPZ-fed mice. Note that non-myelinated axons (red asterisks) are frequent in CPZ-fed mice; D magnified 4,000×, scale bars=2μm. E magnified 10,000×, scale bars=1μm. Data are presented as mean±SEM. *N*=3 per experimental group; experiment repeated two times. $^{**}p<0.01$ and $^{***}p<0.001$ versus the control group; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ versus the CPZ group.

BHB + CPZ group vs. CPZ group, $p < 0.001$, CPZ + BHB group vs. CPZ group, $p < 0.05$, Figure 6B; hippocampus, BHB + CPZ group vs. CPZ group, $p < 0.001$, CPZ + BHB group vs. CPZ group, $p < 0.05$, Figure 6B). Moreover, microglia activation was associated with neuroinflammation during CPZ progression. The expression of IL-1β, IL-6 and TNF-α in the brain extract increased markedly in CPZ-fed mice, while the levels of IL-1β, IL-6 and TNF-α decreased in BHB + CPZ treated mice and CPZ + BHB treated mice ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, Figure 6C). These results suggested that BHB might inhibit the CPZ-induced activation of microglia and decrease inflammatory response.

Effect of BHB on oligodendrocytes

Lee et al. reported that MCT1 (a transporter for monocarboxylic acids such as lactate and ketone bodies) was highly expressed in oligodendroglia, and the disruption of this transporter can lead to axonal damage and neuronal loss (Lee et al., 2012). Fluorescent immunostaining for MCT1 in the corpus callosum revealed that expression of MCT1 was predominantly decreased in CPZ-fed mice, while the expression of MCT1 was significant increased in BHB + CPZ and CPZ + BHB treated mice (BHB + CPZ vs. control, $p < 0.001$; CPZ + BHB vs. control, $p < 0.001$, Figure 7A). This finding indicates that BHB can

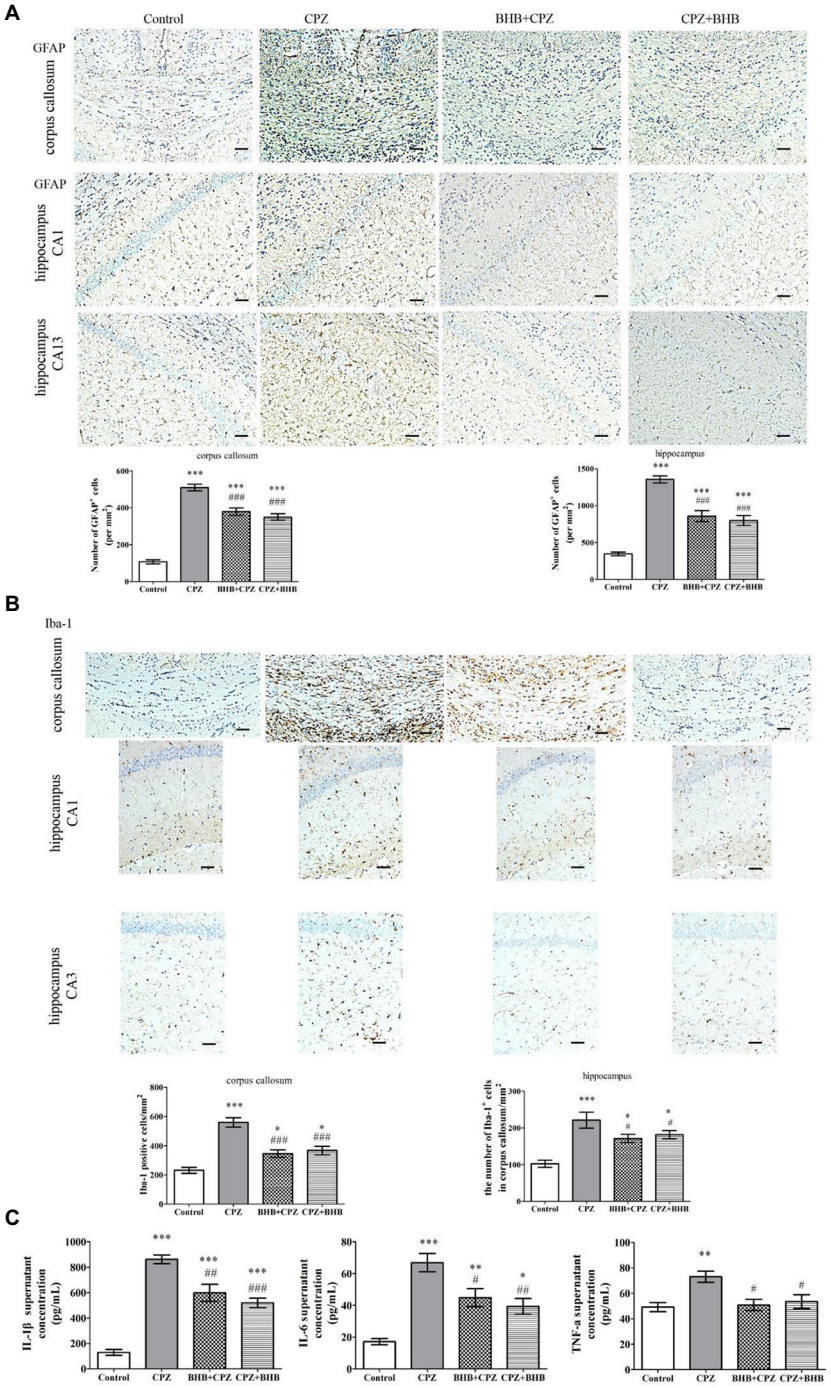

FIGURE 6
Immunohistochemistry for the microglia and astrocytes in the corpus callosum and hippocampus, and the levels of IL-1β, IL-6, and TNF-α in brains of mice at day 35 after CPZ induction. **(A)** Representative GFAP staining and intensity quantification in the corpus callosum and hippocampus of CPZ-fed mice in different groups. **(B)** Representative Iba-1 staining and intensity quantification in the corpus callosum and hippocampus of CPZ-fed mice in different groups. **(C)** IL-1β, IL-6 and TNF-α levels in brains were analyzed by ELISA. Data are presented as mean±SEM. *N*=3 per experimental group; experiment repeated two times. Scale bars=50μm. **p*<0.05, ***p*<0.01, and ****p*<0.001 versus the control group; $^{\#}p$<0.05, $^{\#\#}p$<0.01, and $^{\#\#\#}p$<0.001 versus the CPZ group.

create an environment to meet the higher metabolic needs of myelinating oligodendrocytes by increasing the expression of MCT1.

APC immunolabeling was used to evaluate the oligodendrocyte survival after CPZ induction. As shown in Figure 7B, the density of APC positive cells was dramatically

decreased in the corpus callosum of CPZ-fed mice as compared with the control group (CPZ vs. control, $p < 0.001$, Figure 7B). In contrast, the densities of APC positive cells were increased in the corpus callosum of mice in BHB + CPZ and CPZ + BHB groups. These results suggest that OLs are vulnerable to CPZ-induced damages, and BHB can increase the survival of oligodendrocytes.

It has been reported that up-regulation of PARP promotes the activation of apoptosis-inducing factor (AIF)-mediated OLG apoptosis in old mice during the later stage of CPZ intoxication (Praet et al., 2014). As shown in Figure 7C, WB assay results showed that CPZ induced increased expression of apoptotic proteins, such as PARP1 and AIF, compared to the control group, whereas the expression of PARP1 and AIF was decreased in BHB + CPZ and CPZ + BHB groups (PARP1, BHB + CPZ vs. CPZ, $p < 0.05$; AIF, BHB + CPZ vs. CPZ, $p < 0.01$; PARP1, CPZ + BHB vs. CPZ, $p < 0.001$; AIF, CPZ + BHB vs. CPZ, $p < 0.001$, Figure 7C). These results demonstrate that CPZ induces OLG apoptosis in the corpus callosum of CPZ-fed mice, which could be attenuated by BHB treatment *via* inhibition of PARP and AIF.

Moreover, Sox2 is known to regulate the proliferation of oligodendrocytes (He et al., 2021). First, we detected the expression of Sox2 in the corpus callosum and hippocampus, and found that BHB + CPZ and CPZ + BHB treatment increased Sox2 expression in the corpus callosum (BHB + CPZ vs. CPZ, $p < 0.001$; CPZ + BHB vs. CPZ, $p < 0.001$, Figure 8A). In addition, Sox2 expression showed an increased trend in the hippocampus of mice in the BHB + CPZ and CPZ + BHB groups, although it was not significant (Figure 8A). Next, we measured the expression of Sox2 and GFAP by immunofluorescence double staining. The results showed that PDGF-Ra$^+$ OPCs and GFAP$^+$ astrocytes expressed Sox2 in the corpus callosum of mice (Figure 8B). Moreover, we found that more of the Sox2 deposits co-localized with PDGF-Ra$^+$ cells and GFAP $^+$ astrocytes in the mice of the BHB + CPZ group and the CPZ + BHB group when compared to the CPZ group (Figure 8B).

The deletion of TRPA1 could attenuate CPZ-induced demyelination by reducing the apoptosis of mature oligodendrocytes (Sághy et al., 2016). In our experiments, we found that levels of TRPA1 were significantly reduced in the BHB + CPZ group and CPZ + BHB group as compared with the CPZ group ($^{\#}p < 0.05$, respectively, Figure C). Interestingly, the immunofluorescence double staining showed that both PDGF-Ra$^+$ OPCs and GFAP$^+$ astrocytes expressed Sox2 (Figure 8D), and the density of TRPA1$^+$ oligo2$^+$ and TRPA1$^+$ GFAP$^+$ was reduced in the BHB + CPZ group and CPZ + BHB group than those in CPZ group ($^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$, Figure 8D).

Morphology of the hippocampus

Next, neuropathological changes were investigated. Figure 9A shows Nissl staining in the hippocampal CA1 regions of mice. These staining revealed that neurons in hippocampal CA1 region contained abundant cytoplasm, and were neatly arranged,

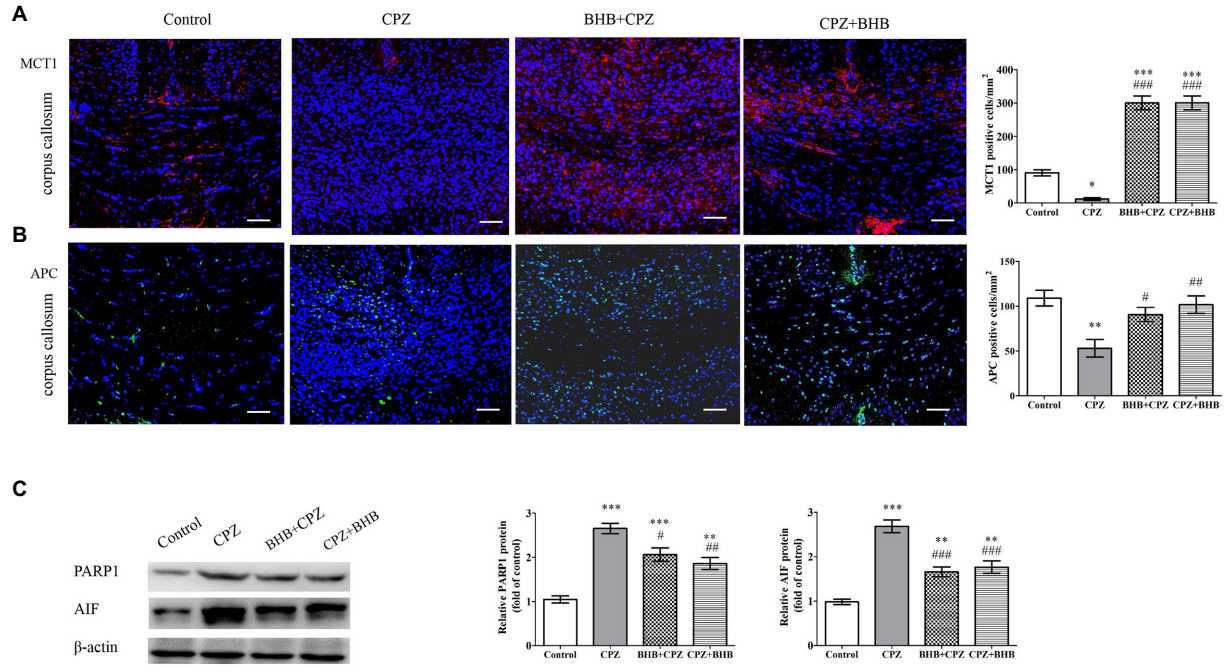

FIGURE 7
Expression of MCT1 and APC, and PARP1 and AIF in the corpus callosum of CPZ-fed mice. The immunofluorescent staining and intensity quantification of MCT1 **(A)** and APC **(B)** in the corpus callosum of mice in different groups. **(C)** WB assays (PARP1, AIF and β-actin as a loading control). Scale bars=50μm. Data are presented as mean±SEM. *N*=3 per experimental group; experiment repeated two times. *p<0.05, **p<0.01, and ***p<0.001 versus the control group; $^{\#}p$<0.05, $^{\#\#}p$<0.01, and $^{\#\#\#}p$<0.001 versus the CPZ group.

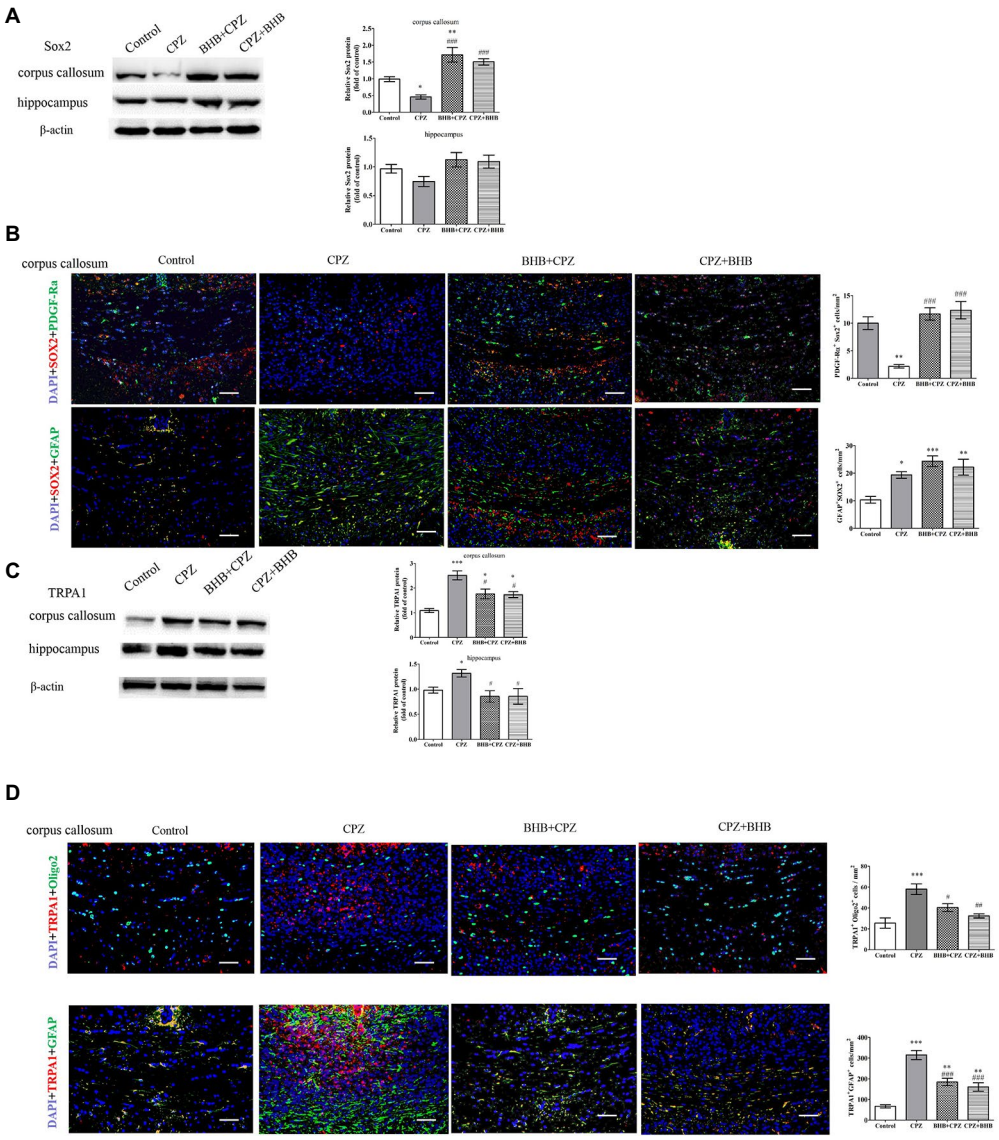

FIGURE 8
Expression of Sox2 and TRPA1 in the corpus callosum shown by immunofluorescence and WB. WB assays indicate the expression of Sox2 **(A)**, TRPA1 **(C)** and β-actin (loading control) in the corpus callosum and hippocampus of mice in different groups. Double immunofluorescence staining for PDGF-Ra$^+$ Sox2$^+$ and GFAP$^+$ Sox2 $^+$ **(B)**, and oligo2$^+$ TRPA1$^+$ and GFAP$^+$ TRPA1$^+$ **(D)** in the corpus callosum of mice. Data are presented as mean±SEM. N=3 per experimental group; experiment repeated two times. Scale bars=50μm. *p<0.05, **p<0.01, and ***p<0.001 versus the control group; $^{\#}p$<0.05, $^{\#\#}p$<0.01, and $^{\#\#\#}p$<0.001 versus the CPZ group.

