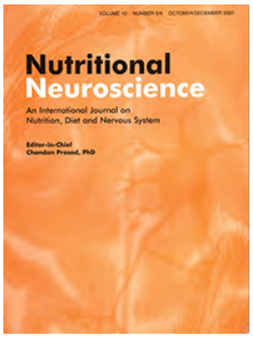

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The effects of walnut supplementation on hippocampal NMDA receptor subunits NR2A and NR2B of rats

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Objectives: Walnuts contain numerous selected dietary factors that have an impact on brain functions, especially learning and memory formation in the hippocampus. Hippocampal *N*-methyl D-aspartate receptors (NMDARs) are involved in the formation of cognitive functions. In this study, we aimed to investigate the molecular effects of walnut supplementation on the hippocampal expressions of NMDARs involved in cognitive functions and lipid peroxidation levels in rats.

Methods: The male Sprague-Dawley rats (6 months old, $n = 24$) were fed with a walnut-supplemented diet (6% walnut diet, $n = 12$) and a control diet (rat food, $n = 12$) as *ad libitum* for 8 weeks. At the end of this period, NMDAR subunits NR2A and NR2B in the hippocampi were assayed by western blotting. Lipid peroxidation levels were measured using the thiobarbituric acid.

Results: The expression of NR2A and NR2B was elevated in the walnut-supplemented rats compared with the control group ($P < 0.05$). In addition, the levels of lipid peroxidation in the walnut-supplemented group were significantly decreased compared with the control group.

Discussion: We suggested that walnut supplementation may have protective effects against the decline of cognitive functions by regulating NMDAR and lipid peroxidation levels in the hippocampus. The study provides evidence that selected dietary factors (polyunsaturated fatty acids, melatonin, vitamin E, and flavonoids) within walnut may help to trigger hippocampal neuronal signal transduction for the formation of learning and memory.

Keywords: Walnut diet, NMDAR, Lipid peroxidation, Hippocampus

Introduction

Certain dietary factors in food are important as they affect the central nervous system in health and disease.[1] Walnuts are valuable food sources, because they contain various dietary factors, such as polyunsaturated fatty acids (PUFAs), melatonin, vitamin E, and flavonoids. These factors have both antioxidant and anti-inflammatory properties and protect brain tissue from oxidative damage.[2] Several studies have shown preventive and therapeutic effects of walnut on age-related motor and cognitive deficits. Those effects were assessed with behavioral experiments such as Morris water maze.[3] Walnuts contain significant amounts of *n*-6 and *n*-3 PUFA, linoleic acid (LA) (18:2*n*-6), and alpha-linolenic acid (ALA) (18:3*n*-3).[3,4] Researches have shown that essential fatty acids regulate a number of cellular processes, including learning and memory in the brain.[5] Growing epidemiological studies suggest that dietary deficiency of *n*-3 PUFA is a candidate risk factor for the development of Alzheimer's disease (AD).[6,7] The amount of dietary *n*-3 PUFA must be sufficient for optimal cognitive performance in several animal species and in an animal model of AD.[7]Several studies using rat models have focused on the ability of dietary alterations or supplementation to ameliorate age-related loss of cognitive function.[8–11] Healthy diets consisting of high amounts of *n*-3 fatty acids can stimulate molecular systems involved in neuronal functions and plasticity in the brain.[1]

N-methyl D-aspartate receptor (NMDAR)-dependent long-term potentiation (LTP) is the major cellular mechanism thought to underlie spatial learning and

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memory in the hippocampus.[8] NMDAR subunits, especially NR2A and NR2B, are essential for LTP induction and maintenance and are required for hippocampal synaptic plasticity. NMDARs contain various combinations of NR1 and NR2 subunits (A–D). The NR2 subunit regulates the duration of Ca^{2+} influx through the NMDA ion channel.[12] Molecular alterations in NMDAR subunits can affect physiological and pathological processes in the hippocampus. NMDARs are involved in numerous physiological processes, including basic neuronal communication, axonal pathfinding, mood regulation, and memory formation.[13] Hyperactivity of NMDARs has been implicated in a variety of neurodegenerative disorders, such as Parkinson's disease and AD.[13] Excessive or deficiency of certain components in foods can result in the overactivation or inhibition of the functions of NMDAR subunits. It has been reported that dietary *n*-3 fatty acid depletion leads to a significant NR2B decrease while *n*-3 fatty acids enrichment results in NR2B increase.[6,7] Keleshian *et al.*[14] evaluated the effects of *n*-3 PUFA deficiency and supplementation of ALA on chronic NMDA-induced changes in rat brain, and found that *n*-3 PUFA deficiency worsened NMDA-induced changes, whereas *n*-3 supplementation did not affect NMDA-induced responses.

