Behavioural Brain Research 320 (2017) 97–112

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

A unique combination of micronutrients rejuvenates cognitive performance in aged mice

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HIGHLIGHTS

- Combinations of specific micronutrients improved active avoidance learning.
- Combinations of micronutrients increased IL-10 expression in the aged hippocampus.
- Combination of micronutrients rejuvenated mtDNA content in the hippocampus.

ARTICLE INFO

Article history:
Received 19 September 2016
Received in revised form 30 October 2016
Accepted 3 November 2016
Available online 2 December 2016

Keywords:
Learning
Memory
Active avoidance
Aging
Diet
Hippocampus

ABSTRACT

It is widely believed that diet can influence the onset and severity of cognitive aging, but the optimal combination of micronutrients and molecular and cellular mechanisms remain elusive. The purpose of this study was to compare the effects of eight distinct diets, consisting of various concentrations of selected micronutrients, on learning and memory as well as markers of neuronal plasticity, and metabolic and neuro-immune status of the aged hippocampus. Eighteen-month-old male and female C57BL/6J mice were fed the diets for 16 weeks, followed by learning and memory trials on the active avoidance task. Number of immature neurons were measured by immunohistochemical detection of doublecortin (DCX^+) in the granule layer of the dentate gyrus. Amount of mitochondrial DNA (mtDNA) and gene expression of molecular markers of mitochondrial biogenesis (Ppargc1α, Sirt1, Tfam), and neuroinflammation (IL-10, Alox15, Ptgs2, IL-1β, IL-6 and Tnf) were assessed by quantitative real time polymerase chain reaction (qRT-PCR) of hippocampal samples. Tissue levels of selected micronutrients and a number of metabolites were measured by liquid chromatography-mass spectrometry. The diet supplemented with RRR d-alpha tocopheryl acetate, citicholine, 5-methyltetrahydrofolic acid, quercetin and the n-3 fatty acid phosphatidylserine-docosahexaenoic acid, improved performance on the active avoidance learning and memory task compared to all the other less-complex diets. This diet also increased IL-10 expression and attenuated the age-related change in mtDNA content in the hippocampus without affecting metabolite levels. Results suggest cognitive benefits of wholesome diets are partially mediated through combined antioxidant and anti-inflammatory activities of optimized mixtures of micronutrients.

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1. Introduction

The life expectancy of elderly individuals continues to climb worldwide, but living longer does not always mean living healthier [1]. One of the most devastating aspects of aging is the inevitable decline in cognitive functioning [2,3]. Certain brain regions which

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http://dx.doi.org/10.1016/j.bbr.2016.11.019
0166-4328/Published by Elsevier B.V.

are critical for forming new memories, such as the hippocampus, are vulnerable to structural decline with age [4,5]. The mechanisms that cause retraction of synapses and loss of cellular components in the hippocampus and other brain areas due to aging are not completely understood, but likely involve a host of features, including increased oxidative stress, altered mitochondrial function [6], perturbed lipid metabolism [7] and intensified inflammatory events [8]. Due to the ubiquitous nature of natural aging and steadily increasing aging population, there is a pressing need for designing novel strategies that can mitigate the adverse effects of natural aging on cognition.

Accumulating recent evidence suggests that manipulating specific micronutrients in the diet can slow the progression of cognitive aging [9–11]. Among the long-chain polyunsaturated fatty acids, docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid, is highly concentrated in cell membranes of the retina and brain [12,13], and its effect on brain function in disease and normal aging has been extensively studied [14–20]. However, results are conflicting [21]. Inconclusive neuroprotective effects of DHA may in part be due to its molecular instability, as DHA is highly susceptible to oxidation because of the multiple double bonds along the fatty acid tails. Therefore, we wanted to investigate whether its supplementation with antioxidants could support a role in enhanced learning and memory function or if it is solely able to have cellular beneficial effects as some animal and clinical studies have claimed [22–25]. Phosphatidylserine enriched with docosahexaenoic acid (PS-DHA) is one of the major phospholipid forms of DHA in brain [26,27]. In clinical studies, PS-DHA has been shown to improve cognitive function in both healthy adults and elderly [28,29], hence we wished to study PS-DHA herein.

Flavonoids are among the largest polyphenolic compounds and have been found to have neuroprotective effects during aging in humans and in rodent models [30,31]. Flavonoids have consistently being shown to provide cellular antioxidant benefits and enhance vascular health via their metabolic derivatives [32–35]. In their own right, flavonoids have been shown to ameliorate, to a degree, age-related decline in measures of learning, memory and neuronal signal transduction in a rat model [36]. Here we selected quercetin, a type of flavonoid, because it has been found to have modulatory effects on various fronts such as mitochondrial biogenesis [37,38] and inflammation [39–41]. Moreover, these modulatory effects extend to maintenance of cognitive function, as demonstrated in animal models of Alzheimer's disease [42], diabetes [43], cerebral ischemia [44], hypoxia [45] and stress [40,46]. For these reasons we were interested in assessing quercetin effects alone or in combination with other micronutrients, especially with DHA, as it may buffer DHA's oxidative metabolism following absorption which may enhance cellular function.

The active form of vitamin B9, 5-methyltetrahydrofolate, is considered a healthier alternative than supplementation with folic acid because of the more direct effect it has on cells [47,48]. Folic acid needs to be converted to 5-methyltetrahydrofolate in the digestive system before it can be used [49]. Hence this compound presented a logical choice as a dietary supplement for aged mice.

Citicoline serves as a choline donor in the biosynthesis of acetylcholine neurotransmitter and neuronal membrane phospholipids such as phosphatidylcholine. It has been suggested to have a mild neuroprotective effects in cerebral ischemia by activating gene modulators of metabolism, resistance to oxidative stress, and in genome maintenance [50], and as a way to enhance choline synthesis in Alzheimer's disease [51]. However, in a seizure model, citicoline was reported to exacerbate neuronal death and microglial activation [52]. We were interested in including it here as a micronutrient that could be potentiated in combination with other micronutrients.

d-alpha-tocopherol is the most biologically active form of vitamin E, and its natural form consists of one isomer [53], which has been hypothesized to assimilate better than synthetic versions [54,55]. Besides being known as a lipid-soluble phenolic cellular antioxidant, d-alpha-tocopherol was demonstrated to have immunomodulatory effect by stimulating cAMP signaling in cell culture [56], and to facilitate recovery from age associated changes in immune function [57–61]. Here we use it as a supplement in its most active biological form to investigate its contribution to enhanced cognitive functioning during normal aging in combination with other micronutrients.

Although published research has suggested that the previously mentioned naturally occurring compounds have neuroprotective effects that can delay cognitive aging, few studies, to our knowledge, have combined micronutrients to determine whether a combination has greater effects than each one alone. It seems reasonable to assume that combinations could be better than single micronutrients given the complexity in which the molecules and their target organs interact. One of the reasons we know less about combinations relative to single micronutrients is that combining multiple micronutrients may hinder the ability to evaluate the contribution of each individual micronutrient. However, the advantage favoring the combination of micronutrients rests in that it could produce larger, more robust and more repeatable effects on brain function. Moreover, because people do not consume single nutrients, but rather a group of nutrients in combination within foods, the concurrent assessment of dietary patterns can offer valuable information regarding associations between diet and improvement in cognitive function. This second aspect is relevant to public health concerns due to increasing divergence in dietary patterns and quality among older individuals in our modern society [62].

Accordingly, the goal of this study was first, to identify combinations of micronutrients capable of improving learning and memory in an aged population of mice; and second, to measure the effects of chronic exposure to the diets on a number of biomarkers of brain function and putative targets of the dietary manipulations: hippocampal neurogenesis, expression of immune regulatory genes, amount of mitochondrial DNA, and expression of genes involved in metabolism of mitochondria in hippocampal sections.

2. Material and methods

2.1. Animals

A total of 200 C57BL/6J mice (100 males and 100 females) were used; 160 of these (equal by sex) arrived at our facility at 4 months of age from The Jackson Laboratory (Bar Harbor, ME) and were aged in house to 18 months. 40 additional mice arrived at our facility at 2 months of age, 2 weeks prior to behavioral testing, to serve as young adult controls. All mice were housed individually in cages that contained plastic toys for environmental enrichment (ball, PVC tubing, paper towel, and a plastic hut). Mice had *ad libitum* access to water and food, and were maintained in a controlled ambient environment (temperature at 23 °C, relative humidity of 45%), on a 12-h light/dark cycle. Animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals and in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign.

2.2. Diets

While mice were aging in house, they were fed *ad libitum* the 8604 Teklad 22/5 rodent diet (Envigo Teklad, Indianapolis, IN). Young mice used for comparison were also fed this Teklad 8604 22/5 diet during acclimation and through behavioral testing. At the

Table 1
Micronutrient composition of the diets.