demonstrating intact morphology in the control group. Specifically, mice in CPZ group have disorganized neuronal layering, while mice both in the BHB + CPZ and the CPZ + BHB groups displayed a recovering effect of the neuronal damage. In addition, it a decrease in the number of DCX$^+$ progenitor cells and neuron-specific nuclear protein (NeuN)$^+$ cells in the hippocampus of mice in the BHB + CPZ and the CPZ + BHB groups comparison to control group was observed (p < 0.001, CPZ vs. control, respectively, Figures 9B,C). Notably, the density of DCX$^+$ cells and NeuN$^+$ cells was increased in the hippocampus of mice following BHB + CPZ and CPZ + BHB treatment, compared to that in the brain of CPZ-fed mice (DCX$^+$, BHB + CPZ vs. CPZ, p < 0.01; DCX$^+$, CPZ + BHB vs. CPZ, p < 0.05; NeuN$^+$, BHB + CPZ vs. CPZ, p < 0.05; NeuN $^+$, CPZ + BHB vs. CPZ, p < 0.05, Figures 9B,C). Immunohistochemical detection of DCX$^+$ (Supplementary data 2) revealed that more DCX$^+$ cells in the mouse dentate gyrus subgranular zone in BHB treated groups than those in the CPZ group, indicating that BHB affect neurogenesis. We also found that the protein expression of BDNF in the CPZ group was significantly lower than that in the control group (CPZ vs. control, p < 0.05, Figure 9D), while the BHB + CPZ and CPZ + BHB groups exhibited significantly higher expression of BDNF than the CPZ group (p < 0.01, respectively, Figure 9D). In parallel with alterations of BDNF levels, its receptor TrkB was also significantly increased in BHB + CPZ and CPZ + BHB groups (BHB + CPZ vs. CPZ, p < 0.001; CPZ + BHB vs. CPZ, p < 0.01, respectively,

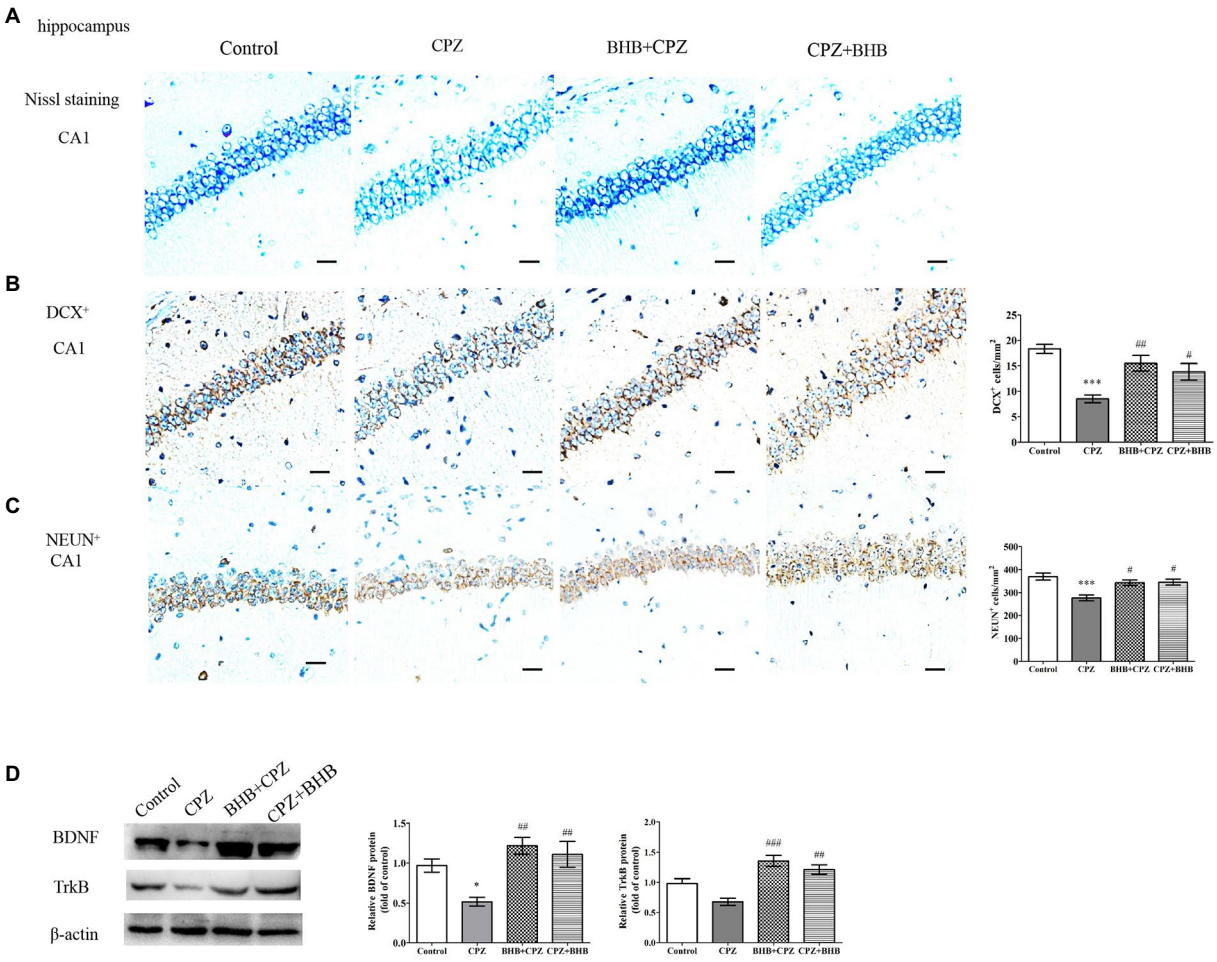

FIGURE 9
Histological images of the hippocampus and WB analysis of expression of BDNF and TrkB. **(A)** Nissl staining of the CA1 in the hippocampus in different treated groups. Distribution of immunoreactive cells for **(B)** DCX$^+$ and **(C)** NeuN$^+$ in the hippocampal CA1 of mice after CPZ exposure. The histograms show the number of immunoreactive cells in the different groups. **(D)** Representative WB of BDNF, TrkB and β-actin in hippocampal protein samples and semi-quantitative analysis of WB results; β-actin served as loading control. Bars=25μm. Data are presented as mean±SEM. N=3 per experimental group; experiment repeated two times. *p<0.05 and ***p<0.001 versus the control group; $^{\#}p$<0.05, $^{\#\#}p$<0.01, and $^{\#\#\#}p$<0.01 versus the CPZ group.

Figure 9D). Thus, experimental results suggest that BHB can create an environment that promotes the regeneration of the central nervous system, such as releasing neurotrophic factor (BDNF) and enhancing the TrkB expression, which could promote the production of neuronal cells.

BHB modulates mitogen-activated protein kinase pathways and PI3K/Akt/mTOR signaling pathway in the corpus callosum of CPZ model

Neurotrophic factors, such as BDNF can promote OPCs survival and maturation, and CNTF is beneficial to axonal sprouting, OLs generation and maturation (Siebert and Osterhout, 2021). In this study, we found that the expressions of BDNF and CNTF were increased in BHB + CPZ and CPZ + BHB groups as compared with the CPZ group (BDNF, BHB + CPZ vs. CPZ, $p < 0.001$, CPZ + BHB vs. CPZ, $p < 0.001$, Figure 10B; CNTF, BHB + CPZ vs. CPZ, $p < 0.05$, CPZ + BHB vs. CPZ, $p < 0.05$; Figure 10C).

It has been reported that CPZ triggers the activation of proapoptotic JNK and p38-MAPK pathways in response to OL death (Sághy et al., 2016). ERK1/ 2 is involved in protecting pre-myelinating oligodendrocytes (PreOLs) and reducing oxidative injury (Cai et al., 2016). Moreover, the PI3K/Akt/mTOR signaling pathway has been confirmed to be involved in the process of remyelination and can increase the number of OLs (Liu et al., 2017). In the present study, CPZ significantly induced the activation of the mitogen-activated protein kinases, phosphorylated p38-MAPK, phosphorylated JNK, phosphorylated ERK1/2, as well as phosphorylated c-Jun when compared with the control group (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, Figures 10D–G). Interesting, BHB treatment

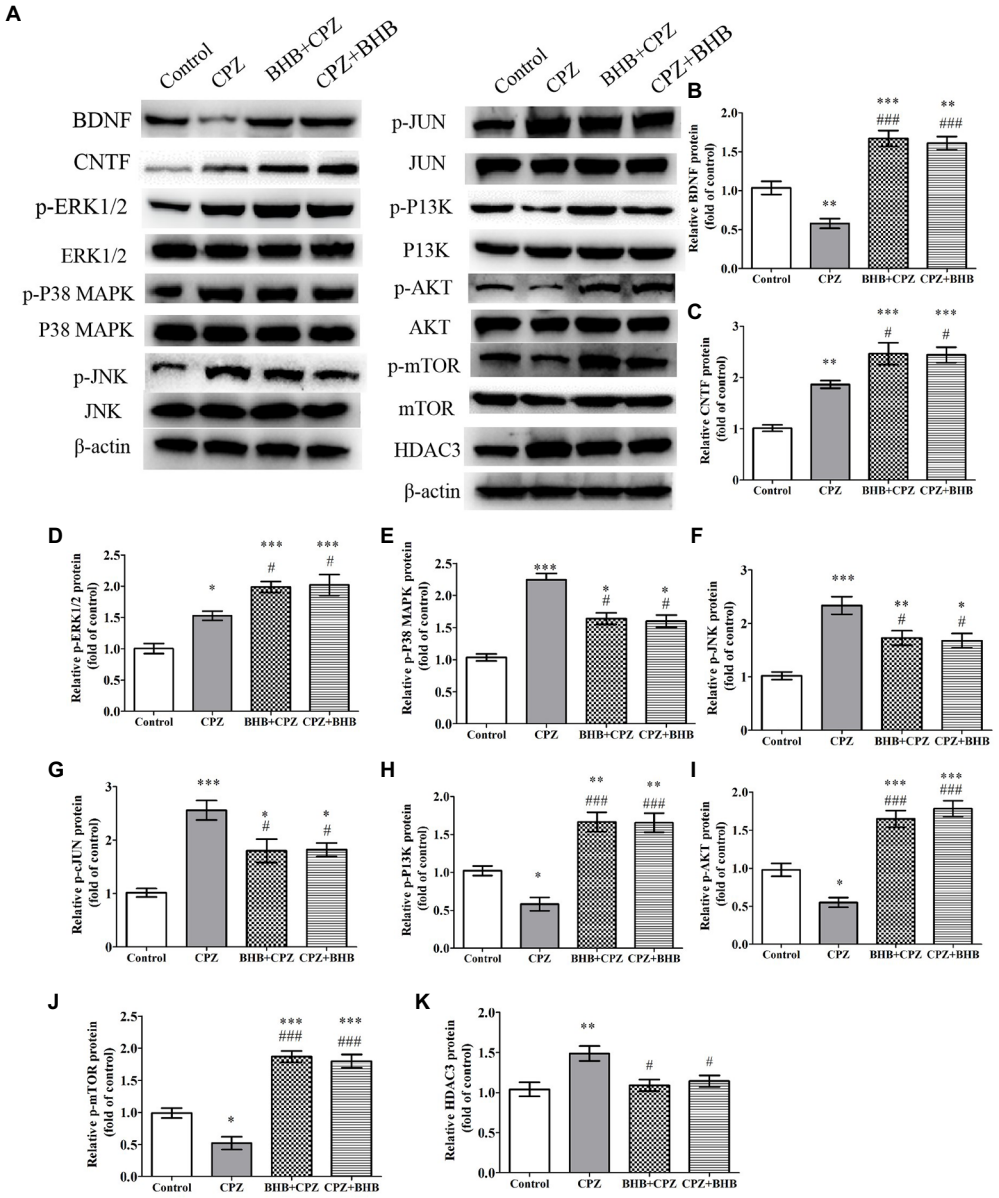

FIGURE 10
The effect of CPZ on the expression of BDNF CNTF and HDAC3, and the phosphorylation state of ERK1/2, p38-MAPK, JNK, c-Jun, PI3K, Akt, mTOR in the corpus callosum of CPZ-fed mice in different groups. **(A)** Representative images of WBs are shown. WBs were performed to detect the **(B)** BDNF, **(C)** CNTF, **(D)** p-ERK1/2, **(E)** p-P38-MAPK, **(F)** p-JNK, **(G)** p-JUN, **(H)** p-PI3K, **(I)** p-PI3K, **(J)** p-mTOR, and **(K)** HADC3 protein levels in CPZ-fed mice. β-actin was used as an internal control. Quantification of WB bands was conducted by ImageJ. The data are presented as mean±SEM. *N*=3 per experimental group; experiment repeated two times. *p<0.05, *p<0.01, and ***p<0.001 versus the control group; $^{\#}p$<0.05 and $^{\#\#\#}p$<0.001 versus the CPZ group.

decreased phosphorylation levels of p38-MAPK, JNK, c-Jun ($^{\#}p < 0.05$) and increased ERK1/2 phosphorylation ($^{\#}p < 0.05$). Moreover, the levels of the phosphorylated PI3K, phosphorylated Akt and phosphorylated mTOR were dramatically reduced in the CPZ group in comparison to the control group, while they were dramatically increased in the BHB + CPZ and CPZ + BHB groups in comparison to the CPZ group ($^{\#\#\#}p < 0.001$, Figures 10H–J). We also found that the HDAC3 expression was markedly decreased in the BHB + CPZ and CPZ + BHB groups when compared with the CPZ group ($^{\#}p < 0.05$, Figure 10K).

Discussion

Aging consider as the ultimate target for prevention of progressive disease course, which is the most important determinant of disability worsening in MS (Zeydan and Kantarci,

2020). Our previous research has demonstrated that KD exerted a neuro-protective effect on demyelination in mice by improving spatial learning, attenuating social anxiety, reducing astrogliosis and microgliosis, as well as inhibiting demyelination and OLs apoptosis. As KD prominent production of ketones (such as BHB and acetoacetate), BHB has been explored as a neuro-protective agent, which has direct effects on specific transcription factors, inflammation, oxidative stress, mitochondria, epigenetic modifications and the composition of the gut microbiome (Gough et al., 2021). Based on these findings, in the present study, we evaluated the neuroprotective effect of BHB on functional and morphological outcomes following CPZ induced demyelination. Our results indicate that both BHB + CPZ and CPZ + BHB treatments (Zhang et al., 2020) improved the exploratory ability, spatial learning and memory (Liu et al., 2020) suppressed astrogliosis and microgliosis (Klein et al., 2018) resisted demyelination, promoted oligodendrocyte differentiation and supported mature oligodendrocyte survival (Manouchehrinia et al., 2017) induced the release of neurotrophic factors in the brain, such as BDNF and CNTF, and enhanced the production or survival of neuronal cells (Stumpf et al., 2019) decreased the expression of HDAC3, TRPA1, and PARP1 and modulated several signaling pathways, such as ERK1/2, JNK, p38 MAPK, and PI3K/AKT/mTOR.

KD and calorie restriction both can produce ketone bodies, and were shown to be beneficial in some myelinopathies, such as multiple sclerosis and Pelizaeus–Merzbacher disease (Brenton et al., 2019; Stumpf et al., 2019). Our previous research also indicated that high levels of ketone bodies are associated with improved neurological injury outcomes (Liu et al., 2020; Zhang et al., 2020). Here we show that BHB treatment could inhibit demyelination in CPZ-fed mice. In this study, OFT and MWM tests indicated that mice showed significant improvement in exploration, spatial memory and the learning ability in the BHB treated groups as compared to the CPZ group. Moreover, TEM evidences revealed that myelinated axon were observed in the BHB + CPZ and CPZ + BHB mice compared to the CPZ-fed mice. These findings indicate that BHB + CPZ/CPZ + BHB treatment exert a therapeutic effect on demyelination of the CPZ model.