Although the beneficial cognitive effects of a walnut-supplemented diet in rats have been documented, the molecular changes contributing to its effects are not fully understood. The aim of this study was to investigate the effect of dietary intake of walnut on NMDAR subunit expression and the level of malondialdehyde (MDA) as a marker of lipid peroxidation in rat hippocampus.

Materials and methods

Animals

Sprague-Dawley male rats (Suleyman Demirel University, Medical Faculty, Animal Experimental Laboratories, Isparta, Turkey), 6–7 months old, weighing between 200 and 240 g, were housed individually to monitor their daily food intake. They were maintained in environmentally controlled rooms (22–24°C, 50–55% humidity) with a 12-hour light/dark cycle. The rats were randomly divided into two groups of 12 animals: one group received a control diet (control group, $n = 12$), and the other group received a diet enriched with walnuts (walnut group, $n = 12$) for 8 weeks. The diets were provided *ad libitum* during the experimental period. The animals' weights were recorded throughout the study, and their food intake during a 24-hour period was assessed. At the end of 8 weeks, each rat was anesthetized separately by injecting intraperitoneal 2% xylazine (10 mg/kg) and then 10% ketamin (80 mg/kg). This anesthesia gave us 1-hour time window to sacrifice the animals.

The experimental protocol of the study was approved by the ethical committee of the Medical Faculty of Suleyman Demirel University. The animals involved in the procedure were maintained and used in accordance with the *Animal Welfare Act* and the *Guide for the Care and Use of Laboratory Animals* prepared by the Suleyman Demirel University Animal Ethical Committee (the number of the document: 04.13.01.06.2006).

Tissue preparation

Rats were decapitated and the brain was rapidly removed. The hippocampi were carefully dissected (within 5 minutes) on an apparatus, which was icy and wetted with phosphate buffer (50 mM) and frozen in the eppendorfs, which were filled with phosphate buffer (50 mM). Samples were stored at −80°C until assayed.

Diets

Walnuts were obtained from Burdur, a city in the Mediterranean region of Turkey. The walnuts were combined with the control diet, and the amount of maize in the control diet was adjusted to compensate for the added volume of walnuts. Control group rats were fed with ordinary food, whereas walnut group were fed with walnut 6% of intake food during the experimental procedure.[3] The standard diet in this study contains 4–6% fat. The metabolizable energy of this diet was 2600 kcal/g for adult rats. The minimum amounts of the required nutrients (protein, amino acid, fat, vitamins, and some minerals) for adult rats are listed in Table 1. The fatty acid compositions of the walnut diets are shown in Table 2. The main PUFAs present in the walnuts were LA (55.30%, omega-6) and ALA (11.83%, omega-3). It is crucial to realize the ideal *n*-6/*n*-3 ratio for optimal cognitive performance in animal species.[3] The ideal ratio was 4:1 in the study. The fatty

Table 1 The chemical constituents of control rat diet

Nutrients	Amount (% of total weight)
Dry element	88 (minimum)
Crude protein	23 (minimum)
Crude cellulose	7 (maximum)
Crude ash	8 (maximum)
Insoluble ash in HCl	2 (maximum)
Calcium	1–1.8 (minimum–maximum)
Phosphorus	0.9 (minimum)
Sodium	0.5–0.8 (minimum–maximum)
Sodium chloride	1 (maximum)
Methionine	0.3
Lysine	1
Vitamin mix	0.2
Fat	4–6
Metabolic energy	2600 kcal/g (minimum)

The nutritive requirements for the rat are listed in the table. The content of each nutrient is expressed as g/100 g dry weight. Control rat diet was purchased from Korkutelim Feed Industry, Antalya, Turkey.