Nutrient	50% Vitamins	Control	150% Vitamins	3-way Combo	Quercetin (0.051 g/kg)	Quercetin (0.102 g/kg)	PS-DHA	5-way Combo
Casein	140	140	140	140	140	140	140	140
L-Cysteine	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
Corn Starch	465.69	465.69	465.69	465.69	465.69	465.69	465.69	465.69
Maltodextrin	155	155	155	155	155	155	155	155
Sucrose[a]	118.69	116.78	114.73	113.83	116.63	116.57	116.09	113.09
Soybean Oil	40	40	40	40	40	40	40	40
Cellulose	50	50	50	50	50	50	50	50
TBHQ, antioxidant	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.008
Choline Bitartrate[a]	1.25	2.5	3.75		2.5	2.5	2.5	
CDP-Choline (Citicholine)[b]				5.382				5.382
Niacin	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Calcium Pantothenate	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016
Pyridoxine HCl[a]	0.0035	0.007	0.0105	0.007	0.007	0.007	0.007	0.007
Thiamin HCl	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006
Riboflavin	0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.006
Folic Acid[a]	0.001	0.002	0.003		0.002	0.002	0.002	
5-methyltetrahydrofolic acid[b]				0.0023				0.0023
Biotin	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002
Vitamin B12 (0.1% in mannitol)[a]	0.013	0.026	0.039	0.026	0.026	0.026	0.026	0.026
Vitamin E dL-alpha tocopheryl acetate (1000 IU/g)[a]	0.0375	0.075	0.112		0.075	0.075	0.075	
d-alpha tocopheryl acetate[b]				0.094				0.094
Vitamin A Palmitate (500,000 IU/g)	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.008
Vitamin D3, cholecalciferol (50,000 IU/g)[a]	0.01	0.02	0.03	0.02	0.02	0.02	0.02	0.02
Vitamin K1, phylloquinone	0.0008	0.0008	0.0008	0.0008	0.0008	0.0008	0.0008	0.0008
Magnesium Oxide[a]	0.425	0.85	1.275	0.85	0.85	0.85	0.85	0.85
Ferric Citrate	0.2121	0.2121	0.2121	0.2121	0.2121	0.2121	0.2121	0.2121
Zinc Carbonate[a]	0.028	0.056	0.084	0.056	0.056	0.056	0.056	0.056
Magnanous Carbonate[a]	0.011	0.022	0.033	0.022	0.022	0.022	0.022	0.022
Cupric Carbonate	0.0109	0.0109	0.0109	0.0109	0.0109	0.0109	0.0109	0.0109
Potassium Iodate	0.0004	0.0004	0.0004	0.0004	0.0004	0.0004	0.0004	0.0004
Sodium Selenate (0.1% Sucrose)[a]	0.18	0.36	0.54	0.36	0.36	0.36	0.36	0.36
Chromium Potassium Sulfate, dodecahydrate	0.0096	0.0096	0.0096	0.0096	0.0096	0.0096	0.0096	0.0096
Lithium Chloride	0.0006	0.0006	0.0006	0.0006	0.0006	0.0006	0.0006	0.0006
Boric Acid	0.0028	0.0028	0.0028	0.0028	0.0028	0.0028	0.0028	0.0028
Sodium Fluoride	0.0022	0.0022	0.0022	0.0022	0.0022	0.0022	0.0022	0.0022
Nickel Carbonate Hydroxyde, tetrahydrate	0.0011	0.0011	0.0011	0.0011	0.0011	0.0011	0.0011	0.0011
Ammonium Meta-Vanadate	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002
Calcium Carbonate	12.50	12.50	12.50	12.50	12.50	12.50	12.50	12.50
Potassium Phosphate, monobasic	8.75	8.75	8.75	8.75	8.75	8.75	8.75	8.75
Potassium Citrate, monohydrate	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98
Sodium Chloride	2.59	2.59	2.59	2.59	2.59	2.59	2.59	2.59
Potassium Sulfate	1.63	1.63	1.63	1.63	1.63	1.63	1.63	1.63
Quercetin Dihydrate[c]					0.051	0.102		0.102
PS-DHA[d]							0.586	0.586

[a] Concentration was manipulated between diets.
[b] Compound added to 3-way Combo and 8-way Combo diets only.
[c] Compound present in Quercetin at 0.051 g/kg or 0.102 g/kg and 5-way Combo diets only.
[d] Compound added to PS-DHA and 5-way Combo diets only.

Table 2
Macronutrient composition of the diets.

	Experimental Diet[a]		Facility Diet[b]	
Macronutrient	% by weight	% Kcal	% by weight	% Kcal
Protein	12.4	13.8	24.3	32.0
Carbohydrate	68.3	76.0	40.2	54.0
Fat	4.1	10.3	4.1	14.0

All diets are formulated by Teklad Harlan Laboratories.
[a] Based on AIN-93M unless modified as specified in Table 1.
[b] Fixed Teklad 8604 22/5 diet for laboratory rodents used for young mice in this study.

start of the chronic dietary experiment, the 18 month-old mice were fed one of eight different diets (10 males and 10 females per diet group; see Table 1). All of the diets were modified using AIN-93M (Harlan Envigo Teklad, Indianapolis, IN) as the base as recommended for maintenance of aged mice [63]. The first diet which hereafter we will refer to as "50% Vitamins" was 50% deficient in vitamins and minerals (Mg, Zn, Mn, Se, vitamin D, vitamin E, choline, folic acid, vitamin B6 and vitamin B12). The second diet which will hereafter be referred to as "Control" was plain AIN-93M. The third diet will be referred to as "150% Vitamins", and was 150% of standard levels of vitamins and minerals. The fourth diet will be referred to as "3-way Combo" and included citicholine, d-alpha tocopheryl acetate and 5-methyltetrahydrofolate. Diets 5 and Diet 6 were supplemented with 0.051 g/kg and 0.102 g/kg concentrations of quercetin, respectively, and will hereafter be referred to as "Quercetin (0.051 g/kg)" and "Quercetin (0.102 g/kg)". Diet 7 was supplemented with 0.586 g/Kg phosphatidylserine-DHA (PS-DHA), and will be referred to as "PS-DHA". Diet 8 used the 3-way Combo as the base but also included 0.586 mg/kg PS-DHA and 0.102 mg/kg quercetin added, and therefore will hereafter be referred to as the "5-way Combo". These compounds were selected for their reported benefits to neuronal health in mice [64–73]. All experimental diets were manufactured by Envigo Teklad (Indianapolis IN), provided in pelleted form, colored per specific diet, and stored in sealed bags at 4 °C prior to use. All experimental diets were formulated for isocaloric content and equal macronutrient (protein, carbohydrate and fat) percentage by weight. The macronutrient contents of the background experimental AIN-93M diet and the standard Teklad 8604 22/5 diet are outlined in Table 2.

2.3. Experimental design

The experiment proceeded in two separate cohorts (80 aged mice and 20 young mice per cohort, with aged mice randomized to all 8 diets in each cohort). Body weights were recorded weekly and food intake monitored three times per week. After the aged mice received 16 weeks of chronic dietary supplementation, both aged and young mice were tested on the active avoidance behavioral task over a 5-day period. All behavior testing was carried out during the dark phase of the light/dark cycle. Animals were euthanized by transcardial perfusion 24 h after the last active avoidance trial.

2.4. Active avoidance test

The GEMINI avoidance system (San Diego Instruments, San Diego, CA) was used which consists of a rectangular avoidance box divided into two chambers of equal size (24 × 20 × 17 cm) by a stainless steel wall with an open door at the base for access to the adjacent compartment. The GEMINI software controlled a cue light, shock grid, and recorded movement of the mouse *via* eight infrared photo-beams in each chamber. On the first day, before starting the trials, each animal was placed in the left chamber, facing away from the door, and allowed to acclimate to the chamber for 2 min in the absence of a stimulus. Each subsequent trial began with the presentation of a cue light (15 W) in the opposite side of the chamber from where the mouse was currently residing which lasted for a duration of 5 s. After 5 s, an electric current is passed through the stainless steel bars of the side of the chamber opposite of the lit chamber for a total of 5 s. If the mouse was present on that side, it would receive a mild foot shock (0.40 mA). An *active avoidance* response was recorded if the mouse moved across into the chamber where the cue light was presented within the 5 s to avoid the electric shock. If the animal moved across chambers during the 5 s electric shock period, the trial is recorded as *escape*. If the mouse remained in the same chamber for the entire duration of the electric shock, the trial was counted as *no response*. For 5 consecutive days, each animal was given 50 trials with a fixed interval of 20 s between trials, in which there was no cue light or electric shock present. The total number of active avoidances per day was used as the response variable for statistical analysis.