Several studies reported that CPZ induced demyelination is associated with increased density of astrocytes and microglia, excessive astrogliosis and microglial activation that could aggravate neuronal damages (Gerhauser et al., 2012; Hansmann et al., 2019). We previously showed that KD treatment reduced the astrocyte and microglia/macrophage recruitment in the corpus callosum and hippocampus of mouse brain at the 5th week of CPZ induction (Liu et al., 2020; Zhang et al., 2020). The current data of BHB treatment is parallel with our previous research that BHB could also improve the neuronal damages. Furthermore, CPZ triggered a neuro-inflammatory response (such as release of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α) in the white matter of the central nervous system, which is detrimental to cell survival. Coincidentally, some reports have demonstrated KD or ketone bodies can decrease the pro-inflammatory response and inhibit the demyelination of mice in CPZ and EAE models (Kim et al., 2012; Zhang et al., 2020; Almeida-Suhett et al., 2022). In addition, BHB induction attenuated expression of IL-18, IL-1β and NLRP3 inflammasome in a rodent model of depression (Kajitani et al., 2020). Our results draw similar conclusions to these published reports, BHB treatment can prevent pro-inflammatory cytokine secretion, including IL-1β, IL-6 and TNF-α in CPZ-fed mice. To our knowledge, these results show for the first time that BHB + CPZ or/and CZP + BHB treatment reduced CPZ-induced over activation of astrocyte and microglia, and inhibited pro-inflammatory cytokine secretion.

In this study, we found that BHB treatment inhibited demyelination of CPZ-fed mice. These findings are consistent with previous results that KD has a protective effect on demyelination (Liu et al., 2020; Zhang et al., 2020). It is known that OPCs proliferated and migrated from the subventricular zone and fornix, and then located to the demyelinated lesions to contribute to remyelination. However, the OPC differentiation and maturation was impeded in the pro-inflammatory microenvironment which was mediated by microglia. This may partly explain why an increased number of $NG2^+$ OPCs was found in the corpus callosum, but still severe demyelination was observed. Our previous study demonstrated that KD decreased inflammatory response and provided an adaptive benefit for remyelination in CPZ model, which related to the inhibition of HADC3 and NLRP3 expression (Liu et al., 2020). Coincidentally, another report indicated that the down-regulation of HDAC3 expression could promote remyelination and functional neurological recovery in lysophosphatidylcholine induced focal demyelinating lesions model (Ding et al., 2020). Our research showed similar results that both BHB + CPZ and CPZ + BHB treatment diminished inflammatory cytokine release, inhibited the activation of microglia, reduced the density of $NG2^+$ OPC cell and decreased the expression of HDAC3, which suggested that BHB treatment could suppress CNS inflammation and inhibited CPZ-induced demyelination might *via* the downregulation of HDAC3 expression. Meanwhile, the higher expression of BDNF and CNTF, and more $MCT1^+$ cells were found in the mice of BHB treated group. BDNF and CNTF are known to induce remyelination, and promote neuronal survival. Moreover, MCT1 also palys an important role for neuron survival and appears to be a fundamental property of oligodendroglia. It regulates the lactate export from oligodendroglia and as an important part of local energy supply to axon, while disruption of this transport balance results in axonal dysfunction (Lee et al., 2012). Thus, we concluded that BHB treatment provide a nutritional support for myelin regeneration. Furthermore, Sox2 expresses in OPCs, and promotes OPCs proliferation and differentiation (Savchenko et al., 2019). Another factor, PDGF-Rα, which has a similar function, is also can modulates several aspects of OPCs biology, such as mediating migration and differentiation of OPCs (Calabretta et al., 2018). Herein, we found that BHB treatment increased the expression of Sox2 and the density of $PDGF\text{-}Ra^+$

Sox2$^+$ cells, exerted a neuroprotective function on demyelination by promoting OPCs proliferation and differentiation.

The increase of PARP activation is observed at the later stage of CPZ induction. Over-activation of PARP inducing AIF transports from the mitochondria to the nucleus, which can promote cell apoptosis (Praet et al., 2014). Inhibition of PARP has been shown to ameliorate OLG loss. Veto et al. have observed the therapeutic effect of PARP inhibitor on demyelination disease of the CPZ model by preventing the weight loss and improving the remyelination (Veto et al., 2010). Herein, we revealed that BHB treatment increased the number of APC cells, decreased the expression of PARP and AIF, which suggested that BHB presents a neuroprotective function against oligodendrocyte death. In addition, it has been reported that TRPA1 also plays an key role in the process of CPZ-induced myelin damage (Sághy et al., 2016). TRPA1 expresses on astrocytes and oligodendrocytes in CNS (Sághy et al., 2016; Bölcskei et al., 2018). Bölcskei et al. confirmed that TRPA1 was activated by CPZ induction and promoted cytosolic Ca^{2+} level in oligodendrocytes, thus induced oligodendrocyte cells apoptosis (Bölcskei et al., 2018). Similarly, Sághy et al., 2016 reported that TRPA1 deficiency attenuated CPZ-induced demyelination by reducing the apoptosis of mature oligodendrocytes (Sághy et al., 2016). In this study, less expression of TRPA1 was identified in the BHB supplementation groups, It can be concluded that BHB treatment targets oligodendrocyte apoptosis by reducing the expression of PARP, AIF and TRPA1, thereby inhibiting demyelination in CPZ model.

MAPK pathway (ERK1/2, JNK and p38-MAPK) plays a crucial role in cell survival or stress, such as apoptosis (Veto et al., 2010). Transcription factor C-jun, as a downstream target of JNK and p38-MAPK, is considered to be a pivotal inducer of apoptosis after various CNS insults (Sághy et al., 2016). Since ERK1/2 activation could promote the oligodendrocyte survival (Veto et al., 2010; Sághy et al., 2016), in present study, we observed that BHB induced ERK1/2 activation and exerted a neuro-protective mechanism against cell death of mature OLs. Some studies demonstrated that CPZ-induced apoptosis was mediated by JNK, p38-MAPK and c-Jun activation in the corpus callosum, which can be attenuated by TRPA1 deficiency and PARP inhibition (Veto et al., 2010; Sághy et al., 2016). In this context, the researcher further verified that TRPA1 deficiency enhanced the activation of ERK1/2 in CPZ model. Thus, we evaluated the effects of BHB on MAPK pathways, the down-regulation of p-JNK, p-P38 MAPK and p-cJun, as well as up-regulation of the p-ERK1/2 expression were found in the present study. These data illustrated that BHB treatment has remarkable neuro-protective effect on promoting oligodendrocyte survival and inhibiting mature OLs apoptosis. Although the protective ERK1/2 signaling pathway activated by CPZ was found in this study, this compensatory mechanism seems to be insufficient to prevent cell apoptosis.

Similarly, another interesting signaling pathway, PI3K/AKT signaling, has been confirmed to regulate a wide variety of cellular functions including cell migration and invasion (Wang et al., 2020). mTOR is a direct substrate of the AKT kinase which promotes cell growth, metabolism and survival. In developing oligodendrocytes, mTOR is required for lipid biosynthesis, and myelin growth, which is activated before/during myelination stage (Liu et al., 2017). Liu et al. suggested that PI3K/AKT/mTOR singling regulated OPCs proliferation and differentiation, and promoted CNS remyelination (Liu et al., 2017). Moreover, Akt activation prevented neuronal apoptosis by inhibiting AIF to the nucleus (Kim et al., 2007), and reduced activity of JNK and p38-MAPK (Veto et al., 2010). Consistent with these studies, our research showed that BHB activated PI3K/AKT/mTOR signaling. Thus, we conclude that BHB treatment is contributed to protecting oligodendrocytes against apoptosis and promoting survival of oligodendrocytes partially mediated by decreasing the activation of JNK/p38-MAPK/c-Jun and increasing the activation of PI3K/AKT/mTOR.

In the hippocampus, radial glia (GFAP positive cells), which co-express with Sox2 and Nestin, was considered as stem cells which has the ability to proliferate and differentiate into neurons (Yan et al., 2018). Moreover, it is known that TRPA1 agonists in the hippocampus lead to the depressive-and anxiolytic-like effects, while pharmacological blockage or TRPA1 gene deletion reduce the depression-and anxiolytic-like symptoms. In addition, BDNF is distributed in the hippocampus and participates in neuronal plasticity and facilitating neurons development (Murawska-Ciałowicz et al., 2021). It can also promote the survival and differentiation of myelin-forming cells (Sakita et al., 2016). BDNF/TrkB axis is involved in long-term potentiation and is essential for growth, differentiation and survival of neurons (Sandhya et al., 2013). Targeting TrkB activation is considered to be a strategy for myelin repair in the brain (Nguyen et al., 2019). Interestingly, the present data showed that BHB treatment promoted MBP, BDNF, TrkB and Sox2 expression, as well as enhancing DCX$^+$ cells and NeuN$^+$ cells production in the hippocampus, which further illustrated BHB favors myelination, promotes cell proliferation and neurogenesis in the hippocampus.

In conclusion, the present study demonstrated the neuro-protective effect of BHB treatment ameliorating behavioral deficits by improving anxiety and alleviating memory impairment in CPZ-fed mice. In addition, BHB treatment could alleviate the demyelination *via* inhibition of the activation of microglia and astrocytes, as well as relieving the neuroinflammatory response, promoting the secretion of neurotrophic factors, preventing oligodendrocyte loss, enhancing OPCs proliferation and differentiation and promoting hippocampal neuron development or persistence in the CPZ-injured mice. Further mechanistic studies showed that BHB exerts neuroprotective effects by down-regulating the expression of TRPA1 and PARP, inhibiting the MAPK pathway (JNK, p38-MAPK and JUN) and activating the ERK1/2 and PI3K/AKT/mTOR signaling pathway. Collectively, our data support BHB as a promising therapeutic agent for future clinical investigations to reduce chronic demyelinating diseases, such as MS.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

All animals received human care in compliance with the guidelines outlined in the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). Moreover, the animal studies in this research were approved by the Animal Ethics Committee of Shandong University (permit number 20191018).

Author contributions

WS: data curation, formal analysis, software, investigation methodology. MW and ML: conceptualization and software. QL and LL: data curation and formal analysis. QW and RZ: resources, investigation methodology, and project administration. GL and H-CS: supervision and writing-review and editing. NZ: conceptualization, funding acquisition, investigation, writing-original draft, and writing-review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2022.1075161/full#supplementary-material

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EDITED BY
Yashi Mi,
University of Arizona,
United States

REVIEWED BY
Shaowei Wang,
University of Southern California,
United States
Rong Fan,
University of Massachusetts Amherst,
United States

*CORRESPONDENCE
Jun Wang
jun.wang@mssm.edu

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Benefits of dietary polyphenols in Alzheimer's disease

Farida El Gaamouch[1,2], Fiona Chen[1], Lap Ho[3], Hsiao-Yun Lin[1,2], Chongzhen Yuan[1,2], Jean Wong[1] and Jun Wang[1,2]*

[1]Department of Neurology, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [2]Geriatric Research, Education and Clinical Center, James J Peters VA Medical Center, Research & Development, Bronx, NY, United States, [3]Department of Genetics and Genomic sciences, Icahn School of Medicine at Mount Sinai, New York, NY, United States

Alzheimer's disease (AD) is an irreversible progressive neurodegenerative disease affecting approximately 50 million people worldwide. It is estimated to reach 152 million by the year 2050. AD is the fifth leading cause of death among Americans age 65 and older. In spite of the significant burden the disease imposes upon patients, their families, our society, and our healthcare system, there is currently no cure for AD. The existing approved therapies only temporarily alleviate some of the disease's symptoms, but are unable to modulate the onset and/or progression of the disease. Our failure in developing a cure for AD is attributable, in part, to the multifactorial complexity underlying AD pathophysiology. Nonetheless, the lack of successful pharmacological approaches has led to the consideration of alternative strategies that may help delay the onset and progression of AD. There is increasing recognition that certain dietary and nutrition factors may play important roles in protecting against select key AD pathologies. Consistent with this, select nutraceuticals and phytochemical compounds have demonstrated anti-amyloidogenic, antioxidative, anti-inflammatory, and neurotrophic properties and as such, could serve as lead candidates for further novel AD therapeutic developments. Here we summarize some of the more promising dietary phytochemicals, particularly polyphenols that have been shown to positively modulate some of the important AD pathogenesis aspects, such as reducing β-amyloid plaques and neurofibrillary tangles formation, AD-induced oxidative stress, neuroinflammation, and synapse loss. We also discuss the recent development of potential contribution of gut microbiome in dietary polyphenol function.

KEYWORDS

polyphenol, Alzheimer, oxidative stress, neuroinflammation, synaptic plasticity

Introduction

AD is a complex disease, which makes its pathophysiology difficult to decipher and consequently challenging to treat or cure. The classical pathologic hallmarks of AD include extracellular accumulation of β-amyloid (Aβ) and intracellular Tau protein aggregation which lead to, respectively, neuritic plaques and neurofibrillary tangles' formation in the brain. The amyloid plaques and tau tangles aggregate trigger successive deleterious events

and other chronic aberrant central nervous system (CNS) features such as hyperactive inflammation, oxidative stress, and sub-optimal energy metabolism which eventually lead to synapse loss and neuronal death. These cellular damages manifest in patients as a progressive neurocognitive impairment accompanied by language alterations and a progressive deterioration of a person's ability to perform everyday activities (Alzheimers Dement, 2020).

Aging is the greatest risk factor for AD. Many genetic risk factors have also been identified, with ApoE4 being the biggest known genetic risk factor. However, there are also lifestyle and environmental risk factors for AD, including lifestyle conditions associated with diabetes and cardiovascular diseases and environmental factors leading to traumatic brain injuries and depression (Armstrong, 2019). Aging and, to a large extent, genetic risk factors, are not amenable to modification (Riedel et al., 2016). In contrast, lifestyle and environmental risks are more readily modifiable. Moreover, there is increasing evidence implicating specific lifestyle factors (e.g., dietary factors such as specific phytochemicals) or environmental factors (e.g., exercise) may protect against AD mechanisms (Xu et al., 2015; Dominguez et al., 2021; Guasch-Ferré and Willett, 2021; Zhang et al., 2021). There is increasing interest in novel AD treatments targeting select relevant lifestyles or environmental factors. In this review, we will focus our discussion on a specific subclass of protective dietary phytochemicals, namely polyphenols, with promise for AD therapeutics.

Dietary components have a direct molecular impact on AD. Over the past decade, a widely distributed subclass of dietary components, polyphenols, have raised great interest in the scientific community for their potential role in protection against AD. Substantial number of clinical trials have been conducted to assess their clinical benefits against AD and associated cognitive impairments using diverse source of polyphenols: either in the form of whole fruit or fruit products such as blueberry, grape juice, pomegranate juice (Krikorian et al., 2010; Krikorian et al., 2012; Bookheimer et al., 2013; Krikorian et al., 2022), or in the form of extracts such as curcumin, grape seed polyphenol extract (GSPE), or pure synthetic material such as resveratrol (Baum et al., 2008; Kennedy et al., 2010; Patel et al., 2011; Mecocci and Polidori, 2012; Ringman et al., 2012; Turner et al., 2015; Moussa et al., 2017). The historical interest in dietary polyphenols is attributed to their high abundance in general food supplies and their antioxidant properties (Scalbert et al., 2005). However, current research of the benefits of dietary polyphenols is largely focused on their interaction and modulation of metabolic pathways regulating inflammation (Maleki et al., 2019; Ansari et al., 2020), endothelial function (Patel et al., 2018; Li et al., 2019; Parsamanesh et al., 2021), fatty acids, amino acids and carbohydrates metabolism (Hanhineva et al., 2010; Wang S et al., 2014; Naveed et al., 2018; Rothenberg et al., 2018; Różańska and Regulska-Ilow, 2018). Collectively, polyphenols' ability to block free radicals' activity, repair DNA damage, modulate the gene expression involved in metabolism, and act as signaling molecules to promote antioxidant defense support the development of dietary polyphenols in AD and other diseases (Azqueta and Collins, 2016; Hussain et al., 2016; Jiang, 2019; Maleki et al., 2019; Prasanth et al., 2019; Xing et al., 2019; Ohishi et al., 2021; Shen et al., 2022).

In this review, we summarize the major molecular mechanisms that correlate the health benefits of dietary polyphenols in AD physiopathology, focusing on the potential effects of these polyphenols to protect against Tau- and Aβ-mediated pathogenesis, oxidative stress, inflammation, synapse loss and memory deterioration.

Polyphenols

Polyphenols are a class of organic compounds characterized by the presence of more than one phenol structural unit (several hydroxyl groups on aromatic rings). These phytochemicals have a protective role in plants involving in defense against ultraviolet radiation or pathogens invasion. They are mainly found in plant-based food diet (Manach et al., 2004; Maraldi et al., 2014). The number of phenol rings in their molecular structure will define the chemical subclass they belong to. More than 8,000 naturally occurring polyphenols exist and can be grouped in 4 chemical subclasses: flavonoids, phenolic acids, stilbenes, and lignans (Manach et al., 2004; Maraldi et al., 2014).

Flavonoids

Flavonoids are the largest and most widespread groups of plant-derived secondary metabolites, with a 15-carbon skeleton, that have been described to exert beneficial effects in the prevention of neurodegenerative diseases (Dai et al., 2006; Kuriyama et al., 2006). Their highly reactive hydroxyl group is largely responsible for their ability to scavenge free radicals and/or chelate metal ions (Kumar et al., 2013; Kumar and Pandey, 2013). Flavonoids can be subdivided into different subgroups: Flavonols, with quercetin and kaempferaol as the representative compounds, are found in all types of food, with higher quantity in onions, broccoli, kale, blueberries and red wine (Herrmann, 1976). In comparison, flavones (luteolin and apigenin) are much less present in fruits and vegetables. Isoflavones are phytoestrogens mainly found in legume such as soya (Coward et al., 1993; Reinli and Block, 1996). Catechin and epicatechin are the basic units of flavanols and they form various oligomers and polymers through C4-C8 or C4-C6 interflavan bonds. Flavanols are found in many types of fruit and in red wine, however green tea and chocolate are the richest sources (Lakenbrink et al., 2000). Anthocyanins (cyanidin, malvidin, etc.) and chalocone (phloretin, arbutin, etc.) are mostly abundant in fruits and vegetables.