Table 2 Fatty acid composition of the walnuts

	Palmitic acid	Stearic acid	Oleic acid	LA	ALA
Walnut (%)	7.50	3.11	22.26	55.30	11.83

The fatty acid composition of the walnuts was measured by GC/MS (Schimadzu QP5050, Japan). The principal fatty acids were LA (linoleic acid) and ALA (alpha-linolenic acid). It is the effective *n*-6/*n*-3 ratio: 1/4 for cognitive functions in the walnut.

acid profile of the walnuts was measured by gas chromatography/mass spectrophotometer (GC/MS) (Schimadzu QP5050, Japan) at the Suleyman Demirel University's Central Laboratory.

Antibodies and chemicals

Anti-glutamate receptor NR2A, anti-glutamate receptor NR2B, monoclonal anti-rabbit IgG alkaline phosphatase conjugate, beta-actin, a pre-stained molecular weight marker kit, nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP), leupeptin, aprotinin, benzamidine, and ethylene glycol-bis (beta-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were all purchased from Sigma (St Louis, MO, USA). All reagents were of an analytical grade or the highest grade available.

Lipid peroxidation assay

MDA, an end product of lipid peroxidation, was assayed by the method of Drapper and Hadley.[15] The principal of the method is spectrophotometric measurement of the color produced during the reaction of thiobarbituric acid (TBA) with MDA. One hippocampus was homogenized (1/5 w/v) in a glass Teflon homogenizer in ice-cold buffer (0.05 M Na_2PO_4/KH_2PO_4 buffer, pH 7.4). The homogenate was centrifuged at $10\,000 \times g$ for 15 minutes at 4°C and used for determining the MDA concentration. For this purpose, 1.25 ml of 20% trichloroacetic acid solution was added to 250 µl of homogenate in a centrifuge tube and placed in a boiling water bath for 15 minutes. After being cooled in tap water, the mixture was centrifuged at $1000 \times g$ for 10 minutes, and 1 ml of the supernatant was added to 0.5 ml of 0.67% TBA solution in a test tube and placed in a boiling water bath for 15 minutes. The solution was then cooled in tap water, and its absorbance was measured spectrophotometrically (Shimadzu UV-1601, Kyoto, Japan) at 532 nm. The concentration of MDA was calculated using the value of the MDA–TBA complex of 1.56×10^5/cm/mol and was expressed as nanomole MDA per milligram protein.

Protein determination

Protein in hippocampus homogenate was assayed by Lowry *et al.*'s (1951) method.[16] To 1.0 ml of supernatant from above, 5.0 ml of alkaline copper sulfate reagent is added and thoroughly mixed. Allow to stand for 10 minutes and then add 0.5 ml of Folin's reagent. To develop color, this is kept standing for 30 minutes. This was followed by recording absorbance in spectrophotometer at 660 nm, against a blank. The blank is prepared by taking 1.0 ml of 0.5 M NaOH in place of sample in a cuvette. Bovine serum albumin is used to draw a standard curve, and the amounts of proteins in different samples are estimated.

Western blot analyses

The other hippocampi were homogenized (1/5 w/v), using a handheld homogenizer in ice-cold buffer (50 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 25 µg/ml leupeptin, 25 µg/ml aprotinin, and 10 µM benzamidine). The homogenate was centrifuged at $10\,000 \times g$, 15 minutes, at 4°C, and aliquot was taken for protein determination using Lowry *et al.*'s method.[20] Equal amounts of protein from each sample (20 µg of protein per lane) were separated by SDS–PAGE on 7.5% minigels, blotted electrophoretically to PVDF membrane (Immobilon P), and incubated in Tris-buffered saline with Tween 20 (TBST) (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20 containing 3% bovine serum albumin for 30 minutes). Blots were incubated overnight with anti-NR2A (1:3000) or anti-NR2B (1:5000) in 1% BSA. The blots were then subjected to three additional 10-minute washings in TBST. The blots were incubated with alkaline phosphatase-conjugated monoclonal anti-rabbit IgG (1:10 000) in 1% BSA for 1 hour at room temperature, and three additional 10-minute washes were carried out with TBST. The membrane was incubated in 20 ml of fresh reagent solution (BCIP/NBT) until color development. Immunoblotting for beta-actin (1:5000) was used as an internal standard to confirm equal protein loading and sample transferring. Images of the immunoblots were analyzed with a computerized image analysis system (Kodak Image MM Station, Ultra-Violet Products Ltd, Cambridge, UK). SDS–PAGE and western blot analyses were performed for three independent hippocampus preparations (two to three animals/group).