2.5. Tissue collection

Mice received 100 mg/kg sodium pentobarbital *ip*, and then were perfused transcardially with a 0.85% saline solution (Thermo Scientific, Waltham, MA). Brains were removed and split along the midline. The left hemisphere was placed in 4% paraformaldehyde for overnight fixation followed by a storage in 30% Sucrose at 4 °C until sectioning for immunohistochemistry. The right hemisphere was placed on an aluminum platform on wet ice and dissected the following way. First the hemisphere was blocked using a razor blade at the caudal and rostral ends. The cerebellum and most of the midbrain and frontal cortex were discarded. The middle section containing the entire hippocampus and surrounding regions was snap frozen in liquid nitrogen and stored at −80 °C until sectioning. The middle section was then reduced by slicing from the rostral end, 4 coronal sections each 50 μm in thickness using a cryostat at −20 °C. A small punch (1 mm in diameter) was taken of the dorsal hippocampus from the remaining section in the cryostat. These pieces were then frozen at −80 °C for ultrahigh performance liquid chromatography and mass spectrometry quantification of selected nutrients (described in *2.9.3. Total protein and total lipid detection*). The remaining section, approximately 30 mg, containing some dorsal and most of the ventral hippocampus, was processed for RNA extraction and qRT-PCR.

2.6. qRT-PCR

2.6.1. Expression of neuroimmune and mitochondria biogenesis genes (mRNA)

Brain sections (~30 mg) containing the hippocampus (see 2.4) were thawed in 1% β-mercaptoethanol/lysis buffer (v/v%) (Qiagen, Valencia, CA) and homogenized at room temperature using an RNase-free disposable pellet pestle (Fisher Scientific, Waltham, MA). Extraction and isolation of RNA was performed with the AllPrep® DNA/RNA/miRNA Kit (Qiagen, Valencia, CA), then quantified with the Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA). Purity of the newly isolated RNA was determined by measuring the ratio of absorbances at 260 nm and 280 nm (260/280) and at 230 nm (260/230) with the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). The average RNA yield was 400 ng/μl per sample with a 260:280 purity ratio of 2.0 or higher. Quality and integrity of RNA was confirmed by 28S/18S rRNA analysis with the Agilent 2100 Bioanalyzer (Santa Clara, CA). Only RNA samples with RNA integrity number (RIN) ≥ 8 were used for cDNA synthesis. Conversion of RNA into cDNA was completed using the iScript™ cDNA synthesis kit (Bio-rad, Hercules, CA) and the following cycling conditions: 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C. After reverse transcription, cDNA sam-

ples were stored at 4 °C for 48 h before two-step quantitative real-time polymerase chain reaction (qRT-PCR) to determine the amount of specific mRNA transcript present in each hippocampal sample. Samples were analyzed for expression levels of neuroinflammatory genes such as IL-1β, IL-6, TNFα, IL-10, Alox15 and Ptgs2; and the mitochondrial biogenesis genes such as Ppargc1α, Sirtui1 and Tfam (see Table 3). Endogenous control genes were: Importin 8 (Ipo8) (qMmuCID0042052), Peptidylprolyl isomerase A (Ppia) (qMmCED0041303) and Hypoxanthine guanine phophoribosyl transferase (Hprt) (qMmuCID0005679). Control genes were selected by preliminary analyses for their analogous expression in samples across all treatment groups and were prepared with each sample in triplicate in a 20 μL reaction. All primers were PrimePCR™ SYBR Green Assays from Bio-Rad (Hercules, CA). The amount of specific mRNA present was determined by utilizing SYBR® Green dye chemistry using a CFX Connect™ Real-Time PCR instrument (Bio-Rad, Hercules, CA), and used the following cycle conditions: 30 s at 95 °C (polymerase activation), 5 s at 98 °C (denaturation), 30 s at 60 °C followed by 40 cycles of 95 °C for 5 and 60 °C for 1 min (melt-curve analysis at 2–5 s/step). The amplification efficiency was equal across all wells for a given gene. For statistical analysis and comparison between groups, the cycle threshold was transformed and normalized to the reference genes using the standard formula: $2^{(CtReferenceGene-CtSampleGene)}$.

2.6.2. Mitochondrial DNA copy number

To quantify concentration of mitochondrial DNA (mtDNA) as a marker for number of mitochondria, total DNA was extracted and isolated from the same hippocampal sample used for RNA isolation with the AllPrep® DNA/RNA/miRNA Kit (Qiagen, Valencia, CA). DNA concentration was measured using the Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA). Purity from phenol and guanidine contamination was determined spectrophotometrically, by the 260/280 and the 260/230 nm absorbance ratios, with the NanoDrop 2000 (Thermo Scientific, Waltham, MA). Only DNA with a 260/280 or 260/230 ratios of 1.8 or above were used. qRT-PCR was performed using 1 ng of DNA as the template; and the mitochondrially encoded cytochrome b (mt-Cytb) (qMmuCED0001514) was used to determine relative expression of mitochondrial gene. The nuclear reference genes β-Actin (Actb) (qMmuCED0027505) and Pepridylprolyl isomerase A (Ppia) (qMmuCED0041303) were used as endogenous references genes. The relative mtDNA to nuclear DNA copy number ratio mt-Cytb: geomean(Actb:Ppia) was calculated and analyzed using the same method as for gene expression of nuclear genes.

2.7. Immunohistochemistry

Right brain hemispheres were sectioned into 40 μm-thick coronal sections using a cryostat set at −20 °C. Sections were kept in tissue cryoprotectant in 24-well plates and stored at −20 °C. A 1-in-6 series of sections throughout the entire rostro-caudal extent of the dentate gyrus were stained with DCX-DAB to estimate total numbers of immature neurons in the granule cell layer of the dentate gyrus at the time of euthanasia. Free floating sections were washed in phosphate-buffering solution (PBS) and then treated with 0.6% hydrogen peroxide in PBS for 20 min. Sections were then blocked with a solution of 0.02% Triton-X and 6% normal donkey serum (NDS) in PBS (PBS-X) for 1 h, followed by incubation in primary antibody, a goat anti-DCX (sc-8066; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:1000 in PBS-X mixed with 3% (NDS) for 48 h at 4 °C. Sections were then washed in a mixture of PBS-X and 3% NDS, then incubated in secondary antibody against goat made in donkey (sc-2042; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution in PBS-X for 90 min at room temperature. After a wash in PBS-X without NDS sections were then treated with the ABC system (Vector Laboratories, Burlingame, CA) for 1 h, followed by a wash in a PBS-X without NDS wash buffer. Sections were then stained using a diaminobenzidine kit (Sigma, St. Louis, MO).

Table 3
Quantitative real-time PCR primer reference.

Gene	Classification	Assay identification[a]
IL-1/3	Pro-inflammatory	qMmCED0005641
IL-6	Pro-inflammatory	qMmCED0005613
Tnf	Pro-inflammatory	qMmCED0004141
Ptgs2	Pro-inflammatory	qMmCED004 7314
IL-10	anti-inflammatory	qMmCED0015452
Alox-5	anti-inflammatory	qMmCED0022324
Ppargc1a	mitochondrial biogenesis	qMmuCI D0006032
Sirt1	mitochondrial biogenesis	qMmuCID0015511
Tfam	mitochondrial biogenesis	qMmuCED0027505
mt-Cytb	mitochondrial Cytochrome b	qMmuCED0001514

[a] Bio-Rad PrimePCR™ SYBR Green gene expression assay identification number.

2.8. Image analysis

The entire unilateral granule layer of the dentate gyrus represented in the 1-in-6 series was photographed using a Zeiss brightfield light microscope (Zeiss, Thornwood, NY), with depth of field sufficient to capture all the cells within the section *via* a high performance axiocam 702 mono high-performance sCMOS microscope camera (Zeiss) interfaced to computer, under 20× (total 200×) magnification. The granule cell layer was outlined using ImageJ and the total number of DCX+ cells counted within the section by an investigator blind to the experimental conditions. Following published methods in our lab [74], the fraction of cells predicted to cross the boundary of the section on one side was removed to produce unbiased estimates.

2.9. Bioanalytical measurements

2.9.1. Reagents

Purified water, chloroform, and methanol (HPLC grade) and concentrated sulfuric acid (analytical grade) were purchased from Fisher Scientific (Pittsburgh, PA). Sodium dodecyl sulfate (SDS) was obtained from Sigma-Aldrich.

2.9.2. Sequential total lipid and protein extraction

Tissue punches, 1 mm in diameter and 2–4 mm long, were taken from the frozen brain samples. The punches were weighed after drying in a lyophilizer (CentriVap Cold Traps, Labconco, MO) under vacuum. Lipids from dried punches were extracted *via* a 2:1 chloroform-methanol mixture. Then, the vials were sonicated in ice for 2 min using an ultrasonic bath sonicator (8891 Ultrasonic Cleaner, Cole-Parmer, Vernon Hills, IL). The lipid extraction solution was taken out of the tube for total lipid analysis and the remaining tissue kept at 4 °C for the subsequent total protein measurement. Tissues were visually completely dissolved after addition of a 2% SDS-water solution and sonication on ice for 20 min. The samples were then incubated in an oven (Lab Companion ON-1E, JEIO TECH, Billerica, MA) for 1 h at 70 °C for follow-up total protein measurements.