Phenolic acids

Phenolic acids are the most abundant group of bioactive compounds present in almost all plants (Rashmi and Negi, 2020).

Phenolic acids are hydroxyl derivatives of benzoic and cinnamic acid. Hydroxybenzoic acid is found in very low quantity in plants. Hydroxycinnamic acids mainly consist of coumaric, sinapic, caffeic and ferulic acids. The richest dietary source of hydroxycinnamic acids are cherries, apples, berries and kiwi (Fleuriet et al., 1990). Caffeic acid is the most abundant hydroxycinnamic acid in many fruits and ferulic acid is mostly found in grains (Krzysztof et al., 1982; Rouau et al., 1997).

Lignans

Lignans are mostly found in plant seeds and are precursors to phytoestrogens. They are a class of secondary plant metabolites. There is a growing interest in lignans in recent years due to their strong bioactivities in antioxidation, anti-inflammation and neuroprotection (Saleem et al., 2005; Teponno et al., 2016). The richest dietary source of lignans are linseeds (Thompson et al., 1991).

Stilbenes

Stilbenes are poorly present in the human diet. The most known and studied is resveratrol, for which anticarcinogenic effects have been shown in medicinal plants screening. Resveratrol is found in wine at a very low quantity (Bertelli et al., 1998; Bhat and Pezzuto, 2002; Vitrac et al., 2005).

Polyphenols modulate Aβ production, oligomerization, and clearance

Senile plaques are mainly composed of β-Amyloid protein (Aβ; Masters et al., 1985) that results from proteolysis of the amyloid precursor protein (APP) by the enzymes β-secretase (BACE) and γ-secretase through amyloidogenic pathway. The non-amyloidogenic process is initiated by α-secretase rather than BACE leading to the formation of soluble APPα and C-terminus fragments and preventing Aβ generation. Both enzymes compete in APP proteolysis and their activities strongly affect Aβ production.

Several studies have demonstrated that polyphenols can modulate Aβ production by either increasing α-secretase activity or inhibiting BACE. (−)-Epicatechin, epigallocatechin, epigallocatechin-3-gallate (EGCG) and curcumin are potent inhibitors of amyloidogenic processing (Wang X et al., 2014; Cox et al., 2015; Guo et al., 2017). *In vitro* experiments conducted in neuronal cell line expressing human APP showed that treatment with EGCG significantly decreased Aβ production (Rezai-Zadeh et al., 2005). These results have been reproduced *in vivo*, where intraperitoneal injection of epigallocatechin-3-gallate in Tg2576 mouse model of AD decreased Aβ levels and favored the non-amyloidogenic α-secretase mediated pathway (Rezai-Zadeh et al., 2005). Another study demonstrated that curcumin treatment increases α-secretase activity (Narasingappa et al., 2012). Curcuminoids and epigallocatechin-3-gallate treatment inhibit BACE activity in neuronal cells (Wang X et al., 2014). While EGCG alone (Cheng et al., 2012) failed to abolish BACE activity *in vivo*, in combination with ferulic acid (FA), a BACE modulator (Mori et al., 2013), epigallocatechin-3-gallate could block BACE activity in APP/PS1 AD mice and reduced amyloidosis and improved cognitive function (Mori et al., 2019). The flavones apigenin (Zhao et al., 2013) and nobiletin (Nakajima et al., 2015) were also shown to significantly reduce soluble and insoluble Aβ as well Aβ deposits in the brain in AD mice (Onozuka et al., 2008). Similarly, chronic administration of the flavone baicalein decreases Aβ production (Zhang et al., 2013). Quercetin (3,5,7,3′,4′-pentahydroxyflavone) is a dietary flavonol widely distributed in plants, fruits and vegetables, and it is also effective at modulating contents of soluble and insoluble Aβ in the brain (Sabogal-Guaqueta et al., 2015; Moreno et al., 2017). Altogether, these results show that select polyphenols can modulate α-secretase or BACE activities and reduce Aβ production both *in vitro* and *in vivo*, however, there has been very few research on mechanisms of action and how select polyphenols promote non-amyloidogenic or inhibit amyloidogenic processing of APP.

Aβ monomers can assemble into soluble and insoluble Aβ oligomers. Insoluble forms of Aβ mostly deposit into extracellular plaques, while the soluble oligomers are now considered the most toxic species in driving Aβ-mediated synaptic toxicity and neuronal death (Kumar et al., 2013). Polyphenols have been shown to prevent Aβ oligomerization or to remodel Aβ oligomers into nontoxic forms. Ehrnhoefer et al. showed that EGCG inhibits Aβ fibrillogenesis leading to unstructured Aβ oligomers. It promotes the assembly of newly formed oligomers into smaller and amorphous nontoxic protein aggregates (Ehrnhoefer et al., 2008). Another group showed (−)-epigallocatechin-3-gallate could bind to preformed fibrils or large oligomers and remodel them into less toxic assemblies (Bieschke et al., 2010). Curcumin can substantially block Aβ oligomerization in a dose dependent manner (Yang et al., 2005; Reinke and Gestwicki, 2007) and it is able to inhibit fibril formation and destabilize preexisting fibrils (Doytchinova et al., 2020). Resveratrol does not prevent Aβ oligomerization, however it can reduce Aβ cytotoxicity by remodeling the oligomers into nontoxic forms (Feng et al., 2009; Ladiwala et al., 2010; Fu et al., 2014). Wang et al., demonstrated that moderate red wine consumption could reduce Aβ aggregation, and improved cognitive function when administered to AD mice (Wang et al., 2006). The same group investigated the specific compounds responsible for Aβ-lowering activity and demonstrated that dietary supplementation with grape seed polyphenolic extract (GSPE), largely composed of catechin and epicatechin monomer, oligomer and polymer, significantly attenuated the development of AD-type Aβ-related cognitive deterioration (Wang et al., 2008). Further investigation revealed that GSPE is a potent inhibitor for the oligomerization of Aβ

peptides. These observations demonstrate that by modulating or remodeling of Aβ oligomers, polyphenols can interfere with the formation of soluble toxic forms of Aβ that are responsible for AD-associated neuronal damages.

Polyphenols can also reduce Aβ pathology by enhancing Aβ clearance. For example, resveratrol was shown to facilitate Aβ clearance *in vitro* (Marambaud et al., 2005; Vingtdeux et al., 2010) but the mechanism remains poorly understood. Several hypothesis have been proposed, for example resveratrol may promote intracellular degradation of Aβ *via* mechanism that involves the autophage and lysosome (Marambaud et al., 2005). Resveratrol may also stimulate the brain insulin-degrading enzyme activity (Rege et al., 2015) which in return will degrade Aβ thereby facilitating Aβ clearance.

Polyphenols modulate Tau phosphorylation

Aberrant aggregation of microtubule-associated protein Tau is another contributor to AD pathology. Tau phosphorylation regulates its ability to bind microtubules. Hyper phosphorylated Tau forms paired helical filaments (PHF) and neurofibrillary tangle (NTF) inclusions that not only alter the cytoskeletal and associated transport system, but also affect cellular signaling and mitochondrial function (Johnson et al., 2016; Bejanin et al., 2017; Kametani and Hasegawa, 2018).

Tau phosphorylation in neuronal cells is regulated by the balance of the dephosphorylation catalyzed mainly by phosphatase 2A (PP2A; Gong et al., 1993) and phosphorylation catalyzed by cdk5, GSK-3β, PKA and other kinases (Medina et al., 2011; Cavallini et al., 2013). Select polyphenols can modulate Tau hyperphosphorylation and subsequent NFTs formation through inhibiting AD-tau kinases or promoting PP2A. Resveratrol has been shown to inhibit the hyperphosphorylation of Tau (He et al., 2016); additionally, in the senescence accelerated mice P8 (SAMP8), resveratrol inhibits Ser^{396} Tau phosphorylation by GSK-3β (Porquet et al., 2013). Resveratrol can also modulate Tau hyperphosphorylation by increasing PP2A activity, which leads to Tau dephosphorylation (Schweiger et al., 2017). Similarly, in an okaidic acid-injection model for AD, curcumin treatment inhibited Tau hyperphosphorylation through activation of GSK-3β pathway (Wang et al., 2019). Oral GSPE supplementation is also effective in significantly modulating Tau-mediated pathogenic phenotypes, including Tau hyperphosphorylation, misfolding into fibrillar polymers and subsequently aggregation into AD-type NFT in various tauopathy mouse models (Ho et al., 2009; Wang et al., 2010; Ksiezak-Reding et al., 2012; Santa-Maria et al., 2012). GSPE is largely composed of proanthocyanidin (PAC) catechin and epicatechin in monomeric, oligomeic and polymeric forms. Bioavailability studies conducted in rats by Ferruzzi et al. (2009) demonstrated that methylated and glucuronidated catechin and epicatechin can be found in the plasm following oral administration of GSPE. Moreover, they also reported that following single oral dosing, these polyphenol metabolites were not found in the brain, however, following repeated dosing, these metabolites could be detected in the brain (Ferruzzi et al., 2009). Similar studies were conducted in AD mouse showing that catechin and epicatechin metabolites can only be found in the brain of the mouse fed with monomeric fraction of the GSPE, but not the polymeric fraction of the GSPE. Moreover, they reported that only the monomeric fraction were effective in reducing amyloid neuropathology and improving cognitive function in AD mice (Wang et al., 2012).

Biophysical studies demonstrated that polyphenols may structurally change Tau protein and prevent its self-association. For example, in the presence of arachidonic acid, Tau self-assembles into β-sheet containing filaments, but in the presence of curcumin, arachidonic acid-mediated filament formation is abolished (Rane et al., 2017). Similarly, epicatechin-3-gallate, myricetin(Taniguchi et al., 2005) and rosmarinic acid could also inhibit Tau β-sheet formation (Cornejo et al., 2017).

Polyphenols modulate AD-associated oxidative stress

The brains of AD patients show significant oxidative stress-associated damage including protein oxidation, lipid peroxidation, DNA damage suggesting the imbalance of free radical generation and antioxidant activity in the brain (Christen, 2000). In AD brain, the main sources of oxidative stress are from the free radicals generated from mitochondria and redox-active metals. Lipid peroxidation occurs when these oxidants attack polyunsaturated fatty acids (Ramana et al., 2014). Free radical-induced lipid peroxidation is widespread in AD brain. Reactive oxygen species (ROS) can also attack amino acid side chains or the protein backbones and generate protein carbonyl derivatives (Butterfield and Stadtman, 1997). Protein carbonyl content was found to be significantly increased in the hippocampus and inferior parietal lobule in AD subjects comparing to normal controls (Hensley et al., 1995; Aksenov et al., 2001). ROS-induced oxidation of key enzymes or structural proteins can significantly impair their cellular function leading to neurodegeneration and cell death (Hensley et al., 1995; Butterfield and Stadtman, 1997; Aksenov et al., 2001). ROS can also leading to base alteration, single and double strand breaks or DNA-protein crosslinkings (Sohal and Weindruch, 1996; DNA Oxidation in Alzheimer's Disease, 2006).

The therapeutic efficacy of flavonoids is historically attributed to their antioxidant potency and natural free radical scavenging properties (Mercer et al., 2005). Chronic administration of nobiletin to AD mice for 2–3 months significantly reduced brain ROS (Nakajima et al., 2015) and other oxidative stress markers (Nakajima et al., 2013). Quercetin has been shown to be strongly effective at scavenging free radicals and preventing oxidant-induced apoptosis (Rice-Evans et al., 1995; Heijnen et al., 2002; Choi et al., 2003). In addition to its high oxygen radical scavenging

properties, quercetin also has the ability to inhibit lipid peroxidation (Fiorani et al., 2010) and to chelate iron and other metal ions that could be detrimental to the brain (Rice-Evans et al., 1995; Salganik, 2001). Chronic administration of the flavone apigenin to an AD mouse model induced a significant decrease of oxidative stress accompanied by increased superoxide dismutase and glutathione peroxidase activities (Rice-Evans et al., 1995; Fiorani et al., 2010; Zhao et al., 2013). Curcumin is another strong antioxidant and can effectively stabilize ROS (Basnet and Skalko-Basnet, 2011). It acts on the inner membrane of mitochondria, facilitating their depolarization, thus preventing the formation of ROS (Zhu et al., 2004). It was also shown to stop the free radical proliferation when administered intravenously to rodent (Jiang et al., 2007). Resveratrol can increase the superoxide dismutase enzyme activity hence reduce ROS formation *in vivo* (Chen et al., 2016). *In vivo* studies have shown that catechin and epicatechin can prevent ROS formation and lipid peroxidation in AD models. A single oral dose of epicatechin could effectively prevent Aβ-mediated lipid peroxidation and ROS formation in hippocampal formation in rats (Cuevas et al., 2009). Blueberry is rich in anthocyanins and proanthocyanidins. Blueberry extract was shown to be able to increase redox buffer glutathione and protect amyloid toxicity through inhibition of MAP kinase and CREB-mediated ROS signaling (Brewer et al., 2010). In humans, it was shown that blueberry supplementation protects against cognitive decline in people with high risk for developing dementia (Krikorian et al., 2022).

Polyphenols modulate AD-associated inflammation

There is increasing consensus that immunological perturbations are major contributors to AD pathogenesis. This is supported by the genome-wide association studies linking myeloid cell-specific genes, such as TYROBP, TREM2 and CD33, with late-onset AD (LOAD). The phenomenon called neuroinflammation is a critical factor in AD pathogenesis. While the exact role of inflammation in AD remains to be investigated, it has been suggested that acute and systemic inflammation, manifested by microgliosis and astrogliosis, can accelerate AD progression and worsen cognitive impairments (Holmes et al., 2009).

Many dietary polyphenols have demonstrated their anti-inflammatory activities both *in vitro* and *in vivo*. Among these, curcumin and resveratrol are the most studied molecules for their potential application in AD treatment. Curcumin was shown to be able to block NF-kappa B action and associated inflammation cascade (Singh and Aggarwal, 1995; Hackler et al., 2016). In addition, curcumin also has the ability to inhibit Aβ-induced pro-inflammatory cytokines and chemokines release (Sundaram et al., 2017). Resveratrol attenuates Aβ-mediated microglia inflammation through inhibition of the TLR4/NF-κB and/or NLRP3 and STAT signaling pathway (Capiralla et al., 2012; Feng and Zhang, 2019). Resveratrol can also activate SIRT1 both *in vitro* and *in vivo* (Herskovits and Guarente, 2014; Favero et al., 2018). SIRT1 is a histone deacetylase that can epigenetically reprogram inflammation. In animal models, resveratrol treatment improved spatial memory, reduced neuroinflammation and increased neurotrophins in the brain of AD mice (Gong et al., 2010; Sun et al., 2019; Broderick et al., 2020). In humans, resveratrol was shown to modulate neuroinflammation and induce adaptive immunity in patients with AD (Moussa et al., 2017). Subjects with mild–moderate AD treated with synthetic resveratrol showed significant decrease of MMP9 in the cerebral spinal fluid (CSF). MMP9 is a protein that interferes with the blood brain barrier (BBB) function. The decrease of CSF MMP9 in AD suggest that resveratrol may mitigate inflammatory responses in the brain by reducing the permeability of CNS and lower infiltration of leukocytes and other inflammatory agents into the brain. Other polyphenols such as flavonoids fisetin, quercetin and luteolin were also shown to decrease inflammation in different AD mouse models and reduce astrogliosis and microgliosis (Sharma et al., 2007; Currais et al., 2014; Currais et al., 2018). Blueberry and its extract have also been shown to be able to inhibit amyloid-mediated microglia activation through attenuation of p44/42 MAPK signaling both *in vitro* and *in vivo* mouse model (Joseph et al., 2003; Zhu et al., 2008).