Statistical analysis

A statistical analysis of the data was performed using SPSS Version 13.0 (Chicago, IL, USA), and the Mann–Whitney *U* test was used for comparison of the control and walnut groups. The significance level was set at $P < 0.05$.

Results

The western blot analyses of the NR2A and beta-actin bands and the graphic of NR2A protein expression are shown in Fig. 1A and B, respectively. The western blot

bands of NR2B and beta-actin and the graphic of NR2B protein expression are shown in Fig. 2A and B, respectively. The protein concentrations of NR2A and NR2B were higher (112 and 158%) in the walnut supplementation group compared with the control group ($P < 0.05$). In addition, the levels of MDA were significantly lower in the walnut group compared with the control group ($P < 0.05$). The mean levels of MDA are shown in Table 3.

Discussion

The study aimed to shed light on the molecular mechanisms underlying the effect of walnut supplementation on hippocampal cognitive functions, such as learning and memory formation, at the experimental level. The primary findings of the study are that walnut consumption has a positive effect on the expression of hippocampal NMDAR subtypes, especially NR2A and NR2B. In addition, we found that a walnut-supplemented diet decreased lipid peroxidation in the hippocampus.

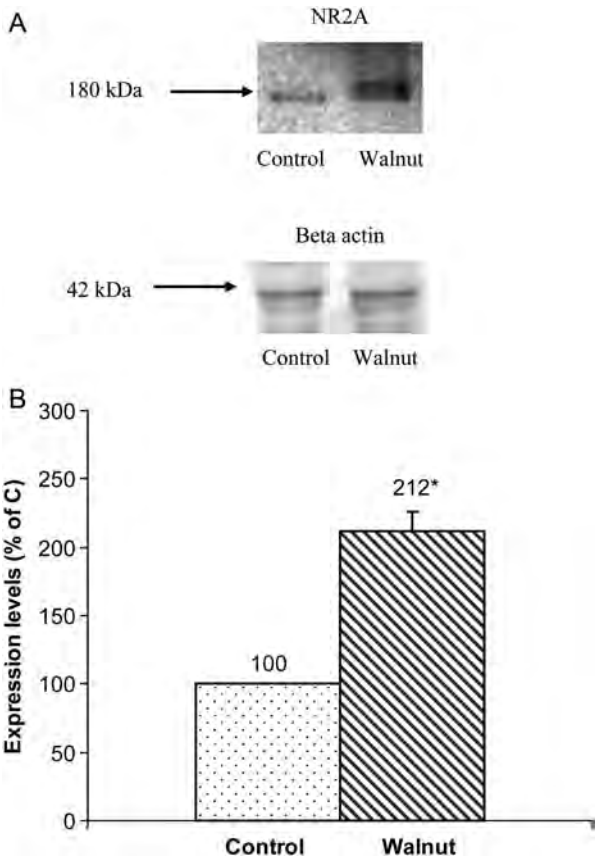

Figure 1 Protein expression of NR2A. (A) Western blotting samples of NR2A and beta-actin. (B) Protein expressions of NR2A. Representative western blotting NR2A bands of all groups from hippocampi. Blotting of beta-actin was used as an internal standard to confirm equal protein loading and sample transferring. Expression of NR2A protein was normalized against that of beta-actin. The density of protein band in the control group was accepted 100% and the walnut group was calculated as a percentage of the control value. Experiments were done on three independent hippocampus preparations (two to three animals/group). Size marker is indicated on the left (alpha-2-macroglobulin, 180 kDa). Walnut supplementation significantly increased NR2A level in the hippocampus. The asterisk indicates significant changes compared with the control group ($P < 0.05$).