2.9.3. Total protein and total lipid detection

For total lipid analysis, the lipid extraction solution was dried with nitrogen gas, dissolved in concentrated sulfuric acid with vortexing, and incubated in the oven for 20 min at 100 °C. After incubation, samples were cooled at room temperature (23–25 °C). Lipid color reagent from a Total Lipid Reagent Set (BQ 045CR, Gen-Way Biotech, San Diego, CA) was added to the samples according to

the manufacturer's instructions and mixed with the sample by vortexing. The reaction was carried out for 15 min in a cold water bath; next, 100 μL of each sample were moved into a 96-well microplate. Total lipid content was determined using UV light absorbance at 530 nm measured with an EPOCH microplate spectrophotometer (BioTek Instruments, Winooski, VT).

For total protein analysis, the 2% SDS-treated samples were supplemented with bicinchoninic acid using a Micro BCA TM Protein Assay Kit (23235, Thermo Scientific, Rockford, IL) according to manufacturer's instructions and mixed by vortexing. Samples were incubated for 1 h at 60 °C. A 100 μL aliquot was moved to a 96-well microplate. The sample's UV absorbance at 562 nm was measured using the EPOCH microplate spectrophotometer.

For total lipid and protein analysis, external standard calibration curves were used to calculate the lipid and protein concentrations in the tissue extracts according to instructions for the analytical kits and the spectrophotometer. Oleic acid and bovine serum albumin (BSA) were used as standards in the total lipid and total protein measurements, respectively.

2.9.4. Elemental analysis

Half of the cerebellum (weighing ~50 mg) was digested by high purity acid combinations in perfluoroalkoxy alkane (PFA) vessels using a Multiwave 3000 Microwave digester (Perkin Elmer, Waltham, MA) equipped with high pressure (60 bar). Digested samples were diluted to around 80 ppb level for each targeted element, and then loaded into a nebulizer and spray chamber *via* the sample introduction system of an inductively coupled plasma (ICP) mass spectrometry (MS) instrument (SCIEX ELAN DRCe ICP-MS, PerkinElmer, Norwalk, CT) for analysis. Mg, Mn, Se and Zn ion signals were monitored. Internal standards were added at a constant rate with the sample solution *via* the third channel of the on-board peristaltic pump to compensate for matrix effects. Target signal intensity was first normalized by internal standard signal intensity. Normalized values were plotted on a related external calibration curve. The concentration of elements are presented in relation to wet weights of tissues.

2.9.5. Metabolite and micronutrient analysis

Individual hippocampal samples (1 mm in diameter and 2–4 mm long) were punched from frozen mouse brain tissues using a pre-chilled (−20 °C) puncher and placed in Eppendorf tubes and stored on dry ice. The length of each tissue punch was measured to calculate sample volumes. Various metabolites were extracted using a multistep extraction procedure as per the following: a 200 μL ethanol solution (200 proof/absolute alcohol) containing 25 mg/L antioxidant butylated hydroxytoluene (BHT) was first added into a tube containing the frozen tissue. Tissue was manually stirred/homogenized with 10–15 strokes of a pestle, incubated for 15 min on ice, and centrifuged at 21 000 *x g* (14 000 rpm) using an Eppendorf 5810R refrigerated benchtop centrifuge (Hauppauge, NY) at 4 °C for 10 min. Supernatant from the first extraction was collected; 100 μL of water containing 25 mg/L BHT was added to the pellet with sample agitation. A 15 min incubation period was followed by 10 min centrifugation at 21 000 × *g*. Supernatant from the second extraction was collected and combined with the supernatant from the first extraction. The last step of analyte extraction from the remaining pellet resembled the first step except that only 50 μL of ethanol containing 25 mg/L of BHT was added. All three supernatants were combined, centrifuged and stored on ice as a final sample extract. The extract was twice diluted with liquid chromatography (LC) mobile phase A (see below) with the addition of 200 ppb of 4-aminobutyric-2,2-d2 acid used as internal standard; 5 μL of the diluted sample extract was used in the LC–MS analysis described below. Unless otherwise indicated, each procedure was performed either on ice or at 4 °C.

Multiple reaction monitoring (MRM) assay was performed for detection of α-tocopherol (α-TCP), γ-aminobutyric acid (GABA), β-alanine (β-Ala), tyrosine (Tyr), glutamate (Glu), aspartate (Asp), vitamin C, dehydroascorbic acid, DHA, serine (Ser), and acetylcholine (Ach), with one quantifier and two qualifier fragment ions detected (Supplemental Table 1). MRM quantitative metabolite analysis was successful applied to brain tissue by us and others [75,76].

The MS analysis was done using an ultrahigh performance liquid chromatography (UHPLC)-atmospheric pressure chemical ionization (APCI) triple quadrupole MS system (EVOQ Elite™ Triple Quadrupole Mass Spectrometer equipped with the Advance™ UHPLC module, Bruker Daltonics, Billerica, MA). A 3 μm Acclaim Mixed-Mode HILIC-1 150 × 4.6 mm internal diameter column (Thermo Fisher Scientific, Waltham, MA) was used for LC analyte separation. Mobile phase A was composed of 50 mM ammonium acetate (pH 4) and acetonitrile (5/95 v/v). Mobile phase B was composed of acetonitrile, water and 50 mM ammonium acetate (pH 4) at a ratio of 50/40/10 v/v. The flow rate was set to 600 μL/min. The LC conditions were as follows: 0–3.30 min 95% A; 3.31–5.15 min 100% B; 5.16–8.00 min 95% A.

The analysis was performed using both positive and negative ionization polarity. The parameters used for the mass spectrometer were as follows: (+) spray current 15.0 μA; (−) spray current 15.0 μA; cone temperature 250 °C; cone gas flow 10 aU (arbitrary units); heated probe temperature 450 °C; probe gas flow 15 aU; nebulizing gas flow 60 aU; collision gas pressure 1.5 mTorr (Argon); 0.7 unit resolution for both Q1 and Q3 quadrupoles. Exhaust gas was turned on to continuously clean the APCI source internal space from excess solvent vapor and eliminate the recirculation of nebulized gases so as to reduce chemical noise and keep the system cleaner.

The stock solutions of glutamate, aspartate and GABA were prepared in water, whereas others were prepared in ethanol in the presence of BHT; solutions were diluted with mobile phase A (described above), creating a set of samples with concentrations of the analyte standards ranging from low ppb to low ppm. Standard solutions as well as biological sample extracts were analyzed with LC–MS in sequence with a 5 μL injection. Absolute concentrations of the metabolites and micronutrients were determined based on the external calibration curves obtained using standard compounds. The 4-aminobutyric-2,2-d2 acid internal standard was used for monitoring the overall performance of the system. For each sample, two technical replicates were acquired and averaged.

The data were processed using Bruker software (Bruker MS Workstation, version 8.1.2, for the EVOQ™). Data were first normalized to tissue volume. To reduce the influence of batch effects, likely related to sample collection and duration of storage differences between different cohorts, the resulting data were normalized to means of the aged male group fed the control diet in each cohort. The data sets were sorted into groups based on the sex, age and treatment, and processed using statistical methods described below.

2.10. Statistical analyses

Data were analyzed using SAS software version 9.2. In all analyses, $P < 0.05$ was considered statistically significant. Body weight and food intake were analyzed using repeated measures ANOVA with week as the within subjects and diet as the between subject variables. These data were also analyzed by multiple linear regression including sex and interactions between sex and the other factors in the model. The number of DCX+ cells, qRT-PCR gene expression, and mass spectrometry molecular analysis were analyzed by 3-way ANOVA with age (adult or aged), diet, and sex as factors. The total number of active avoidances per day, were analyzed using repeated measures ANOVA with day as the

within-subjects factor and sex, age, and diet as between-subjects factors. Additional analyses were conducted including only aged subjects. To determine which diets or micronutrients were driving the dietary effects among the aged subjects, separate analyses were conducted comparing each diet to the Control. Further, an additional three separate analyses were conducted collapsing the diets that contained similar micronutrients. For these analyses, the Control diet was compared to 1) the diets that contained quercetin, *i.e.*, Quercetin (0.051 g/kg) and Quercetin (0.102 g/kg), 2) the diets that contained PS-DHA, *i.e.*, PS-DHA and 5-way Combo, and 3) the diets containing the triad of citicholine, metafolin, and RRR alpha tocopherol (3-way Combo and 5-way Combo). Two subjects were removed from these analyses because they displayed abnormal behavior, *i.e.*, either they continued to make no responses by the end of the trial, or the data were corrupted. Data were power transformed to improve homogeneity of variance and reduce skewness. The following variables were transformed: number of active avoidances per day were raised to the power of 4, concentration of beta alanine and aspartate were raised to the power of 0.5, tyrosine and glutamine were log transformed. For vitamin C and acetylcholine, one very high outlier was removed. For manganese and zinc, 2 outliers were deleted and then remaining data were log transformed. For selenium, 2 high values were deleted and then remaining data raised to power of 0.5.

3. Results

3.1. Food intake and body weight

Average food consumption collapsed across the 16 weeks was similar for the different diets (Fig. 1A). Females ate approximately 8 g more food than males per week after correcting for differences in body mass ($F_{1,145} = 14.9$, $P = 0.0002$). As expected, average body mass increased by approximately 2 g from week 1 to week 16 ($F_{1,134} = 33.2$ $P < 0.0001$), and males were approximately 9 g heavier than females ($F_{1,133} = 214.6$, $P < 0.0001$), but no differences were detected between the dietary treatments (Fig. 1B). No other main effects or interactions were significant.