Polyphenols modulate synaptic function and memory

Selective polyphenols also showed promising effects in rescuing cognitive function in transgenic AD mouse models. For example, old 5xFAD mice chronically treated with 7,8-dihydroxyflavone (7,8-DHF) reduced synapse loss in the brain and performed better at the working memory Y maze test (Devi and Ohno, 2012; Zhang et al., 2014). Oral administration of apigenin led to increased activation of ERK/CREB signaling and learning and memory improvement in 2xFAD mice (Zhao et al., 2013). Learning and memory were improved in both the 1 × FAD and 3xFAD mouse models of AD following treatment with nobiletin (Onozuka et al., 2008; Nakajima et al., 2015). Mice treated with fisetin showed increased activation of ERK/MAPK signaling, increased expression of synaptic proteins and improved cognitive function (Currais et al., 2014; Ahmad et al., 2017; Currais et al., 2018). Flavonoid rutin, a quercetin molecule with the addition of disaccharide rutinose, was also found to be effective in improving cognitive function through increased expression of brain derived neurotrophic factor (BDNF) in rats injected with Aβ (Moghbelinejad et al., 2014). Green tea contains high levels of EGCG can prevent the loss of synaptic proteins and cognitive impairments in a 1xFAD mouse model (Walker et al., 2015). On the same note, anthocyanins were also found to be able to reduce synaptic protein loss and improve memory function (Ali et al., 2017; Kim et al., 2017). Anthocyanin from grape juice was shown to rescue oligomeric Aβ-induced long term potentiation

(LTP) deficit in hippocampal slices (Wang et al., 2014a). Wang et al. explored the effect of cocoa flavanols on AD pathogenesis (Wang et al., 2014b). In their study they showed that catechin and epicatechin enriched cocoa extracts interfered with Aβ oligomerization and prevented synaptic deficits. They demonstrated that application of cocoa extracts on mice hippocampal slices could prevent Aβ-induced LTP deficit (Wang et al., 2014b). Cocoa was also shown to prevent Aβ oligomer-induced neurite dystrophy by activating BDNF in neuronal cultures (Cimini et al., 2013). These observations have also been corroborated by clinical studies demonstrating that cocoa flavanols enhance the dentate gyrus function and reduces cognitive decline in humans (Crews et al., 2008; Desideri et al., 2012; Scholey and Owen, 2013; Brickman et al., 2014). Blueberry was shown to improve memory function in APP/PS1 AD mice through increase of ERK signaling and neural sphingomyelin-specific phospholipase C activity (Joseph et al., 2003).

Polyphenols and gut microbiome

The biological activity of dietary polyphenols largely depends on the bioavailability of the bioactive forms of the parent compounds in the target organs. Once ingested, polyphenols are absorbed and metabolized first in the gastrointestinal tract and then are further modified in the liver through glucuronidation, sulfonation, or methylation, before entering the blood stream. The biological activities of their metabolites can be very different from the parent compounds. For example, Serra et al. explored this relationship between dietary polyphenols and gut metabolism using an anthocyanin-rich extract obtained from Portuguese blueberries and a simulated gastrointestinal digestion process. Both the digested and non-digested extracts displayed different chemical compositions and had different effects on neuroinflammation (Serra et al., 2020). Gut microbiome is receiving increased attention due to their potential role in health and disease (Durack and Lynch, 2019). Recent studies have demonstrated strong links between polyphenol metabolism and the gut microbiome (Hervert-Hernández and Goñi, 2011; Fraga et al., 2019). The gut microbiota can influence the process and metabolism of polyphenols, which may influence the production and diversity of polyphenol metabolites; Polyphenols have the ability to influence the intestinal environment, which allows them to modulate the composition of gut microbiome (De Bruyne et al., 2019). Moreover, there is also bidirectional communications between gut microbiota and the central nervous system, the so-called gut-brain axis and currently, the gut-brain axis is one of the favorable targets for therapeutic treatment of neurodegenerative disorders including AD due to their bidirectional interactions that may affect brain function (Carabotti et al., 2015; Reddy et al., 2020). Curcumin, for example, exhibits beneficial effects against AD despite having limited blood–brain barrier penetration. It is postulated that curcumin becomes a more effective neuroprotective agent after undergoing metabolism by gut microbial and its interaction with the gut-brain axis also allows it to react indirectly with the CNS and exerts its neuroprotective activity (Di Meo et al., 2019; Reddy et al., 2020).

Conclusion

Pathological mechanisms involved in Alzheimer's pathogenesis include both Aβ and Tau toxicity. Therapeutic strategies aiming at targeting one or the other continue to fail in clinical trials. Polyphenols offer a new approach that can simultaneously target Aβ, Tau, neuroinflammation and oxidative stress, which could lead to better outcomes.

Dietary factors and diet composition can play a critical role in AD prevention. Our review lists few of the numerous polyphenols, considered as confirmed or promising therapeutic candidates due to their potent anti-inflammatory, antioxidant properties and AD-disease modifying activities (Table 1). AD is a multifactorial disease and the current lackluster performance of clinical studies is, in part, due to the prevailing approach targeting individual pathogenic mechanisms. Most of the polyphenolic compounds have exhibited pleiotropic

TABLE 1 Role of polyphenols in modulating AD-type neuropathology.

Polyohenol	Activity	Mechanism	Model	Dose	Reference
Epicatechin	Aβ↓	BACE↓	TASTPM	15 mg/day	Cox et al., 2015
	ROS↓	-	Aβ injected rat	30 mg/kg	Cuevas et al., 2009
EGCG	Aβ↓	α-secretase ↑	Tg2576	20 mg/kg	Rezai-Zadeh et al., 2005
	Aβ↓/memory↑	BACE↓	SAMP8	15 mg/kg	Guo et al., 2017
	Aβ fibril↓	-	*in vitro*	-	Ehrnhoefer et al., 2008; Bieschke et al., 2010
Curcumin	Aβ ↓	BACE↓	Drosophila Melanogster	1 mM	Wang et al., 2014
	Aβ oligomer↓	Aβ aggregation↓	Tg2576	25 mg/kg	Yang et al., 2005
	Tau phosphorylation↓	GSK-3β↑	okadaic acid AD model	10 μg i.p.	Wang et al., 2019
	neuroinflammation↓	NF-κB↓	*in vitro*	-	Singh and Aggarwal, 1995
	neuroinflammation↓/Memory↑	CDK5↓	p25Tg	0.8 g/kg	Sundaram et al., 2017
Apigenin	Aβ↓/ROS↓/memory↑	BACE↓	APP/PS1	40 mg/kg	Zhao et al., 2013

(Continued)

TABLE 1 (Continued)

Polyohenol	Activity	Mechanism	Model	Dose	Reference
	ROS↓	SOD↑/GPx↑	*in vitro*	-	Rice-Evans et al., 1995; Fiorani et al., 2010
Nobiletin	Aβ↓/ROS↓/memory↑	-	3xTg	30 mg/kg	Nakajima et al., 2015
	Aβ↓/memory↑	ERK phosphorylation↑	APP-SL 7–5 Tg	10 mg/kg i.p.	Onozuka et al., 2008
	Tau phosphorylation↓/ROS↓/memory↑	-	SAMP8	10–50 mg/kg	Nakajima et al., 2013
Quercetin	Aβ↓/memory↑	BACE↓	3xTg	25 mg/kg	Sabogal-Guaqueta et al., 2015
	neuroinflammation↓/memory↑	-	SAMP8	25 mg/kg	Moreno et al., 2017
	ROS↓	-	*in vitro*	-	Choi et al., 2003; Heijnen et al., 2002; Rice-Evans et al., 1995; Fiorani et al., 2010
Resveratrol	Aβ oligomer toxicity ↓	-	*in vitro*	-	Feng et al., 2009; Ladiwala et al., 2010; Fu et al., 2014
	Aβ ↓	-	*in vitro*	-	Marambaud et al., 2005
	Aβ clearance ↑	AMPK/mTOR autophage	*APP/PS1*	350 mg/kg	Vingtdeux et al., 2010
	Aβ clearance ↑	IDE↑	*in vitro*	-	Rege et al., 2015
	Tau phosphorylation↓/memory↑	ADAM-10↑/GSK-3β↑/CDK5↓	SAMP8	1 g/kg	Porquet et al., 2013
	neuroinflammation↓/Aβ oligomer↓/memory↑	NF-κB↓	SAMP8	4 g/kg	Broderick et al., 2020; Gong et al., 2010; Sun et al., 2019
Fisetin	neuroinflammation↓/ROS↓/memory↑	CDK5↓/SAPK/JNK↓	SAMP8	25 mg/kg	Currais et al., 2018
	neuroinflammation↓/memory↑	CDK5↓	APPswe/PS1dE9	25 mg/kg	Currais et al., 2014
GSPE	Aβ oligomer↓/memory↑	Aβ aggregation↓	Tg2576	200 mg/kg	Wang et al., 2008
	tau aggregation↓	-	*in vitro*	-	Ho et al., 2009; Ksiezak-Reding et al., 2012
	Tau phosphorylation↓	ERK1/2↓	TMHT	200 mg/kg	Wang et al., 2010
	Tau phosphorylation↓/tau aggregation↓/motor function↑	-	*JNPL3*	150 mg/kg	Santa-Maria et al., 2012

EGCG: epigallocatechin-3-gallate; GSPE: grape seed polyphenolic extract.

bioactivities (Table 1) which may have advantages over conventional pharmaceutical drugs for the treatment of AD. In spite of their multi-targeting features, clinical development of polyphenols for AD is hampered by their poor absorption and limited brain bioavailability. Moreover, most of the available polyphenol metabolite forms, following digestive and hepatic activity, may not have the same biological activity as the native compound. Therefore, the *in vitro* biological activities of "parental" polyphenol forms may not relevant to biological activities *in vivo*, which is the ultimate arbitrator of therapeutic benefit. Future advancement of polyphenols in AD prevention and/or treatment will largely rely on the development of select polyphenols or their derivatives with better brain bioavailability while preserving their multi-targeting bioactivities.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Zhigang Liu,
Northwest A&F University, China

REVIEWED BY
Rong Fan,
University of Massachusetts Amherst, United States
Xiaoshuang Dai,
Beijing Genomics Institute (BGI), China

*CORRESPONDENCE
Emmanuelle Le Bars
✉ e-le_bars@chu-montpellier.fr

†These authors have contributed equally to this work and share last authorship
‡Members are listed in the "MAPT/DSA group" section

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Impact of multidomain preventive strategies on functional brain connectivity in older adults with cognitive complaint: Subset from the Montpellier center of the ancillary MAPT-MRI study

Lisa Perus[1,2,3,4], Jean-François Mangin[4,5], Jérémy Deverdun[3], Laure-Anne Gutierrez[2], Emmanuelle Gourieux[4], Clara Fischer[4], Liesjet E. H. Van Dokkum[3], Clara Manesco[6], Germain Busto[1,2], Sophie Guyonnet[7,8], Bruno Vellas[7,8], Audrey Gabelle[1,2†], Emmanuelle Le Bars[3*†] and the MAPT/DSA group[‡]

[1]Memory Resources and Research Center, Department of Neurology, Gui de Chauliac Hospital, Montpellier, France, [2]INM, Univ Montpellier, INSERM, CHU Montpellier, Montpellier, France, [3]Institut d'Imagerie Fonctionnelle Humaine, I2FH, Department of Neuroradiology, Gui de Chauliac Hospital and University of Montpellier, Montpellier, France, [4]CATI, US52-UAR2031, CEA, ICM, SU, CNRS, INSERM, APHP, Ile de France, France, [5]Université Paris-Saclay, CEA, CNRS, Neurospin, UMR9027 Baobab, Gif-sur-Yvette, France, [6]Laboratoire Charles Coulomb (L2C), University of Montpellier, CNRS, Montpellier, France, [7]Inserm UMR 1295, University of Toulouse III, Toulouse, France, [8]Gérontopôle, Department of Geriatrics, CHU Toulouse, Toulouse, France

Introduction: The impact of multi-domain preventive interventions on older adults, in particular on those with higher risk to develop Alzheimer's disease (AD), could be beneficial, as it may delay cognitive decline. However, the precise mechanism of such positive impact is not fully understood and may involve brain reserve and adaptability of brain functional connectivity (FC).

Methods: To determine the effect of multidomain interventions (involving physical activity, cognitive training, nutritional counseling alone or in combination with omega-3 fatty acid supplementation and vs. a placebo) on the brain, longitudinal FC changes were assessed after 36 months of intervention on 100 older adults (above 70 year-old) with subjective cognitive complaints.

Results: No global change in FC was detected after uni or multidomain preventive interventions. However, an effect of omega-3 fatty acid supplementation dependent on cognitive decline status was underlined for frontoparietal, salience, visual and sensorimotor networks FC. These findings were independent of the cortical thickness and vascular burden.

Discussion: These results emphasize the importance of patient stratification, based on risk factors, for preventive interventions.

KEYWORDS

magnetic resonance imaging (MRI), resting-state functional MRI (rs-fMRI), multidomain intervention, exercise, cognitive training, omega-3 fatty acids

1. Introduction

The appearance of clinical symptoms leading to a loss of autonomy in neurodegenerative disorders takes about 15 years after the development of brain physiopathological lesions. This leaves a large time-window to initiate preventive treatments to slow down cognitive decline, or hopefully impede the onset of Alzheimer's disease (AD) (Bhatti et al., 2020). Still, the most impactful prevention strategies and the right populations to be targeted remain to be identified. Clinical trials have evaluated the impact of multiple interventions on older adults' cognition, including physical activity, cognitive stimulation, mediterranean diet, cardiovascular prevention, or nutritional supplementation (Brini et al., 2018; Kivipelto et al., 2018; Bott et al., 2019; Buckinx and Aubertin-Leheudre, 2021). Nutritional interventions have focused on multiple nutrients, although some supplements, such as omega-3 fatty acids, have received particular attention. Indeed, they show protective effects against age-related processes such as neuroinflammation (Joffre et al., 2020), oxidative stress (Mora et al., 2022), and blood-brain barrier dysfunction (Barnes et al., 2021). Omega-3 fatty acid supplementation has been associated with reduced memory (Yurko-Mauro et al., 2015) and cognitive (Marti del Moral and Fortique, 2019) impairment and a reduced risk of developing dementia (Zhang et al., 2015). More recently, various studies have evaluated the impact of multidomain preventive strategies, that combine different interventions, as it has been hypothesized that their effect might be optimal as they simultaneously target multiple risk factors associated with AD (Kivipelto et al., 2018). Several studies suggest that multi-domain interventions are indeed more effective than single-domain interventions on the cognition of older adults with mild cognitive impairment (MCI) (Salzman et al., 2022). Positive results from the FINGER trial have prompted the creation of a global initiative to evaluate and optimize the effect of multidomain lifestyle interventions (Word-Wide FINGERS) (Kivipelto et al., 2020). If promising, the results of interventional studies are however disparate (Solomon et al., 2021). In addition, the question of how interventions impact the brain remains unanswered.

Magnetic resonance imaging (MRI) of the brain can reveal localized, subtle and functional alterations. Anatomical MRI data has been predominantly used to evaluate interventions related with physical activity (Haeger et al., 2019), multidomain interventions (Stephen et al., 2019), as well as diets and/or effect of nutrition patterns on the brain (Bos et al., 2016; Rodrigues et al., 2020). Taken together, they indicate for instance that omega-3 fatty acid supplementation, nutritional patterns or red blood cell levels are associated with larger brain (Conklin et al., 2007; Pottala et al., 2014; Witte et al., 2014; Berti et al., 2015; Prinelli et al., 2019) and hippocampus (Samieri et al., 2012; Pottala et al., 2014; Witte et al., 2014) volumes and with increased white matter integrity (Tan et al., 2012; Virtanen et al., 2013; Witte et al., 2014). More recently, resting-state functional MRI (rs-fMRI) has been identified as a biomarker of interest. It characterizes brain regions functional similarity, and can describe neurodegenerescence across the continuum of AD (Hohenfeld et al., 2018), as well as brain alterations in the early asymptomatic phase of subjective cognitive decline (Viviano and Damoiseaux, 2020). Moreover, functional connectivity (FC) in middle-aged or older adults has been shown to be impacted by interventions. It is modified by physical activity (Chen et al., 2020), and can be altered by nutrition (Rodrigues et al., 2020). Omega-3 fatty acid nutritional patterns have for instance been associated with enhanced functional networks efficiency (Zwilling et al., 2019), and differences in FC have been associated with differences in omega-3 fatty acid red blood cell levels (Talukdar et al., 2019) and supplementation (Park et al., 2020). Cognitive training also modifies neural networks related to important cognitive functions. It induces opposing cognitive patterns to the ones typically associated with aging and neuro-degeneration. On the one hand, it increases the within-network connectivity of the Default Mode Network (DMN) and on the other, the anticorrelation between the DMN and the frontoparietal network (FPN) (van Balkom et al., 2020).

Despite these promising results, no study has yet addressed the impact of a multidomain intervention combining physical activity, cognitive training, nutritional counseling and an omega-3 fatty acid supplementation on the FC of an older population with cognitive complaints and thus at-risk to develop AD. Such intervention has been implemented in the Multidomain Alzheimer Preventive Trial (MAPT) (Vellas et al., 2014). It showed no effect on the cognitive decline of older adults (>70 years' old) at-risk for AD (Andrieu et al., 2017). We aim to decipher whether, despite this apparent lack of effect on cognition, the intervention may impact brain FC, which is a sensitive marker of brain integrity (Charroud et al., 2016; Conti et al., 2021).