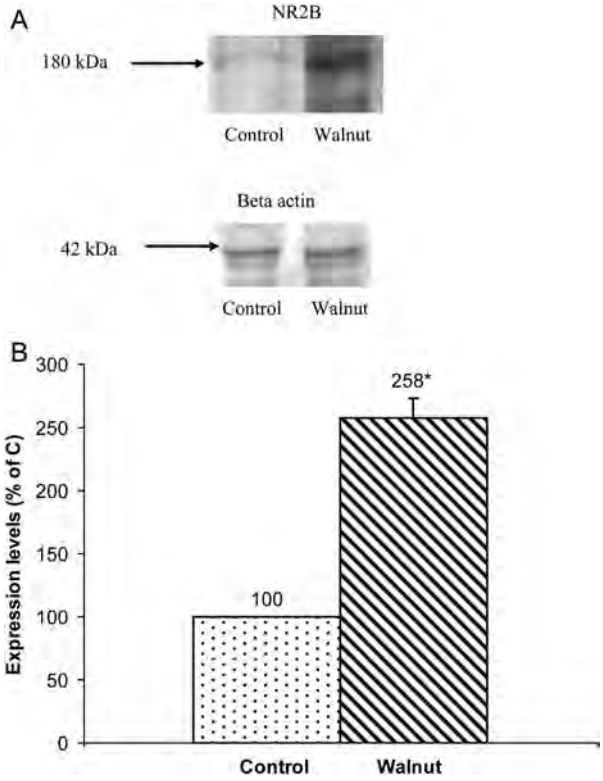

Figure 2 Protein expression of NR2B. (A) Western blotting samples of NR2B and beta-actin. (B) Protein expressions of NR2B. Representative western blotting NR2B bands of all groups from hippocampi. Blotting of beta-actin was used as an internal standard to confirm equal protein loading and sample transferring. Expression of NR2B protein was normalized against that of beta-actin. The density of protein band in the control group was accepted 100% and the walnut group was calculated as a percentage of the control value. Experiments were done on three independent hippocampus preparations (two to three animals/group). Size marker is indicated on the left (alpha-2-macroglobulin, 180 kDa). Walnut supplementation significantly increased NR2B level in the hippocampus. The asterisk indicates significant changes compared with the control group ($P < 0.05$).

Increasing evidence indicates that dietary factors have an influence on cognitive functions in aging and neurodegenerative disorders, such as AD.[1,5,7] A number of dietary factors, such as saturated fatty acids, higher calorie intake, and excessive alcohol, have been reported to increase the risk of dementia and AD.[17] In contrast, antioxidants, fish, methionine-rich proteins, and vitamins were shown to be protective against the disease. Several cross-sectional studies suggested a relationship between particular nutrients and the presence of cognitive changes.[17–18]

NMDARs are required for hippocampal-dependent learning and memory.[19] The activation of NMDARs can be either toxic to neurons or promote their survival and plasticity.[20] Aberrant NMDAR activity can

Table 3 Levels of MDA in the hippocampus in walnut (*n*:12) and control groups (*n*:12)

	Control	Walnut	*P*
MDA (nmol/mg protein)	177 ± 18.6	109 ± 18.3*	0.020

The results are expressed as mean ± SD.
*Significant change compared with the control group. Walnut diet significantly decreased the levels of MDA compared with the control groups in the hippocampus.

cause excitotoxicity, resulting in uncontrolled Ca entry via NMDAR channels.[20] On the other hand, the activity of synaptic NMDARs is crucial for the survival of neurons.[20] The degree of activation of NMDARs is important for hippocampal-dependent cognitive functions. Sufficient expression of NMDARs is needed for LTP induction in the hippocampus. Excess activation of NMDARs causes generation of reactive oxygen species (ROS), and increased ROS can alter NMDAR-dependent calcium influx, ultimately resulting in oxidative damage in the hippocampus.[21] Long-term oxidative stress led to a decrease in LTP formation, and the supplementation of antioxidant-rich dietary factors was shown to reverse the impaired LTP response.[8,21,22] In the study, the beneficial effects of the walnuts were dependent on several factors, one of which was the ratio of ALA and LA in the walnuts. The walnuts studied in the study were found to contain LA and ALA at a ratio of 1:4.7 (55.0 and 11.83%, respectively). This finding was also concordant with the previously suggested optimal ratio of ALA and LA (4:1), which improved cognitive performance.[3] PUFAs can activate membrane-bound receptors, such as the NMDAR subtypes, NR2A and NR2B, which are involved in cognitive functions. The flexibility of the membrane is crucial for the function of membrane-embedded receptors, such as NR2B, and signal transduction.[23] In addition, PUFAs can contribute to synaptic membrane fluidity, reduce oxidative stress, and modulate signaling mechanisms. Several studies reported that enriching the diet of old rats with PUFAs reverses age-related impairments in LTP in the hippocampus.[24,25] Dyall *et al.*[24] showed that dietary supplementation with *n*-3 PUFA reversed age-related decreases in GluR2 and NR2B subunits in the prefrontal cortex, striatum, and hippocampus. It has been shown that the extracts of flavonoid-rich plant or specific molecules, such as blueberry,[7] grape,[8,26] and baicalein[27], can prevent cognitive dysfunctions and can achieve NMDAR-mediated LTP response by enhancing in the rat hippocampus. Results showed that another dietary factor in walnut is melatonin, which has had the effects on the expressions of hippocampal NMDARs for cognitive functions. Our previous study demonstrated that melatonin regulated the hippocampal expressions of NR2A and NR2B in a dose-dependent manner without causing lipid peroxidation.[28] Delibas *et al.*[29] showed that NR2A and NR2B concentrations in hippocampus of rats maintained in dark showed significant increases compared with the control and functional pinealectomy groups. There was no significant increase in lipid peroxidation while the NMDAR concentration increased significantly.[29] These results suggest that melatonin regulates on the hippocampal expressions of NMDARs. Melatonin and flavonoids in walnut can contribute to modulate the expressions of NMDARs. These effects may vary depending on the levels of the components in walnut.