3.2. Active avoidance

All the mice learned the task as indicated by significant increases in number of active avoidances across the 5 days (Fig. 2, $F_{4,776} = 1073.9$, $P < 0.0001$). The young mice learned faster and outperformed the aged mice as indicated by significant effect of age ($F_{1,189} = 48.9$, $P < 0.0001$), and significant age by day interaction ($F_{4,776} = 12.0$, $P < 0.0001$). Among only the aged mice, we observed a significant effect of day ($F_{28,555} = 472.9$, $P < 0.0001$) and interaction between treatment and day ($F_{28,555} = 1.5$, $P = 0.044$). No main effect of treatment was detected. Separate analyses comparing each diet with the Control indicated that aged mice fed the 5-way Combo learned faster than mice fed the other diets (Fig. 2D). This was indicated by a significant main effect of treatment for the analysis that included only the Control diet and the 5-way Combo ($F_{1,34} = 5.0$, $P = 0.03$) and marginally non-significant interaction between day and treatment ($F_{4,136} = 2.1$, $P = 0.08$). Although the aged mice fed 5-way Combo were slower to learn than the young mice, they eventually reached young-levels of performance by days 4 and 5, a feat accomplished by none of the other aged groups. No other main effects or interactions were detected for any of the other single diet comparisons (Fig. 2).

The analyses that collapsed diets based on presence of specific micronutrients identified a significant effect of quercetin on improving learning in the aged mice. This was indicated by a main effect of day ($F_{4,580} = 466.0$, $P < 0.0001$), quercetin ($F_{1,145} = 5.5$, $P = 0.02$) and interaction between quercetin and day ($F_{4,580} = 3.7$, $P = 0.006$), with the quercetin animals showing steeper learning than others. No other diets with isolated micronutrients were significantly different from the Control diet.

3.3. Gene expression

No significant differences were found in levels of gene expression of the mitochondrial enzymes between ages or diets (Fig. 3A). However, aged mice displayed approximately 26% increased concentration of mt-Cytb, indicator of total number of mitochondria as compared to young mice ($F_{1,51} = 4.8$, $P = 0.03$; Fig. 3B). Posthoc analysis indicated that this effect was driven mainly by elevated mt-Cytb in aged mice fed the Control diet as aged mice fed the Control diet differed significantly from young mice ($P = 0.026$) whereas aged mice fed the 5-way Combo did not differ from young mice. As expected, the aged mice displayed significantly elevated levels of pro-inflammatory cytokines, IL1β ($F_{1,50} = 22.3$ $P < 0.0001$), IL6 ($F_{1,50} = 42.8$, $P < 0.0001$) and TNFα as compared to young mice ($F_{1,50} = 18.8$, $P < 0.0001$; see Fig. 3C). Diet had no influence on the pro-inflammatory cytokines. However, the 5-way Combo significantly increased the anti-inflammatory cytokine, IL10, relative to the young mice. This was indicated by significant treatment effect in the 1-way ANOVA ($F_{2,39} = 3.3$, $P = 0.049$) and significant posthoc pair-wise comparison between the 5-way Combo and the young control ($P = 0.01$). No other pair-wise differences were significant.

3.4. Adult hippocampal neurogenesis

As expected aged mice displayed nearly 2 orders of magnitude fewer dcx+ cells as compared to young mice ($F_{1,83} = 12{,}705.7$, $P < 0.0001$), and female aged mice displayed approximately 29% greater numbers of dcx cells than males ($F_{1,57} = 11.2$, $P = 0.0015$; see Fig. 4A). No effect of dietary treatment was detected.

3.5. Hippocampal metabolites

No effect of diet was observed for any of the metabolites (see Table 4). Alpha tocopherol was 2.8 fold higher in aged mice than in young mice ($F_{1,190} = 212.7$, $P < 0.0001$), and 20% higher in females than in males ($F_{1,190} = 30.6$, $P < 0.00011$). Levels of β-alanine decreased in old relative to young males but this age difference was not observed in females. This was indicated by a significant interaction between sex and age ($F_{1,189} = 5.2$, $P = 0.02$). Main effects of age and sex were not significant. Posthoc analyses indicated that in males, β-alanine decreased with age by approximately 20% ($P = 0.02$), whereas in females it remained constant. Levels of magnesium decreased in old relative to young females, but this age difference was not observed in males. This was indicated by a significant interaction between sex and age ($F_{1,99} = 6.5$, $P = 0.01$). The main effects of age and sex were not significant. Posthoc analyses indicated that in females, magnesium decreased with age by approximately 26% ($P = 0.01$), whereas in males it remained constant. Lipid content increased in old relative to young males, but this age difference was not observed in females. This was indicated by a significant interaction between sex and age ($F_{1,99} = 6.0$, $P = 0.02$). Age was also significant ($F_{1,99} = 6.1$, $P = 0.02$), but not sex. Posthoc analyses indicated that in males, lipid content increased with age by approximately 28% ($P = 0.0008$), whereas in females it remained constant.

4. Discussion

The major finding of the study is the combination of naturally occurring micronutrients α-tocopherol, citicholine, 5-MTHF,

Table 4
Hippocampal metabolites.