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; APOE, apolipoprotein E; CAIDE, cardiovascular risk factors, aging and dementia; CDR, clinical dementia rating; CSF, cerebrospinal fluid; DHA, docosahexaenoic acid; DMN, Default Mode Network; EPA, eicosapentaenoic acid; FC, functional connectivity; FINGER, Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability; FLAIR, fluid attenuated inversion recovery; FPN, FrontoParietal Network; GM, gray matter; IADL, instrumental activity of daily living; MAPT, Multidomain Alzheimer Preventive Trial; MCI, mild cognitive impairment; MMSE, Mini Mental State Examination; MI, multidomain intervention; MRI, magnetic resonance imaging; Om3, omega-3; PET, positron emission tomography; rs-fMRI, resting-state functional MRI; SMN, Sensorimotor Network; SN, Salience Network; SPC, Spatial Pairwise Clustering; TIV, total intracranial volume; VIS, Visual Network; WM, white matter; WMH, white matter hyperintensity.

We therefore aim:

- To investigate the impact of a multidomain preventive intervention on the FC of MAPT trial participants using whole brain rs-MRI data analysis;
- To study the effect of the intervention in subgroups defined by specific patient characteristics such as clinical dementia rating score (CDR; CDR = 0 vs. CRD = 0.5) or Fried's frailty criteria (Fried et al., 2004).

2. Materials and methods

2.1. General study design

All participants were recruited from the ancillary MAPT MRI study, which is fully described elsewhere (Vellas et al., 2014). Briefly, participants with either spontaneous memory complaint, limitation in one instrumental activity of daily living (IADL), slow gait speed ($\leqslant$0.8 m/s), or a combination of these factors, were included in a multi-center randomized controlled trial designed to assess the efficacy of omega-3 fatty acid supplementation (Om3) and multidomain intervention (MI) alone, or in combination (Om3 + MI) against a placebo (Pl). The multidomain intervention combined cognitive training, physical activity and nutritional counseling (e.g., recommendation to increase fruit and vegetable consumption) (Hercberg et al., 2008), and was applied during 36 months. Omega-3 fatty acid or placebo supplementations were delivered daily during 36 months: participants consumed two capsules containing either 400 mg docosahexaenoic acid (DHA) and a maximum amount of 112.5 mg per capsule of eicosapentenoic acid (EPA) or a placebo. Three hundred and eighty participants underwent MRI imaging, demographical, clinical and cognitive evaluations at baseline that were repeated at 36 months (M36). Participants were excluded from the trial if they were diagnosed with (a) dementia, (b) a Mini Mental State Examination (MMSE) score (Folstein et al., 1975) lower than 24, (c) any difficulty in basic living activity or (d) if they were already taking an omega-3 fatty acids supplementation. The demographic characteristics included, amongst others, age, sex, and educational level. Intervention efficacy was primarily assessed in the parent study by using a cognitive composite score. Participant subgroups were defined by different risk profiles at baseline (Andrieu et al., 2017). The MAPT study protocol was approved by the Advisory Committee for the Protection of Persons participating in Biomedical Research of the Toulouse University Hospital, and was authorized by the French Health Authority. The protocol (NCT00672685) can be found on a public access clinical trial database (www.clinicaltrials.gov). This ancillary study was approved by the scientific committee of the MAPT study group.

2.2. Specific MRI based data selection and acquisition

The MAPT-MRI study included 188 participants with rs-fMR scans available at both baseline and follow-up (M36). On average, the baseline scan was performed at 114 days (3–569) after the beginning of the intervention. The follow-up scan was performed on average at 986 days (695–1388) after the first MRI scan. Only participants passing the rs-fMRI quality control were included for further analysis. Quality control included a combined analysis of automatic metrics and visual inspection (head coverage, intensity inhomogeneity, ghosting artifacts, etc.). Motion was evaluated using Framewise Displacement (FD) and DVARS metrics to characterize volumes with excessive motion, as suggested by Power et al. (2012). Their respective thresholds were 0.2 mm and 0.5%ΔBOLD signal change. Images with more than 15% of volumes exceeding both of these thresholds were discarded, as were images exceeding a threshold of 2 degrees of rotation or a translation of more than a voxel size. We excluded 44 participants with strong motion, 24 with other artifacts, two with failed preprocessing and finally we included only participants scanned in Montpellier to eliminate biases induced by artificial differences like scanner type and center effects. This resulted in a total of 100 participants being eligible for further evaluation (see the study flowchart, Figure 1).

All imaging was performed with an 1.5T Siemens AVANTO scanner. rs-fMRI parameters were: voxel size = 3 $\times$ 3 $\times$ 5 mm^3, repetition time (TR) = 2,400 ms, echo time (TE) = 50 ms, flip angle = 90° and slice number = 28. The scanning lasted for 8.07 min. Anatomical 3DT1 MPRAGE parameters were: voxel size = 1 $\times$ 1 $\times$ 1 mm, TR = 2,100 s, TE = 1.4 ms, flip angle = 15°, inversion time (TI) = 1,100 ms, and slice number = 160. FLAIR sequence acquisition parameters were: voxel size = 0.9 $\times$ 0.9 $\times$ 5 mm, TR = 8s, TE = 109 ms, TI = 2.5 s, Turbo Factor = 21, and slice number = 27.

2.3. Resting-state functional MRI data preprocessing

Resting-state functional MRI data was preprocessed using the SPM12 toolbox (https://www.fil.ion.ucl.ac.\uk/spm/). The preprocessing steps were as follows: the first 10 volumes of the functional images were discarded to reach a steady-state, then we performed subsequently slice time correction, motion correction with a six-parameters rigid-body spatial transformation, normalization to MNI space, and smoothing with a 6 mm kernel. Anatomical images were segmented into white matter (WM), gray matter (GM) and cerebrospinal fluid (CSF). In addition, using the Conn toolbox (v19c) (Whitfield-Gabrieli and Nieto-Castanon, 2012), functional scans were de-spiked, and physiological artifacts (WM and CSF

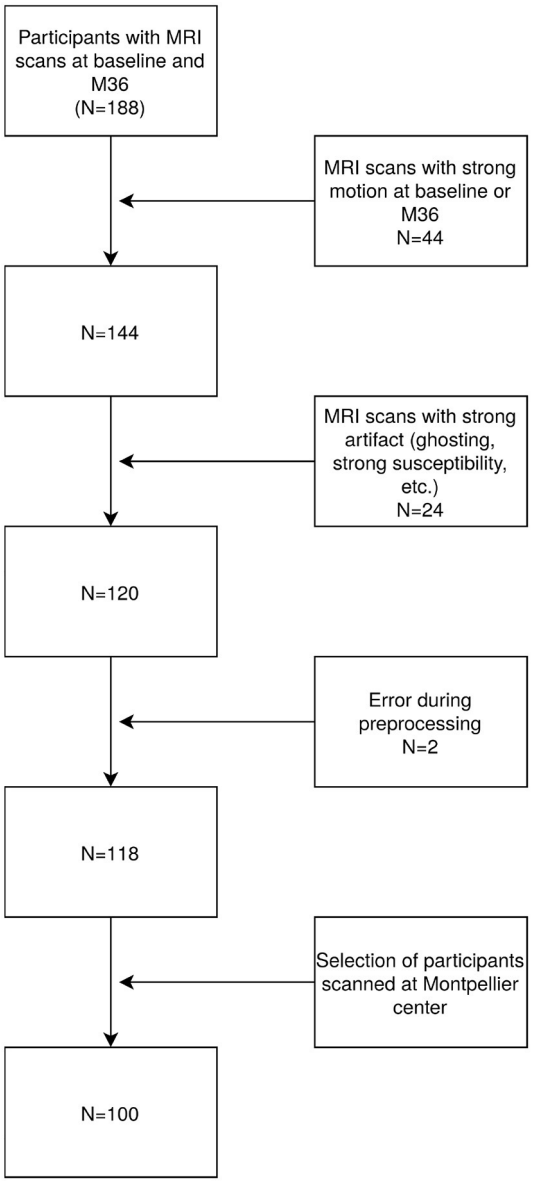

FIGURE 1
Chart of participants exclusion process.

mean global signals) and residual subject movement (six motion parameters and their derivatives) were removed with linear detrending and a band-pass filter of (0.008, 0.09) Hz was applied.

2.4. Computation of functional connectivity metrics

FC scores were computed between networks of the Bootstrap Analysis of Stable Clusters (BASC)–Cambridge atlas (Urchs et al., 2015), using the Conn toolbox. Each score is defined as the Fisher-transformed bivariate correlation coefficient between a pair of networks' mean time series. The BASC Cambridge atlas is a multiresolution atlas with multiple levels of whole-brain network parcellations ranging from seven to 444 networks. The coarser parcellation with seven networks (R7) comprises well-defined large-scale networks, such as the DMN (Badhwar et al., 2017). We selected for this analysis the parcellation with 36 networks (R36–630 connections) (Urchs et al., 2015). This parcellation has notably been used by Badhwar et al. (2017) to accurately pinpoint regions associated with AD in a review of rs-fMRI studies. For the purpose of clarity, we refer to the R36 networks as "subnetworks," as they are finer entities than the well-known networks defined in R7. Furthermore, in order to name and define these subnetworks, we report the larger brain networks from R7 with which they overlap. For instance, a subnetwork of R36 which overlaps with the R7 DMN network will be qualified as "a subnetwork of the DMN." Note that the overlap between a R36 subnetwork and R7 network is not necessarily total and that a subnetwork can overlap with different networks from R7. In that case, we report the R7 network with which the R36 subnetwork overlaps the most. We also report the main anatomical structures (e.g., middle frontal gyrus) that compose the subnetworks to name them.

2.5. Statistical methodology for functional connectivity analyses

All main statistical analyses were computed using non-parametric cluster statistics on the 630 subnetwork to subnetwork connections in the Conn toolbox. The Spatial Pairwise Clustering (SPC) method, as implemented in Conn, was used to identify related sets of connections sharing similar effects. Briefly, depending on the test, T or F statistics are computed for all connections, resulting in a matrix of statistical values. All subnetworks from this matrix are then sorted automatically using a hierarchical clustering procedure (Bar-Joseph et al., 2001) based on functional and anatomical similarity, and all connections are thresholded with an individual connection threshold. The thresholded connections are gathered into sets of non-overlapping clusters, that are characterized by their mass, that is, the sum of squared statistics over all their connections. The distribution of cluster mass values under the null hypothesis is estimated using permutations iterations on the data, and each cluster mass is compared to this distribution, resulting in a cluster uncorrected p-value representing the likelihood of having a randomly-selected cluster with a similar or larger mass under the null hypothesis. Family-Wise Error (FWE) correction is applied to all individual

cluster p-values, to control for false positives. Conn default individual connection threshold for the SPC method ($p < 0.01$) was applied and clusters were kept for p-FWE < 0.05. As specified above, the SPC method sorts input regions, here the R36 subnetworks, using a hierarchical clustering procedure based on functional and anatomical similarity. This measure of similarity and thus the clustering procedure depends on participants' data. In order to keep the same and most stable clustering for all statistical tests, we always used the clustering derived from data with all included subjects at both timepoints.

In cases when the Conn toolbox analyses revealed significant between-group differences or pairwise group differences needed to be assessed, we performed *post-hoc* analysis outside the toolbox. Functional connectivity values were extracted at baseline and 36 months for all significant connections as determined by the SPC method. *Post-hoc* analyses assessed changes of each connection from baseline to 36 months using R (version 3.6.1) multcomp package (version 1.4-10).

2.6. Statistical group analyses on functional connectivity

Participants' characteristics were described using mean (standard deviation) [min–max] for quantitative variables, and percentages for qualitative variables. The effects of the MAPT study interventions were assessed on the whole population using a repeated measure ANOVA adjusted for age, sex and level of education (binarized as $<$ University level or $\geq$ University level). Significant results using the unadjusted model were reported in the Supplementary material.

Additional subgroup analyses were also performed, and interaction effects between interventions and risk-factor based subgroups were added to the previous model. Two criteria defined in a previous research work on the MAPT cohort (Andrieu et al., 2017) were examined to define these subgroups: the CDR status (CDR = 0 vs. CRD = 0.5), and on the Fried's frailty criteria (none vs. at least one frailty criteria) (Fried et al., 2004).

It should be mentioned that the previous research work on the MAPT cohort defined other subgroups (Andrieu et al., 2017). These subgroups were based on: the MMSE score, red blood cell DHA and EPA concentrations, dementia risk (CAIDE score) (Kivipelto et al., 2006), APOE $\epsilon 4$ genotype, brain Aβ load [florbetapir PET scan (Fleisher, 2011)]. We could not test an effect of interventions on these subgroups due to limited sample sizes. Characteristics of these subgroups are described in Supplementary Table S1.

2.7. Structural brain characteristics: Cortical thickness and white matter hyperintensities load

It has been reported that WMH load and cortical thickness can have an impact on resting state FC for MCI participants (Wang et al., 2020; Vettore et al., 2021). Both WMH load and cortical thickness were thus computed and analyzed to ensure that the observed effects on FC were not driven by these factors. Cortical thickness was computed when the BASC-Cambridge atlas subnetworks displayed significantly modified FC for the above-defined tests. All subnetworks were first resliced with the SPM12 toolbox to the anatomical 2iso MNI brain template provided by FSL v6.0.0 (Jenkinson et al., 2012). The brain template was then registered to Freesurfer "fsaverage" subject space and the registration was used to transform the resliced subnetwork images (Freesurfer v6) (Fischl and Dale, 2000). Participants' cortical thickness was computed with the Freesurfer recon-all pipeline at baseline and 36 months and mapped to the fsaverage surface. Subnetworks' cortical thickness was then extracted and averaged over left and right hemispheres for each participant and at both timepoints. Difference of cortical thickness between both timepoints was eventually computed. Freesurfer participants' GM segmentation and subnetworks' mapping to fsaverage surface were visually inspected. The WMH load was evaluated using the White matter Hyperintensities Automated Segmentation Algorithm (WHASA) on available FLAIR and 3DT1 data. See Samaille et al. (2012) for a full description of the WHASA method. Volumes of WMH were evaluated at baseline and 36 months and the difference of volume between both timepoints was computed. All participants WMH volumes were expressed relative to the total intracranial volume (TIV) at baseline. Visual quality control excluded participants with poor quality segmentation. The participants' cortical thickness data and WMH volumes were computed by the CATI Platform.

All statistical analyses on cortical thickness and WMH load were adjusted for age, sex and level of education and performed using permutation statistics (N = 5,000 permutations) with the Palm software (v119) (Winkler et al., 2014). For cortical thickness analyses, false discovery rate correction (p-FDR < 0.05) was applied to take into account the multiple tests on all subnetworks.

TABLE 1 Whole MAPT MRI subsample characteristics and comparison between the different intervention groups.

	MRI subsample (N = 100)	Om3 + MI (N = 27)	Om3 (N = 24)	MI (N = 24)	Pl (N = 25)	p-value*
Age, years (mean, (SD)/range)	74.26 (3.74)/ [70.00; 84.00]	74.59 (4.01)/ [70.00; 83.00]	73.92 (3.34)/ [70.00; 81.00]	74.79 (3.83)/ [70.00; 82.00]	73.72 (3.58)/ [70.00; 84.00]	0.756
Sex (F)	63 (63%)	16 (59%)	18 (75%)	14 (58%)	15 (60%)	0.580
Education level (≥University level)	49 (49%)	10 (37%)	15 (62%)	10 (42%)	14 (56%)	0.231
Composite score (mean, (SD)/range)						
At baseline	0.16 (0.51)/ [−1.60; 1.27]	0.16 (0.52)/ [−0.84; 1.23]	0.22 (0.34)/ [−0.62; 0.93]	0.03 (0.66)/ [−1.60; 1.27]	0.23 (0.45)/ [−0.54; 1.24]	0.522
Difference from baseline to 36 months	0.14 (0.49)/ [−1.61; 1.11]	0.09 (0.46)/ [−1.26; 0.94]	0.29 (0.39)/ [−0.53; 1.11]	0.08 (0.49)/ [−1.61; 0.88]	0.09 (0.56)/ [−1.58; 1.05]	0.388
Mini Mental State Examination (mean, (SD)/range), /30						
At baseline	28.01 (1.38)/ [24.00; 30.00]	28.04 (1.37)/ [25.00; 30.00]	28.29 (1.24)/ [24.00; 30.00]	28.00 (1.29)/ [25.00; 30.00]	27.72 (1.54)/ [24.00; 30.00]	0.491
Difference from baseline to 36 months	0.44 (1.73)/ [−5.00; 6.00]	0.30 (1.54)/ [−4.00; 2.00]	0.41 (1.85)/ [−3.00; 6.00]	0.38 (1.87)/ [−5.00; 4.00]	0.68 (1.64)/ [−3.00; 5.00]	0.904
Slow gait speed (≤0.8 m/s)[a]	11 (11%)	2 (7%)	4 (17%)	2 (8%)	3 (12%)	0.719
Exploratory subgroups						
Clinical dementia rating at baseline						0.260
0	52 (52%)	15 (56%)	16 (67%)	11 (46%)	10 (40%)	
0.5	48 (48%)	12 (44%)	8 (33%)	13 (54%)	15 (60%)	
Clinical dementia rating evolution from baseline to 36 months						0.002
0– 0	36 (36%)	6 (22%)	14 (58%)	6 (25%)	10 (40%)	
0.5–0.5	25 (25%)	9 (33%)	1 (4%)	9 (38%)	6 (24%)	
0–0.5	16 (16%)	9 (33%)	2 (8%)	5 (21%)	0 (0%)	
0.5–0	23 (23%)	3 (11%)	7 (29%)	4 (17%)	9 (36%)	
Fried's frailty criteria						0.487
No frailty criteria	57 (60%)	16 (62%)	16 (73%)	13 (54%)	12 (52%)	
At least one frailty criteria	38 (40%)	10 (38%)	6 (27%)	11 (46%)	11 (48%)	

*Comparison between intervention groups. Kruskall–Wallis and Anova or Chi-2 tests were used for quantitative and qualitative variables respectively.