It has been suggested that the oxidative stress plays a major role in the pathogenesis of neurodegenerative disease and aging.[30] MDA, a marker of oxidative tissue damage, is by-product of lipid peroxidation induced by free radicals.[8] Dietary antioxidants help to protect against free radical-induced damage in the brain. Walnuts contain a variety of important dietary factors with strong antioxidative properties, such as melatonin, vitamin E, and flavanoids.[3,4] In our study, we used walnuts obtained from Burdur city in the Mediterranean area of Turkey. We analyzed the percent content of fatty acids in the walnuts. According to the results of the analysis, the walnuts were rich in essential fatty acids, especially omega-6 vs. omega-3 PUFA. A limitation of the study is that we did not conduct a detailed analysis of the content of other dietary components in the walnuts. In the study, the hippocampal levels of MDA decreased significantly in the walnut supplementation group compared with the control group. This result demonstrated that the production of the lipid peroxidation in the hippocampus is prevented by dietary factors in walnut. Dietary factors in walnuts protect against oxidative stress-induced damage by reducing the generation of free radicals and inhibiting membrane lipid peroxidation. Essential fatty acids (*n*-3 PUFA) in walnuts may prevent lipid peroxidation by changing the composition of membrane lipids and membrane fluidity. Another protective factor, flavonoids can affect transcriptional upregulation of antioxidant enzymes, such as glutathione synthesizing enzymes.[18] Walnut supplementation increases the total antioxidant capacity of blood by increasing melatonin levels.[31] Other nutrients in walnuts, such as vitamin E, melatonin, and polyphenols, may prevent NMDAR-mediated excitotoxicity by increasing antioxidant capacity and decreasing lipid peroxidation, thereby contributing to the effects of walnut's antioxidant. These components can also cause inhibition of stress signals and activation of protective signals. Walnut polyphenols include ellagitannins, which have been shown to inhibit oxidative stress and to modulate cell-signaling cascades.[23]

In the study, in addition to inducing the expression of hippocampal NMDARs, walnut supplementation decreased lipid peroxidation in the rat hippocampus. Considering some dietary factors within walnut, the mechanism of effect on hippocampal-dependent functions may be associated with the cumulative effects of PUFAs, and above-mentioned factors can help to trigger the mechanism.

Disclaimer statements

Contributors H.H.: the planning of the study, tissue preparation, western blotting, and imaging. H.V.: the planning of the study, statistical analysis, and interpretation of the results. N.D.: western blotting and interpretation of results. R.S.: imaging and evaluation of the bands. F.G.: the determination of the protein and evaluation of the results. N.Y.: tissue preparation and the determination of the lipid peroxidation.

Funding None.

Conflicts of interest None.

Ethics approval I have read and have abided by the statement of ethical standards for manuscripts submitted to the nutritional neuroscience. The animals involved in the procedure were maintained and used in accordance with the *Animal Welfare Act* and the *Guide for the Care and Use Laboratory Animals* prepared by the Süleyman Demirel University Ethical Committee. The study was approved by the Ethics Committee for Animal Experiments, Süleyman Demirel University's Medical Faculty.

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