Diet	Sex	n	α-TCP[b, c]	n	GABA	n	β-Ala[d]	n	Tyr	n	Glut	n	Asp	n	Vit C	n	Oxi-Vit C	n	DHA	n	Ser	n	Ach	n	Mg[a, e]	n	Mn[a]	n	Se[a]	n	Zn[a]	n	protein	n	lipid[f]
Young	male	28	0.41 ± 0.04	28	1.20 ± 0.09	28	1.18 ± 0.19	28	1.16 ± 0.12	28	1.18 ± 0.14	28	0.76 ± 0.17	28	0.90 ± 0.06	18	1.10 ± 0.07	18	0.91 ± 0.22	18	0.83 ± 0.01	18	0.78 ± 0.11	18	178.2 ± 8.6	17	2.12 ± 1.13	18	0.10 ± 0.03	18	6.30 ± 0.75	18	41.0 ± 0.78	18	68.2 ± 4.31
	female	20	0.51 ± 0.40	20	1.14 ± 0.07	20	0.91 ± 0.13	20	1.00 ± 0.07	20	1.05 ± 0.13	20	0.98 ± 0.21	20	0.77 ± 0.05	11	0.90 ± 0.07	11	0.51 ± 0.12	11	0.90 ± 0.14	11	1.04 ± 0.23	11	263.6 ± 36.0	11	1.73 ± 0.52	11	0.15 ± 0.04	11	6.71 ± 0.68	11	41.5 ± 2.34	11	79.8 ± 6.43
50%	male	9	0.99 ± 0.08	9	1.00 ± 0.05	9	1.00 ± 0.14	9	1.00 ± 0.08	9	1.00 ± 0.12	9	1.00 ± 0.29	5	1.00 ± 0.06	5	1.00 ± 0.09	5	1.00 ± 0.22	5	1.00 ± 0.22	5	1.00 ± 0.28	5	194.5 ± 21.1	5	1.24 ± 0.72	5	0.11 ± 0.04	5	8.63 ± 2.51	5	40.5 ± 2.75	5	83.9 ± 8.52
Vitamins	female	7	1.14 ± 0.06	7	0.89 ± 0.02	7	1.09 ± 0.21	7	0.85 ± 0.08	7	0.99 ± 0.15	7	0.89 ± 0.35	3	0.78 ± 0.11	3	0.95 ± 0.12	3	0.85 ± 0.14	3	0.99 ± 0.18	3	0.93 ± 0.09	3	168.7 ± 9.9	3	1.00 ± 0.38	3	0.13 ± 0.06	3	5.66 ± 0.46	3	48.2 ± 3.18	3	99.2 ± 17.18
Control	male	10	1.15 ± 0.09	10	1.12 ± 0.97	10	1.17 ± 0.20	10	1.20 ± 0.16	10	1.07 ± 0.22	10	0.94 ± 0.38	4	0.88 ± 0.04	5	1.23 ± 0.18	5	1.00 ± 0.21	5	1.05 ± 0.27	5	1.02 ± 0.28	5	232.0 ± 31.0	5	1.84 ± 1.16	5	0.15 ± 0.42	5	6.45 ± 0.37	5	38.6 ± 4.76	5	85.3 ± 11.94
	female	9	1.51 ± 0.15	9	1.13 ± 0.09	9	0.89 ± 0.13	9	0.96 ± 0.11	9	0.89 ± 0.12	9	1.12 ± 0.25	4	0.81 ± 0.08	4	1.09 ± 0.19	4	1.09 ± 0.24	4	0.73 ± 0.36	3	0.65 ± 0.12	4	194.8 ± 20.9	4	1.02 ± 0.26	4	0.04 ± 0.04	4	5.70 ± 0.35	4	41.5 ± 2.27	4	84.2 ± 8.16
150%	male	10	1.22 ± 0.13	10	1.05 ± 0.07	10	0.63 ± 0.12	10	1.02 ± 0.11	10	1.01 ± 0.16	10	0.88 ± 0.23	5	0.84 ± 0.08	5	1.12 ± 0.06	5	0.43 ± 0.23	5	0.97 ± 0.22	5	0.58 ± 0.15	5	198.4 ± 22.6	5	2.20 ± 1.63	5	0.13 ± 0.08	3	6.45 ± 2.45	5	44.2 ± 3.94	5	96.9 ± 13.29
Vitamins	female	8	1.54 ± 0.14	8	1.03 ± 0.09	8	0.85 ± 0.27	8	0.83 ± 0.09	8	0.81 ± 0.18	8	1.00 ± 0.33	3	0.64 ± 0.04	3	0.89 ± 0.10	3	0.30 ± 0.18	3	1.04 ± 0.14	3	0.95 ± 0.04	3	253.8 ± 46.7	3	0.81 ± 0.24	3	0.15 ± 0.01	3	6.21 ± 0.08	3	39.6 ± 3.87	3	80.7 ± 22.05
3-way	male	10	1.23 ± 0.10	10	1.21 ± 0.08	10	0.59 ± 0.13	10	1.27 ± 0.19	10	0.89 ± 0.12	10	1.10 ± 0.12	5	0.83 ± 0.06	5	1.12 ± 0.11	5	0.57 ± 0.35	5	1.08 ± 0.17	5	0.63 ± 0.11	5	160.9 ± 7.2	4	1.69 ± 0.94	5	0.12 ± 0.07	5	5.29 ± 0.59	5	41.2 ± 2.85	5	84.2 ± 12.66
Combo	female	9	1.56 ± 0.15	9	1.16 ± 0.15	9	0.96 ± 0.10	9	0.98 ± 0.08	9	1.03 ± 0.14	9	1.36 ± 0.33	5	0.70 ± 0.05	5	1.03 ± 0.11	5	0.35 ± 0.32	5	1.14 ± 0.27	5	0.63 ± 0.14	5	188.9 ± 19.5	5	2.63 ± 1.24	4	0.12 ± 0.01	5	7.26 ± 1.31	5	39.5 ± 2.47	5	73.5 ± 7.50
Quercetin	male	10	1.13 ± 0.11	10	1.10 ± 0.07	10	0.94 ± 0.15	10	1.28 ± 0.15	10	0.94 ± 0.09	10	1.04 ± 0.29	5	0.87 ± 0.13	5	1.21 ± 0.11	5	0.68 ± 0.31	5	1.20 ± 0.26	5	0.69 ± 0.19	5	184.1 ± 19.8	5	2.63 ± 2.16	5	0.14 ± 0.07	5	9.82 ± 3.50	5	43.0 ± 2.50	5	94.8 ± 15.00
(0.051 g/kg)	female	9	1.30 ± 0.11	9	0.93 ± 0.07	9	0.93 ± 0.13	9	0.88 ± 0.06	9	0.81 ± 0.14	9	0.74 ± 0.20	5	0.74 ± 0.05	5	0.97 ± 0.10	5	0.84 ± 0.35	5	1.00 ± 0.24	5	0.91 ± 0.23	5	193.9 ± 21.7	5	1.41 ± 0.79	5	0.08 ± 0.04	5	5.53 ± 0.41	5	39.1 ± 2.20	5	70.9 ± 4.86
Quercetin	male	10	1.29 ± 0.12	10	1.23 ± 0.14	10	0.96 ± 0.22	10	1.20 ± 0.14	10	1.12 ± 0.18	10	0.76 ± 0.26	5	0.86 ± 0.05	5	0.96 ± 0.10	5	0.74 ± 0.26	5	0.85 ± 0.28	5	0.79 ± 0.30	5	211.2 ± 37.2	5	0.84 ± 0.26	5	0.10 ± 0.04	5	6.95 ± 0.79	5	40.0 ± 1.50	5	86.2 ± 5.28
(0.102 g/kg)	female	8	1.35 ± 0.10	8	1.11 ± 0.11	8	0.98 ± 0.20	8	0.91 ± 0.10	8	0.89 ± 0.11	8	1.14 ± 0.36	5	0.72 ± 0.08	5	1.03 ± 0.19	5	0.74 ± 0.28	5	1.01 ± 0.23	5	0.89 ± 0.23	5	170.4 ± 50.3	5	2.53 ± 1.65	5	0.11 ± 0.04	5	5.73 ± 0.38	5	38.4 ± 1.75	5	84.2 ± 5.34
PS-DHA	male	10	1.07 ± 0.04	10	1.09 ± 0.09	10	0.67 ± 0.17	10	1.10 ± 0.20	10	0.76 ± 0.12	10	0.67 ± 0.15	5	0.91 ± 0.14	5	0.99 ± 0.10	5	0.96 ± 0.20	5	0.75 ± 0.20	5	0.71 ± 0.10	5	197.8 ± 55.8	5	0.58 ± 0.14	5	0.11 ± 0.05	5	6.03 ± 0.38	5	40.9 ± 1.52	5	90.1 ± 7.61
	female	9	1.36 ± 0.12	9	1.10 ± 0.06	9	0.94 ± 0.12	9	0.89 ± 0.12	9	0.83 ± 0.16	9	1.09 ± 0.36	5	0.77 ± 0.06	5	0.10 ± 0.11	5	0.77 ± 0.26	5	1.25 ± 0.32	5	0.66 ± 0.17	5	186.6 ± 26.6	5	0.68 ± 0.08	5	0.10 ± 0.04	5	6.28 ± 0.47	5	44.2 ± 1.29	5	83.2 ± 6.17
5-way Combo	male	9	1.16 ± 0.05	9	1.16 ± 0.14	9	0.69 ± 0.15	9	1.10 ± 0.15	9	0.76 ± 0.18	9	0.67 ± 0.13	4	0.82 ± 0.03	4	0.83 ± 0.10	4	1.02 ± 0.31	4	0.95 ± 0.18	4	1.02 ± 0.38	4	234.9 ± 20.3	4	1.09 ± 0.41	3	0.10 ± 0.05	4	6.16 ± 0.27	4	41.9 ± 2.02	4	83.2 ± 7.48
Combo	female	9	1.42 ± 0.11	9	1.12 ± 0.09	9	1.10 ± 0.20	9	1.04 ± 0.21	9	0.99 ± 0.21	9	1.62 ± 0.43	5	0.70 ± 0.03	5	0.90 ± 0.03	5	0.63 ± 0.36	5	1.20 ± 0.25	5	0.74 ± 0.14	5	221.9 ± 53.9	5	0.62 ± 0.07	5	0.11 ± 0.04	5	6.66 ± 0.73	5	39.0 ± 1.89	5	72.7 ± 6.73

All units are μg/μL unless indicated.

[a] Expressed in parts per million (ppm).

[b] Significantly higher content in aged mice (in all diets) than in young mice ($p < 0.0001$).

[c] Significantly higher content in female mice than male mice across all groups ($p < 0.0001$).

[d] Significantly higher content in young male mice than aged male mice ($p < 0.02$).

[e] Significantly higher content in young female mice than in aged female mice (#$p < 0.01$).

[f] Significantly lower content in young male mice than aged male mice in all dietary groups (ψ$p < 0.0008$).