[a]All participants presented spontaneous memory complaints but none of them were impeded in instrumental activities of daily living.

Percentages were calculated with the number of participants for whom data was available for each variable. Percentages were rounded to the nearest value. This rounding can result in a loss of accuracy and the sum of the percentages may be close but not equal to 100%.

Om3 + MI, omega-3 fatty acid supplementation and multidomain intervention; Om3, omega-3 fatty acid supplementation; MI, multidomain intervention; Pl, placebo.

3. Results

3.1. Baseline characteristics of the participants

All one hundred participants [37% male, mean (SD [min–max]) age 74.3 (±3.74, [70–84]) years] described spontaneous memory complaints but were not limited in instrumental activities of daily living and in addition about 11% showed reduced walking speed (below or equal to 0.8 m/s). At baseline, the mean cognitive composite score was 0.16 (±0.51, [−1.60; 1.27]) and the mean MMSE score was 28.01 (±1.38, [24; 30]), with 87% scoring below 30 at baseline. Furthermore, 38% showed at least one frailty criteria and 52% had a baseline CDR score of 0. A detailed description is presented in Table 1. Note that some participants show a change in CDR score over time, especially in the placebo and Om3 arms with a CDR-score evolution from 0.5 to 0. No significant difference is observed between included and excluded participants concerning age, sex and level of education, but

TABLE 2 Significant connections for interaction over 36 months between intervention group and baseline CDR status.

Individual connection statistics*	Subnetwork (main network) 1	Subnetwork (main network) 2
$F = 6.05$ $p < 0.001$	L/R Cun/SOG (VIS)	L/R MFG/MSFG (FPN)
$F = 5.64$ $p = 0.001$	L/R SMG/Post CG (SN)	L/R MFG/SMG (FPN)
$F = 5.38$ $p = 0.002$	L/R Pre CG (SMN)	L/R MFG/SMG (FPN)
$F = 5.06$ $p = 0.003$	L/R Sup Pre/Post CG (SMN)	L/R MFG/MSFG (FPN)
$F = 4.90$ $p = 0.003$	L/R Sup Pre/Post CG (SMN)	L/R MFG/SMG (FPN)
$F = 4.85$ $p = 0.004$	L/R Pre/Post CG (SMN)	L/R MFG/SMG (FPN)
$F = 4.84$ $p = 0.004$	L/R SPL (SN)	L/R MFG/SMG (FPN)
$F = 4.60$ $p = 0.005$	L/R SMG/Post CG (SN)	L/R MFG/MSFG (FPN)

*Results presented are significant after SPC FWE-cluster correction. All significant connections are part of the same cluster.
The model was adjusted for age, sex and level of education.
VIS, Visual network; FPN, Frontoparietal network; SN, Salience network; SMN, Sensorimotor network; L/R Cun/SOG, left/right cuneus/superior occipital gyrus; L/R MFG/MSFG, left/right middle frontal gyrus/superior frontal gyrus medial segment; L/R SMG/Post CG, left/right supramarginal gyrus/post central gyrus; L/R MFG/SMG, left/right middle frontal gyrus/supramarginal gyrus; L/R Pre CG, left/right pre central gyrus; L/R Sup Pre/post CG, left/right superior pre/post central gyrus; L/R Pre/Post CG, left/right pre/post central gyrus; L/R SPL, left/right superior parietal lobule; SPC, spatial pairwise clustering; FWE, family-wise error.

participants included improved more on average during 36 months on the MMSE and cognitive composite scores than excluded participants. There is however no difference on CDR status between included and excluded participants (see Supplementary Table S2).

3.2. Main effects of intervention and interactions with risk-factor based subgroups on FC

No difference of FC was found between the intervention groups over time. There was neither a main effect of time, indicating that the FC of participants combined across intervention groups did not differ between baseline and after 36 months of intervention, nor a main effect of groups, showing that FC did not differ between groups at pre and post intervention states. No effect was found for the unadjusted model either.

However, a significant interaction between baseline CDR status and intervention groups over time was found (Table 2, see Supplementary Table S3 for unadjusted model). These connections associated the frontoparietal (FPN) subnetworks with subnetworks of the sensorimotor (SMN), salience (SN) and visual (VIS) networks. Subnetworks from the FPN were mainly centered on the middle frontal gyrus. The structure and main network affiliation of all subnetworks that are significant for this interaction and for all results reported thereafter are further described in Supplementary Table S4.

Post-hoc analyses highlighted that FPN-SMN, FPN-SN and FPN-VIS connectivity was different between interventions with omega-3 fatty acid supplementation (Om3 and Om3 + MI) compared to interventions without omega-3 (MI and placebo), though this difference was dependant on participants baseline CDR status and displayed opposite directions between CDR0 and CDR0.5 subgroups of participants (Table 3, Figure 2). When examining directly the raw mean connectivity, it appeared that for participants with a CDR0 at baseline, the Om3 supplementation induced a stability or slight decrease of the FC compared to the placebo where FC was increased (Supplementary Table S3, Supplementary Figure S1). For the participants with CDR0.5 at baseline, FC also remained stable or increased after Om3 supplementation, but it systematically decreased more after placebo intake (Supplementary Table S3, Supplementary Figure S1). It is interesting to note that when we tested for an interaction between baseline CDR status and omega-3 fatty acid supplementation (Om3 and Om3 + MI) vs. no omega-3 fatty acid supplementation (MI and placebo), the same FPN-SMN, FPN-SN and FPN-VIS connections were significant, though new significant connections between the FPN-SMN, FPN-VIS, FPN-SN, DMN-VIS, and DMN-SMN were revealed (Supplementary Table S5). This shows that the observed effect between intervention groups and baseline CDR status is driven by omega-3

TABLE 3 Post hoc tests on significant connections between subnetworks (main networks) for interaction intervention group × baseline CDR status × time.

	CDR0 at baseline (*t*; *p* value*) Om3 + MI (N = 15) ≠ Om3 (N = 16) ≠ MI (N = 11) ≠ Pl (N = 10)						CDR0.5 at baseline (*t*; *p* value*) Om3 + MI (N = 12) ≠ Om3 (N = 8) ≠ MI (N = 13) ≠ Pl (N = 15)					
	Om3 + MI > Om3	Om3 + MI > MI	Om3 > MI	Om3 + MI > Pl	Om3 > Pl	IM > Pl	Om3 + MI > Om3	Om3 + MI > MI	Om3 > MI	Om3 + MI > Pl	Om3 > Pl	IM > Pl
Connections from subnetwork L/R MFG/SMG (FPN) to subnetworks												
L/R SMG/Post CG (SN)	$T = 0.40$ $p = 0.978$	$T = -2.38$ $p = 0.094$	**$T = -2.80$ $p = 0.036$**	**$T = -2.67$ $p = 0.049$**	**$T = -3.10$ $p = 0.017$**	$T = -0.35$ $p = 0.984$	$T = -0.14$ $p = 0.999$	$T = 1.18$ $p = 0.638$	$T = 1.36$ $p = 0.527$	$T = 1.42$ $p = 0.490$	$T = 1.36$ $p = 0.527$	$T = 0.22$ $p = 0.996$
L/R SPL (SN)	$T = 0.49$ $p = 0.961$	$T = -1.44$ $p = 0.478$	$T = -1.93$ $p = 0.231$	$T = -2.03$ $p = 0.190$	$T = -2.53$ $p = 0.069$	$T = -0.61$ $p = 0.928$	$T = 1.11$ $p = 0.685$	**$T = 2.83$ $p = 0.034$**	$T = 1.22$ $p = 0.617$	$T = 2.20$ $p = 0.139$	$T = 0.71$ $p = 0.891$	$T = -0.70$ $p = 0.894$
L/R Pre CG (SMN)	$T = -0.04$ $p = 1.000$	$T = -2.01$ $p = 0.199$	$T = -2.01$ $p = 0.197$	**$T = -3.52$ $p = 0.005$**	**$T = 3.58$ $p = 0.004$**	$T = -1.50$ $p = 0.444$	$T = 1.03$ $p = 0.734$	$T = 1.83$ $p = 0.273$	$T = 0.48$ $p = 0.963$	$T = 1.91$ $p = 0.237$	$T = 0.55$ $p = 0.945$	$T = 0.05$ $p = 1.000$
L/R Sup Pre/Post CG (SMN)	$T = -0.05$ $p = 1.000$	$T = -2.28$ $p = 0.118$	$T = -2.28$ $p = 0.119$	**$T = -2.88$ $p = 0.029$**	**$T = -2.92$ $p = 0.027$**	$T = -0.66$ $p = 0.912$	$T = 1.70$ $p = 0.334$	$T = 1.59$ $p = 0.392$	$T = -0.38$ $p = 0.981$	$T = 2.22$ $p = 0.133$	$T = 0.10$ $p = 1.000$	$T = 0.60$ $p = 0.929$
L/R Pre/Post CG (SMN)	$T = 1.39$ $p = 0.509$	$T = -1.35$ $p = 0.533$	**$T = -2.67$ $p = 0.049$**	$T = -2.5$ $p = 0.074$	**$T = -3.82$ $p = 0.002$**	$T = -1.13$ $p = 0.672$	$T = 0.82$ $p = 0.842$	$T = 2.38$ $p = 0.097$	$T = 1.13$ $p = 0.674$	$T = 1.48$ $p = 0.453$	$T = 0.40$ $p = 0.978$	$T = -0.96$ $p = 0.773$
Connections from subnetwork L/R MFG/MSFG (FPN) to subnetworks												
L/R Cun/SOG (VIS)	$T = 0.32$ $p = 0.988$	$T = -1.87$ $p = 0.256$	$T = -2.20$ $p = 0.138$	$T = -1.91$ $p = 0.236$	$T = -2.21$ $p = 0.124$	$T = -0.11$ $p = 1.00$	$T = 0.54$ $p = 0.947$	**$T = 2.97$ $p = 0.024$**	$T = 1.89$ $p = 0.247$	$T = 2.06$ $p = 0.182$	$T = 1.18$ $p = 0.638$	$T = -0.99$ $p = 0.753$
L/R Sup Pre/Post CG (SMN)	$T = 0.75$ $p = 0.876$	$T = -1.02$ $p = 0.739$	$T = -1.73$ $p = 0.318$	$T = -2.05$ $p = 0.185$	**$T = -2.78$ $p = 0.038$**	$T = -1.01$ $p = 0.742$	$T = 1.75$ $p = 0.310$	**$T = 3.05$ $p = 0.020$**	$T = 0.76$ $p = 0.869$	**$T = 3.10$ $p = 0.017$**	$T = 0.81$ $p = 0.850$	$T = -0.01$ $p = 1.000$
L/R SMG/Post CG (SN)	$T = 0.67$ $p = 0.908$	$T = -0.78$ $p = 0.865$	$T = -1.41$ $p = 0.497$	$T = -1.45$ $p = 0.476$	$T = -2.09$ $p = 0.171$	$T = -0.66$ $p = 0.909$	$T = -1.23$ $p = 0.610$	$T = 0.99$ $p = 0.756$	$T = 2.01$ 0.198	$T = 1.57$ $p = 0.402$	***$T = 2.63$*** ***$p = 0.055$***	$T = 0.57$ $p = 0.938$

Significant results are outlined in bold (near significant are in bold and italics).

*The model was adjusted for age, sex and level of education.

Om3 + MI, omega-3 fatty acid supplementation and Multidomain intervention; Om3, omega-3 fatty acid supplementation; MI, multidomain intervention; Pl, placebo; VIS, Visual network; FPN, Frontoparietal network; SN, Salience network; SMN, Sensorimotor network; L/R Cun/SOG, left/right cuneus/superior occipital gyrus; L/R MFG/MSFG, left/right middle frontal gyrus/superior frontal gyrus medial segment; L/R SMG/Post CG, left/right supramarginal gyrus/post central gyrus; L/R MFG/SMG, left/right middle frontal gyrus/supramarginal gyrus; L/R Pre CG, left/right pre central gyrus; L/R Sup Pre/Post CG, left/right superior pre/post central gyrus; L/R Pre/Post CG, left/right pre/post central gyrus; L/R SPL, lef/right superior parietal lobule.

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fatty acid supplementation. In contrast, no interaction effects were found between intervention groups and Fried's frailty criteria.

3.3. CDR status and omega-3 fatty acid supplementation

3.3.1. The effect of CDR status and omega-3 fatty acid supplementation on cortical thickness and WMH load: Implications for FC

Brain structural alterations may drive the differences of FC observed between participants with different CDR status and with or without an omega-3 fatty acid supplementation. To explain the observed interaction effect between omega-3 fatty acid supplementation and CDR status, we first tested whether the FC changes of the FPN with the SMN, SN and VIS networks (Table 2) could be explained by differences of cortical thickness between the groups. For each subnetwork, the evolution of cortical thickness between baseline and 36 months was compared between CDR0 participants supplemented with Om3 (Om3 and Om3 + MI: $N = 29$) and without (MI and placebo: $N = 20$) and between CDR0.5 participants supplemented with Om3 (Om3 and Om3 + MI: $N = 20$) and without ($N = 28$) after exclusion of participants that did not reach quality control for cortical thickness analysis. The same evaluation was performed for the evolution of WMH load between CDR0 participants supplemented with Om3 (Om3 and Om3 + MI: $N = 26$) and without (MI and placebo: $N = 17$) and between CDR0.5 participants supplemented with Om3 (Om3 and Om3 + MI: $N = 17$) and without ($N = 23$) after excluding participants with erroneous WMH segmentation. No difference was detected, suggesting that these structural characteristics do not drive the FC changes observed for the CDR0 or CDR0.5 participants supplemented with Om3.

3.3.2. Baseline differences in FC between omega-3 fatty acid and placebo supplementations according to participants' CDR status

We observed an effect of intervention linked to baseline CDR status on FC evolution. To determine if this effect could be attributed to baseline FC differences, we assessed the interaction between intervention groups and baseline CDR status on baseline FC. No significant difference was found, implying that FC is not different at baseline according to CDR status and group assignment. There was neither a significant difference when we pooled participants into larger groups of subjects supplemented or not with omega-3 fatty acids (Om3 & Om3 + MI vs. MI & placebo) and tested for an interaction with baseline CDR status on baseline FC.

3.3.3. The role of the progression of the CDR on FC

Two profiles may exist: stable cognitive participants with a CDR0 or CDR0.5 at both time-points and participants that show cognitive changes, marked by a CDR0 at baseline and CDR0.5 at follow-up or vice-versa (Table 1). No significant difference on the evolution of FC was found after testing the effect of Om3 supplementation on subgroups of participants with stable CDR0 or CDR0.5 status. There was however a trend for stable CDR0 participants ($p = 0.067$; Supplementary Table S6) for the same FPN-SMN and FPN-SN connections that were significant when testing the effect of Om3 supplementation in CDR0 participants (Table 3). This suggests that this subset of stable participants may be equally responsive to Om3 intake. A trend for participants with stable CDR0.5 ($p = 0.078$; Supplementary Table S7) was also detected, but on VIS-SN connections that differed from the ones previously observed (Table 2). We thus cannot conclude if we observe a true effect of Om3 intake on stable CDR0.5 participants.

4. Discussion

This study aimed to evaluate the impact of preventive uni or multidomain intervention strategies during a 3-years period on older people with cognitive complaints by analyzing longitudinal FC changes. While reasonably large imaging data sample that included quality control procedures was assessed, no FC changes could be revealed for any intervention (MI including physical activity, cognitive training, and nutritional counseling alone, vs. omega-3 fatty acid supplementation alone, and MI combined with omega-3 fatty acid supplementation) . When targeting cognitive status at baseline, interestingly, an effect of the omega-3 fatty acid supplementation on longitudinal FC emerged, suggesting an effect of this supplementation on selected populations.

Participants with a normal CDR at baseline had stable FPN-VIS, FPN-SMN, and FPN-SN FC through time when receiving omega-3 fatty acid supplementation, while their FC would naturally increase without this supplementation (i.e., in the placebo group). This observation was also partially confirmed for the subset of participants with a stable good cognitive profile over time (CDR0 at baseline and follow-up), endorsing the fact that this process is also associated with a good prognosis. For participants with slight cognitive impairment (CDR0.5) the FPN-SMN, FPN-VIS and FPN-SN FC either remained stable or slightly increased with omega-3 fatty acid supplementation, while it decreased with placebo intake.