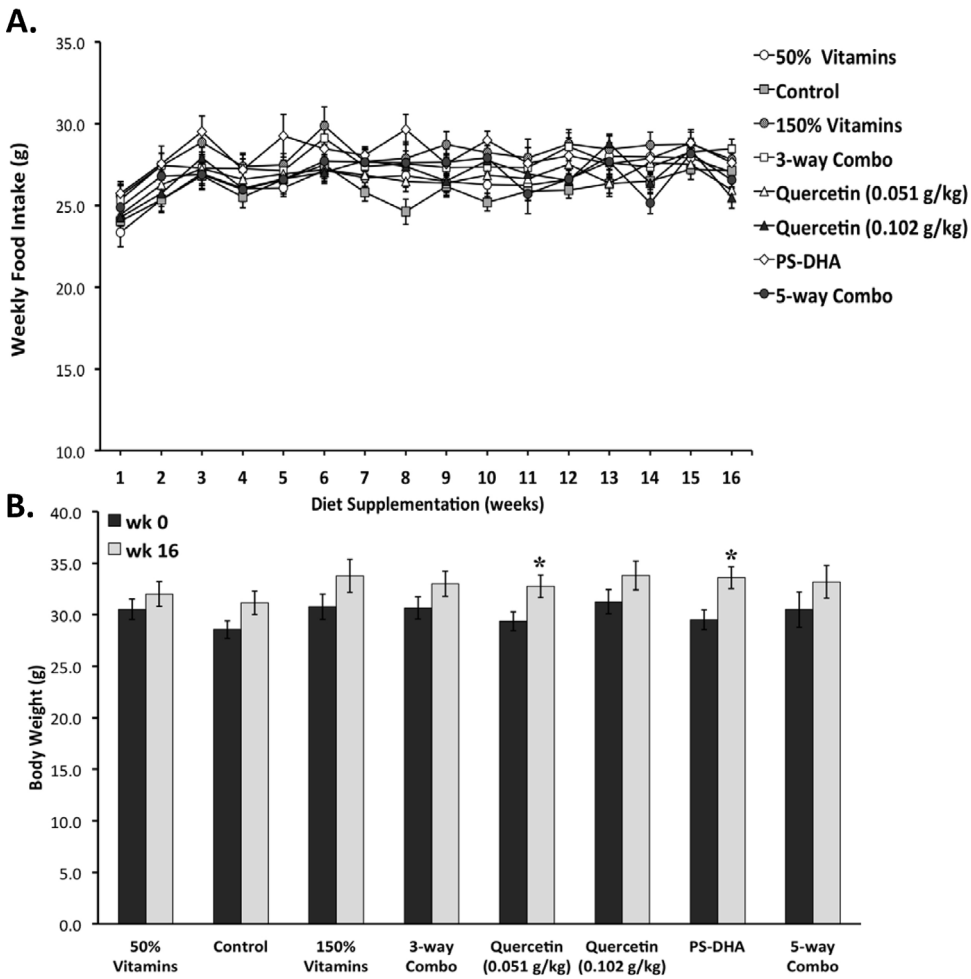

Fig. 1. Food intake and body weight. A) Average weekly food intake is shown for all 8 diets over the course of the study, collapsed across sex. B) Body weight is shown at the beginning and end of the study for each of 8 diets collapsed across sex. No differences between diets were detected. Mice gained a few grams during the study. Standard error bars shown (n = 19 or 20 per diet group). *indicates significant difference by paired *t*-test ($p < 0.05$).

quercetin and the n-3 fatty acid containing PS-DHA reliably rejuvenates cognitive performance in mice. This was established in two separate cohorts, in a large number of aged mice, in both males and females. Administering these micronutrients alone or in simpler diets was not sufficient–the cocktail was the key. Results highlighted the importance of including the flavonoid quercetin in the combination, as diets containing quercetin collapsed together also outperformed the Control diet. Quercetin, a dietary flavonoid ubiquitously present in vegetables, fruits, tea and wine, has been suggested to be a strong antioxidant [9,77,78] and an anti-inflammatory agent in the hippocampus and other brain areas [79].

The efficacy of combining quercetin with other antioxidants or neuroprotective compounds within a diet has been demonstrated in previous studies. The ingestion of a cluster compounds of similar properties can result in improved glycosylation which hastens absorption kinetics rates *via* transporters in the small intestine [80,81]. Previous studies demonstrated that the simultaneous ingestion of quercetin with vitamin C, folate, and additional flavonoids improves absorption rates and bioavailability compared to single flavonoids such as isoquercetin, which have a higher absorption rate [82,83]. Therefore, it is possible that the addition of the vitamins and minerals, combined with the other micronutrients in the 5-way Combo, increased the bioavailability of quercetin, which facilitated learning. However, this hypothesis would need to be confirmed by direct serological analysis of the nutrient metabolites.

Tissue concentrations of selected micronutrients were measured to provide evidence as to whether the different diets affected bioavailability or absorption of micronutrients that could explain the pro-cognitive effects. However, the results revealed no specific effects of diets and only a few mild sex differences on concentration of metabolites in the brain section containing the hippocampus and surrounding regions (Table 4). It is not clear why we were not able to detect increased concentrations of α-tocopherol, vitamin C, DHA, and other selected vitamins and minerals even though these were added to some of the diets. It could be that most of the absorption, accumulation, and/or metabolism of the studied entities occurred in other tissues, or the brain region we analyzed was actively maintaining a set level of the compounds. Alternatively, it could be that the pro-cognitive effects were secondary and not related to the accumulation of the specific micronutrient, but rather more closely related to derivatives, metabolites, or physiological events that may occur in other tissues. Nevertheless, we did observe some interesting age and sex differences in the metabolites that deserve some attention. The most relevant findings from this analysis were an age-related increase in lipid content in male mice only, and the lower content of α-tocopherol in the hippocampus of aged mice, which was more pronounced in males. It is widely thought that α-tocopherol acts as an antioxidant in the brain during lipid metabolism [58,71,84]. Adequate absorption and bioavailability of

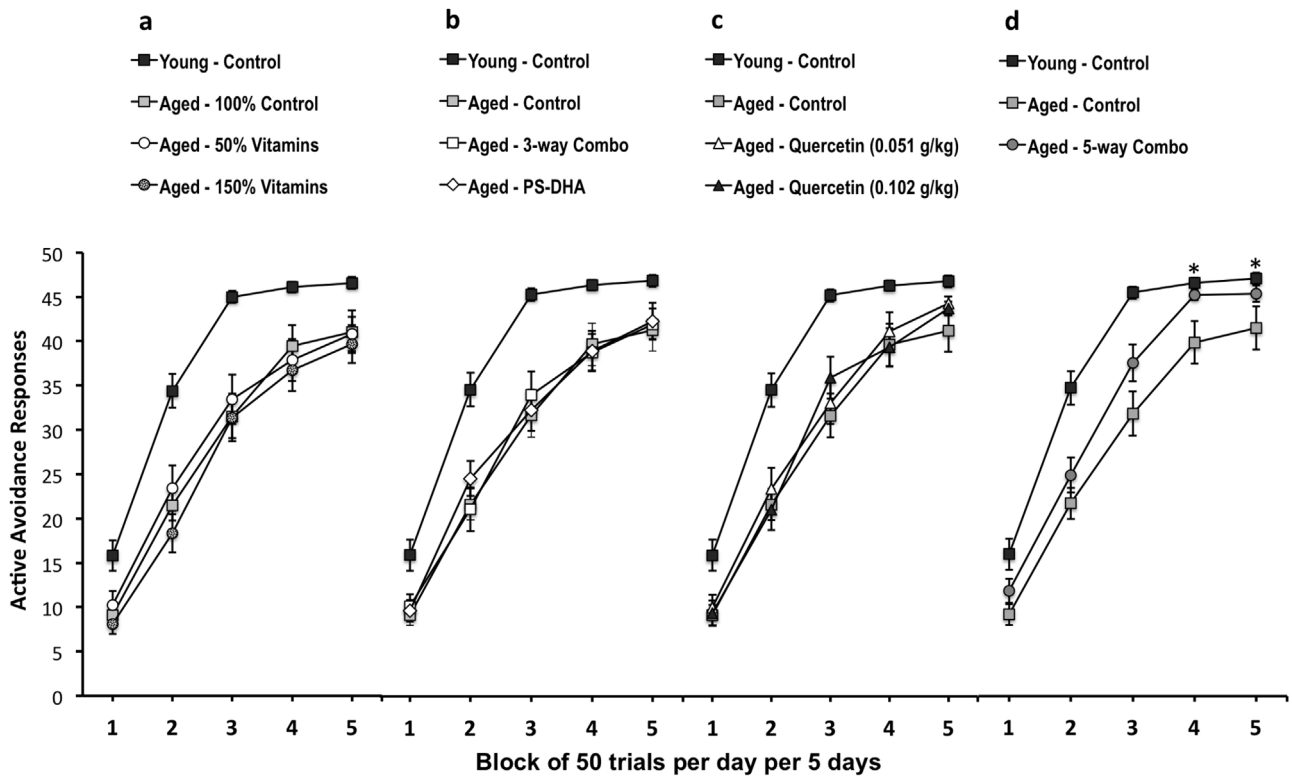

Fig. 2. Cognitive performance on the active avoidance task. Average number of active avoidances out of 50 trials per day plotted across days for each of the 8 diets. A–D) Graphs are separated to facilitate comparisons of 1 or 2 treatment groups at a time to the aged Control diet and young mice. Standard error bars shown (n = 19 or 20 per diet group). Only the 5-way Combo (panel D, the diet with the most micronutrients) displayed significantly improved performance over the aged Control. *indicates significant main effect of the 5-way Combo diet and interaction between the 5-way Combo diet and day compared to the aged Control ($p < 0.05$).

α-tocopherol in old age is important to prevent the build-up of specific lipid derivatives associated with mild cognitive impairments [85,86]. Although females did not show differences α-tocopherol content in the brain across age in our measurements, results for males suggest that effects of age on oxidative status during lipid metabolism may partly influence cognitive function, as old mice, regardless of which diet they received, learned at a slower rate than young mice in the active avoidance task. This assertion needs to be confirmed with a comprehensive analysis of fatty acids in the hippocampus and other brain regions supporting cognitive function to determine which subtype(s) are associated with age-related cognitive dysfunction.