For participants with normal CDR, the lack of omega-3 fatty acid supplementation induces an increased between-network connectivity that is similar to what is observed in the aging process (Betzel et al., 2014; Geerligs et al., 2015). This could

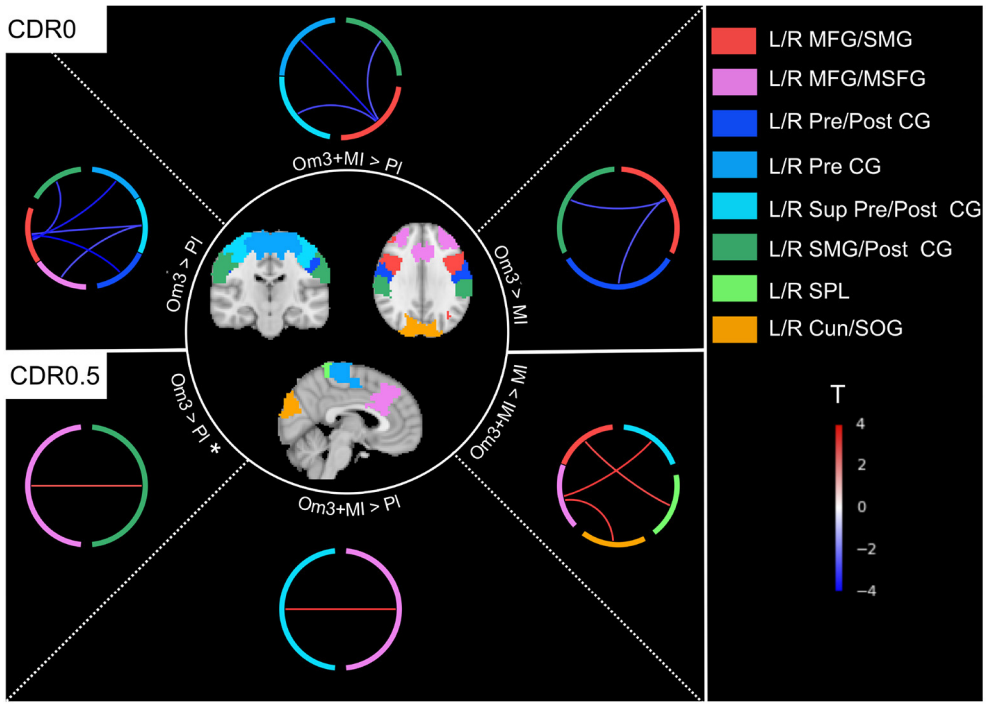

FIGURE 2
Connections showing a difference over the evolution of FC between interventions within each subgroup of baseline CDR0 and CDR0.5 (Table 3). Almost all connections were significant except for the L/R MFG/MSFG - L/R SMG/Post CG connection for the comparison CDR0.5 Om3 > Pl that was close to significance ($p < 0.06$)*. Om3 + Ml, Omega-3 fatty acid supplementation and multidomain intervention; Om3, omega-3 fatty acid supplementation; Ml, multidomain intervention; Pl, placebo; L/R Cun/SOG, left/right cuneus/superior occipital gyrus (visual network); L/R MFG/MSFG, left/right middle frontal gyrus/superior frontal gyrus medial segment (frontoparietal network); L/R SMG/Post CG, left/right supramarginal gyrus/post central gyrus (salience network); L/R MFG/SMG, left/right middle frontal gyrus/supramarginal gyrus (frontoparietal network); L/R Pre CG, left/right pre central gyrus (sensorimotor network); L/R Sup Pre/Post CG, left/right superior pre/post central gyrus (sensorimotor network); L/R Pre/Post CG, left/right pre/post central gyrus (sensorimotor network); L/R SPL, left/right superior parietal lobule (salience network); *T*, *t*-value for *post hoc* tests (Table 3), red connections represent a positive difference between mean connectivity of interventions group with omega-3 fatty acid supplementation vs. no omega-3 fatty acid supplementation, blue connections represent a negative difference.

be imputed to either a loss of functional specificity from the different resting-state networks, or to a compensatory process in which networks act conjointly to maintain stable cognitive functions within time (Jockwitz and Caspers, 2021). In this light, omega-3 fatty acid supplementation could stabilize FC between networks. For participants with impaired cognition (CDR0.5), FC evolves differently. Viviano and Damoiseaux (2020) note in their review that various and inconsistent results about the FC of populations with subjective memory complaints have been reported, and propose a nonlinear model of FC evolution to explain this variability, though specifically for DMN and medial temporal regions. FC would first increase, due to noisy signals or compensatory mechanisms, to later decrease as neurodegeneration spreads. It could be hypothesized that CDR0.5 participants are either at the beginning or in the middle of this latter phase of decline. They would, when ingesting the placebo and depending on the connection, either have stable or decreasing between-network FC whereas omega-3 fatty acid supplementation would result in an opposite process by respectively increasing or preserving inter network connectivity. This interpretation should be taken with caution, as the model proposed by Viviano et al. is specific to the DMN and medial temporal regions and may not apply to other regions or networks.

The mechanisms linked to omega-3 fatty acid supplementation are not fully understood and do not seem in our study to be modulated by the cortical thickness nor the WMH load. An absence of change in cortical thickness was not expected as omega-3 fatty acid supplementation or fish-oil supplements have been shown to reduce atrophy in the temporal and parietal cortex, and more particularly in the hippocampus, for healthy older adults (Conklin et al., 2007; Raji et al., 2014; Witte et al., 2014; Daiello et al., 2015) and MCI and AD participants (Daiello et al., 2015). In addition, natural red blood cell DHA and EPA concentrations equally reshape GM in healthy older people (Walhovd, 2014). Concerning FC, a direct association was found between these omega-3 blood cell levels and FC of regions including the prefrontal cortex,

hippocampus, precuneus and amygdala (Talukdar et al., 2019). When assessed indirectly through nutrient patterns, omega-3 fatty acid supplementation impacted the FC of the visual network and influenced the relation between the FC of the FPN and intelligence (Zwilling et al., 2019). The modifications we observe in FC of regions centered around the middle frontal gyrus and subnetworks of the FPN are congruent with the hypothesis that frontal regions are affected by cognitive impairment and neurodegenerative disease, like AD (Bayram et al., 2018).

We did not find any effect of the MI, alone or combined with omega-3 fatty acid supplementation for the overall population or risk-based subgroups. Interestingly, when looking at the main MAPT study as well as other brain imaging studies on MI, mixed results are reported. The ancillary FDG PET MAPT Trial showed that MI had no impact on global brain metabolism at 12 months (Delrieu et al., 2020). However, an effect on brain morphometry was detected through a specific deformation-based method, whereas classic segmentation based analyses did not yield any result (Sivera et al., 2020). This underscores the importance of methodological choices for the analysis of brain imaging data. Other trials such as the FINGER and PREDIVA studies failed to detect an effect of MI on either gray matter structure (Stephen et al., 2019) or WMH volumes (van Dalen et al., 2017; Stephen et al., 2019). They however showed that these interventions were more beneficial for participants with higher baseline cortical thickness in regions affected by AD (Stephen et al., 2019), or with higher baseline WMH volumes (van Dalen et al., 2017). This is concordant with our findings which suggest that the effect of interventions on the brain may be dependent on participants' baseline profiles.

The differences between studies examining the effect of preventive interventions and our findings, whether it concerns the effect of omega-3 fatty acid supplementation associated with participants' CDR status or the lack of benefit from MI, can be explained by multiple factors. First, inclusion criteria differed between studies, leading to populations with different cognitive profiles (cognitively healthy, at-risk to develop AD, frail, early AD, etc). Furthermore, beyond cognitive status, there is variability in the response of individuals to interventions, and many factors can modulate the effect of an intervention. Previous analyses suggest for instance that the impact of exercise on older people with mild AD depends on their APOE genotype (Jensen et al., 2019) or that the effect of omega-3 fatty acid supplementation on cognitively impaired older adults is influenced by participants' baseline plasma homocysteine levels (Jernerén et al., 2019). Second, the methodological choices to evaluate MRI data metrics and in particular FC varied between studies. It is instructive to note that in their review on cognitive training, van Balkom et al. (2020) identify the use of seed-based approaches as one of the major limitations in FC studies, as the results are highly dependent on the selected source seed. Similarly, the choice of brain atlas used to parcelate the brain might bias findings. Here we used one parcellation with 36 regions (630 connections) from a multiresolution atlas that was previously used in a meta-analysis to identify precise regions affected by AD (Badhwar et al., 2017). Other methodological choices concerning the integration of confounding risk factors such as APOEϵ4 or amyloid brain deposits can also explain the variability observed between studies (Rakesh et al., 2017; Bhatti et al., 2020; Yao et al., 2020). Eventually, differences between studies can be attributed to the intervention design that may vary in length, modality, frequency, and intensity. Also, it should be mentioned that while we mostly compare our findings to the results of interventional studies, some of the studies concerning omega-3 fatty acids are observational. Within our study, training intensity for MI differed over three-years. Participants underwent twelve sessions during the first two months, but this frequency was then reduced to one session per month. It could be argued that by the time participants were scanned at follow-up, any impact of the initial high intensity intervention had been wiped out, and that effects could have been observed at an earlier stage of the intervention. We did detect, in contrast, an effect of omega-3 fatty acid supplementation, and it is interesting to note that this specific intervention intensity was maintained during the three-year period.

This study present strengths but also limits. The study strength includes a substantial amount of MRI data, a quality control of imaging data, multimodal data, a longitudinal design and the complementary nature of MI. Some limitations should however be considered. Missing data (17%) might have limited the interpretation of WMH load effects on FC. Also, the small number of subjects in the subgroup analyses (CDR, Fried's frailty criteria), the even smaller number of subjects for some subgroups (APOE ϵ4 carriers, participants with low levels of omega-3, with high risk dementia, with low MMSE score) and missing information (amyloid status) unfortunately limited further detailed analysis.

Our study should be interpreted in the light of what is known about the amyloid status. Longitudinal analyses pointed out that the evolution of connectivity for cognitively intact older adults differed according to their amyloid status (Lin et al., 2020) and amyloid status appeared to modify FC over a wide range of studies including participants in the spectrum of AD (Hasani et al., 2021). Participants' amyloid load may thus be an important factor mediating the effect of interventions on FC. In our case, we reasoned that intervention groups presented similar cognitive profiles at baseline and were homogeneous on factors such as APOE ϵ4 status that are associated with increased amyloid burden (Sánchez-Juan and Seshadri, 2017; Sperling et al., 2020; Janssen et al., 2021). We hypothesize that it should limit the differences of amyloid load between the groups. Given the low number of APOE ϵ4 subjects in the sample it is also possible that few participants had a high amyloid burden. Nevertheless, the information about amyloid status should be taken into account in future studies.

In addition to replicating the results on CDR status, future studies on preventive interventions should aim to explore the effects of interventions on MRI biomarkers in potential subgroups of interest and to scan large samples of participants to this effect.

5. Conclusion

Older people with cognitive complaints did not show any change in FC after 36 months of multidomain or unidomain interventions. However an effect of omega-3 fatty acid supplementation on the FC of the frontoparietal, sensorimotor, visual and salience networks was detected if participants cognitive decline was considered. This effect was located on the frontoparietal network, known to be involved in neurodegenerative and aging processes, and showed opposite patterns of connectivity for participants with CDR0 or CDR0.5 at baseline. This effect was independent of cortical thickness and WMH load. Overall, our findings suggest that preventive strategies should consider participants' cognitive status and the heterogeneity of target populations when designing future studies on preventive interventions.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Advisory Committee for the Protection of Persons participating in Biomedical Research of the Toulouse University Hospital and authorized by the French Health Authority. The patients/participants provided their written informed consent to participate in this study.

MAPT/DSA group

MAPT study group

Principal investigator: Bruno Vellas (Toulouse); Coordination: Sophie Guyonnet; Project leader: Isabelle Carrié; CRA: Lauréne Brigitte; Investigators: Catherine Faisant, Françoise Lala, Julien Delrieu, Hélène Villars; Psychologists: Emeline Combrouze, Carole Badufle, Audrey Zueras; Methodology, statistical analysis and data management: Sandrine Andrieu, Christelle Cantet, Christophe Morin; Multidomain group: Gabor Abellan Van Kan, Charlotte Dupuy, Yves Rolland (physical and nutritional components), Céline Caillaud, Pierre-Jean Ousset (cognitive component), Françoise Lala (preventive consultation). The cognitive component was designed in collaboration with Sherry Willis from the University of Seattle, and Sylvie Belleville, Brigitte Gilbert, and Francine Fontaine from the University of Montreal.

Co-investigators in associated centers: Jean-François Dartigues, Isabelle Marcet, Fleur Delva, Alexandra Foubert, Sandrine Cerda (Bordeaux); Marie-Noëlle-Cuffi, Corinne Costes (Castres); Olivier Rouaud, Patrick Manckoundia, Valérie Quipourt, Sophie Marilier, Evelyne Franon (Dijon); Lawrence Bories, Marie-Laure Pader, Marie-France Basset, Bruno Lapoujade, Valérie Faure, Michael Li Yung Tong, Christine Malick-Loiseau, Evelyne Cazaban-Campistron (Foix); Françoise Desclaux, Colette Blatge (Lavaur); Thierry Dantoine, Cécile Laubarie-Mouret, Isabelle Saulnier, Jean-Pierre Clément, Marie-Agnès Picat, Laurence Bernard-Bourzeix, Stéphanie Willebois, Iléana Désormais, Noëlle Cardinaud (Limoges); Marc Bonnefoy, Pierre Livet, Pascale Rebaudet, Claire Gédéon, Catherine Burdet, Flavien Terracol (Lyon), Alain Pesce, Stéphanie Roth, Sylvie Chaillou, Sandrine Louchart (Monaco); Kristel Sudres, Nicolas Lebrun, Nadège Barro-Belaygues (Montauban); Jacques Touchon, Karim Bennys, Audrey Gabelle, Aurélia Romano, Lynda Touati, Cécilia Marelli, Cécile Pays (Montpellier); Philippe Robert, Franck Le Duff, Claire Gervais, Sébastien Gonfrier (Nice); Yannick Gasnier and Serge Bordes, Danièle Begorre, Christian Carpuat, Khaled Khales, Jean-François Lefebvre, Samira Misbah El Idrissi, Pierre Skolil, Jean-Pierre Salles (Tarbes).

MRI group: Carole Dufouil (Bordeaux), Stéphane Lehéricy, Marie Chupin, Jean-François Mangin, Ali Bouhayia (Paris); Michèle Allard (Bordeaux); Frédéric Ricolfi (Dijon); Dominique Dubois (Foix); Marie Paule Bonceour Martel (Limoges); François Cotton (Lyon); Alain Bonafé (Montpellier); Stéphane Chanalet (Nice); Françoise Hugon (Tarbes); Fabrice Bonneville, Christophe Cognard, François Chollet (Toulouse).

PET scans group: Pierre Payoux, Thierry Voisin, Julien Delrieu, Sophie Peiffer, Anne Hitzel, (Toulouse); Michèle Allard (Bordeaux); Michel Zanca (Montpellier); Jacques Monteil (Limoges); Jacques Darcourt (Nice). Medico-economics group: Laurent Molinier, Hélène Derumeaux, Nadège Costa (Toulouse). Biological sample collection: Bertrand Perret, Claire Vinel, Sylvie Caspar-Bauguil (Toulouse). Safety management: Pascale Olivier-Abbal.

DSA group: Sandrine Andrieu, Christelle Cantet, Nicola Coley.

Author contributions

LP, JD, L-AG, EG, CF, and EL contributed to data analyses and manuscript writing. J-FM, CM, GB, LV, SG, BV, and AG

contributed to manuscript writing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2022.971220/full#supplementary-material

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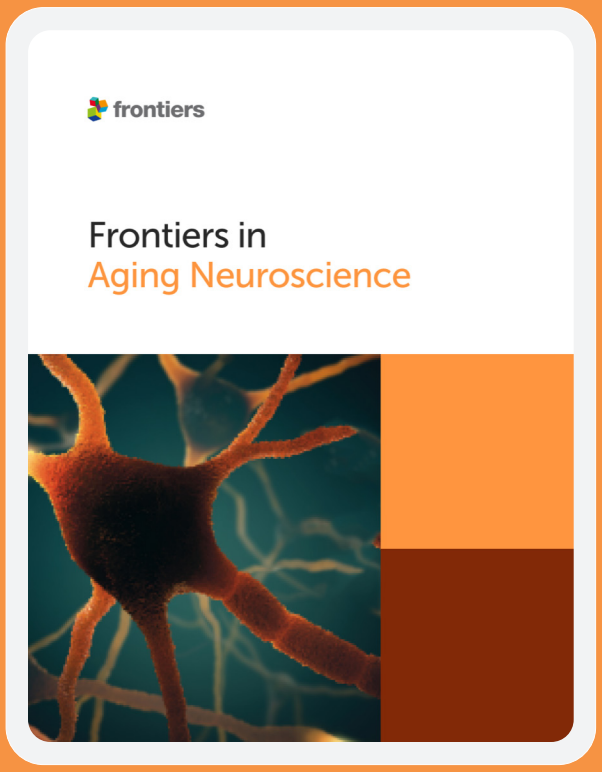

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