The learning performance data presented here clearly show the importance of "background" nutrient insufficiencies on the impact of putative neuroprotective candidate compounds (compare the 5-way Combo performance to all the other diets). Our data strongly suggest that a dietary intervention that tests specific neuroprotective compounds while ignoring nutrient status changes (especially micronutrients) associated with aging is not enough. This observation might help explain some inconsistencies in the literature on the pro-cognitive effects of DHA supplementation [87,88]. Both the animal and clinical studies have attributed pro-cognitive effects of DHA to antioxidant, anti-inflammatory and anti-apoptosis properties, by virtue of its capacity to support neuronal cell membrane structure, enhancing fluidity and influencing important metabolic signaling events that maintain neuronal health [89–92]. Recent clinical studies investigating the long-term effects of DHA on cognitive function in healthy aged adults have found mixed results and have suggested that factors such heredity and environment complicate the story. A study in England found no effects of a 6-month dietary supplementation with n-3 PUFAs, alone or in combination with other micronutrients, on the cognitive performance of elderly humans [93]; whereas a 12-month DHA-enriched meal regime improved cognitive function and mental health in Japanese octogenarians [94]. Part of the inconsistent results could come from the fact that the compounds are taken in the background of other micro- and macronutrients in the diet, which may work synergistically.

A possible mechanism responsible for the pro-cognitive effects of the 5-way Combo at the cellular level may be enhanced protection of DHA from oxidation by dietary antioxidants such as α-tocopherol and quercetin. This can translate into more efficient production of DHA-derived metabolites, which are important key cellular protective factors against oxidative stress [95]. In aged rodents, high incorporation of n-3 fatty acids (mainly DHA) can lead to phospholipid hydroperoxide accumulations by oxidative reactions at any 6 double bonds in their long polyunsaturated fatty acid tail when exposed to cellular reactive oxygen species [96]; and this oxidation can be cytotoxic and genotoxic [97]. Quercetin, and like other flavonoids of similar structure, may localize to the lipid membrane and provide redox protection to key fatty acids [98,99]. Recent data demonstrated cell membrane co-localization of both α-tocopherol and lutein, ((a plant-derived) carotenoid with some chemical structure similarities as quercetin), with free DHA in brain regions controlling cognition (*e.g.,* the prefrontal cortex, cerebellum and striatum) of *Rhesus Macaque* [100]. Once co-localized in the cell membrane, quercetin can enhance its oxidative actions by increasing its lipophilicity, from its absorbed moderate hydrophilic flavonoid glycoside form to its hydrophobic form *via* esterification with membrane-bound fatty acids *in situ* [101–103]. A recent *in vitro* experiment demonstrated that esters of quercetin-3-*O*-glucoside (Q3G) display significantly higher inhibition of Cu^{2+}- and peroxyl radical-induced low-density lipoprotein (LDL) oxidation in comparison to Q3G alone [104]. Taken together, these data

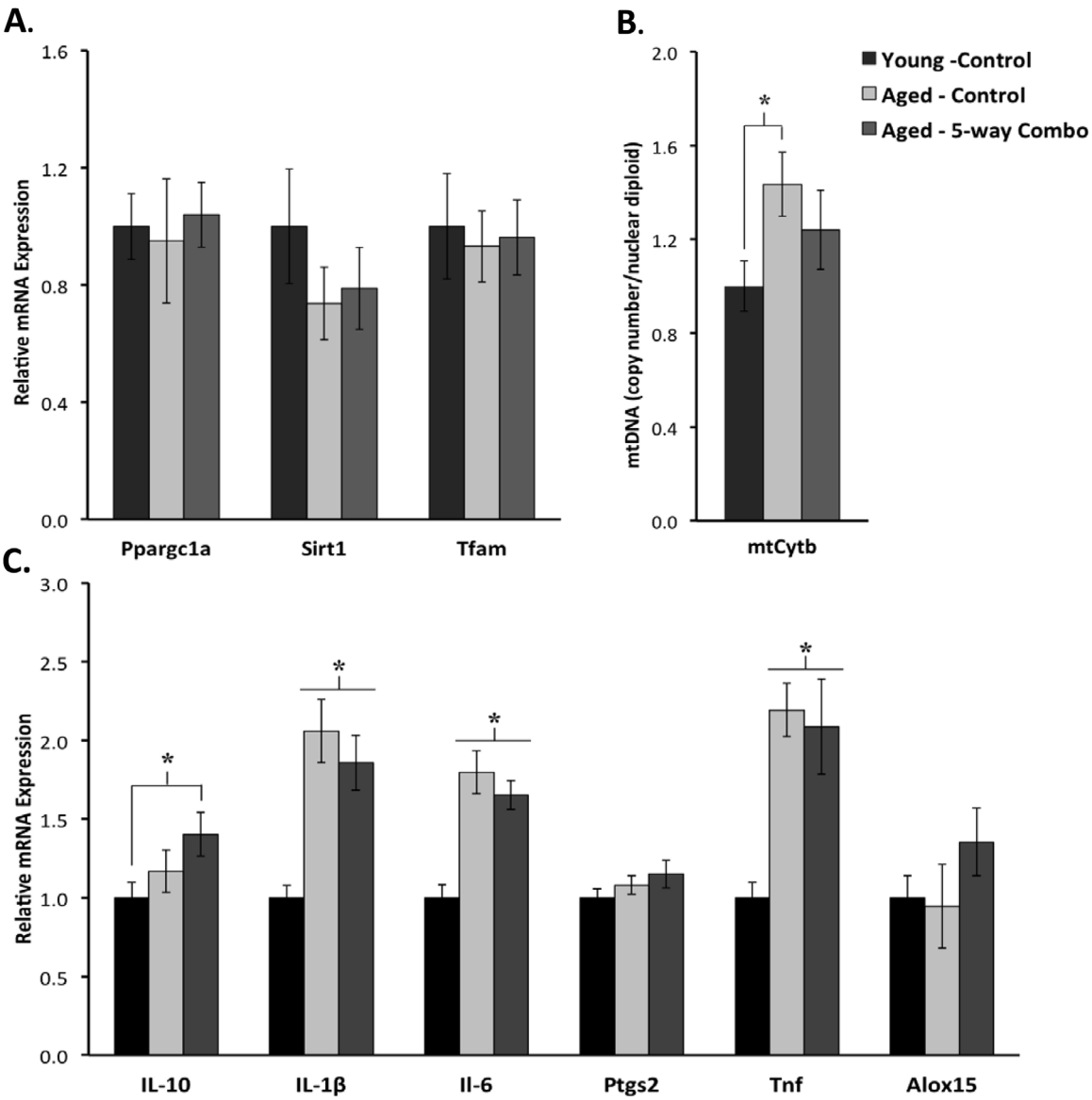

Fig. 3. Mitochondrial biogenesis and neuro-immune gene expression. A section of the brain containing the hippocampus and surrounding regions was analyzed and compared between aged mice fed the Control diet, the 5-way Combo, and young mice. Results are expressed as proportion of average expression levels in the young control group (Young-Ctrl Diet). A) Relative mRNA expression of mitochondrial biogenesis genes. No group differences were detected. B) Relative copy number of mitochondrial cytochrome b DNA (mt-Cytb), as indicator of number of mitochondria. Aged mice fed the Control diet displayed higher levels than young mice. C) mRNA expression of neuro-immune cytokines. Expression of proinflammatory cytokines IL-1β, IL-6 and TNFα were significantly elevated in aged relative to young mice, but no effect of diet was detected. Expression of the anti-inflammatory cytokine IL-10 expression was higher in mice fed the 5-way Combo compared to young control mice. No other differences were detected. Standard error bars shown (n = 19 or 20 per diet group). *indicates significant difference relative to the young control group ($p < 0.05$).

support co-supplementation of antioxidants such as α-tocopherol, quercetin or other flavonoids to augment the benefits of n-3 fatty acids, such as DHA, in food matrices. The outcome can be expected to enhance cellular protection against damage from reactive oxygen species (ROS) and the maintenance of neuronal health [78,105], which can translate into improved cognitive function in aged mouse models.

Mitochondrial ROS are key culprits of age-associated cognitive impairment, and a reduction of mitochondrial ROS could improve cognition by maintaining mitochondrial health and mitochondrial biogenesis processes [106–108]. Accordingly, we measured the effects of the 5-way Combo on the expression of three markers of mitochondrial biogenesis: a) peroxisome proliferator-activated receptor-gamma, co-activator 1, alpha (Ppargc1α), a transcriptional co-activator required for mitochondrial biogenesis [109], b) sirtuin 1 (Sirt1), a mammalian transcriptional co-activator, an important regulator of energy homeostasis in response to nutrient availability [110], and c) mitochondrial transcription factor A (TFAM), essential for mtDNA transcription and a key regulator of mtDNA copy numbers in mammals [111]. However, we found no effects of diet or age on the expression of any of the three genes of mitochondrial biogenesis, but there was an age-related increase in mtDNA content that was reduced by the 5-way Combo. The analysis of mtDNA content and its association with age has yielded conflicting results, such as a decrease of mtDNA content in aged mice [112], or an increase with age in mice [113–115] and senescent rats [114]. Furthermore, a longitudinal comparative study in various tissues in 24 month-old C57BL/6 mice found no significant increase in mtDNA content [115]. A possible explanation for the discrepancies could be the differential methodology used as well as the different regions of the brain measured [112]. Despite these differences, the literature agrees that an increase in mtDNA content is

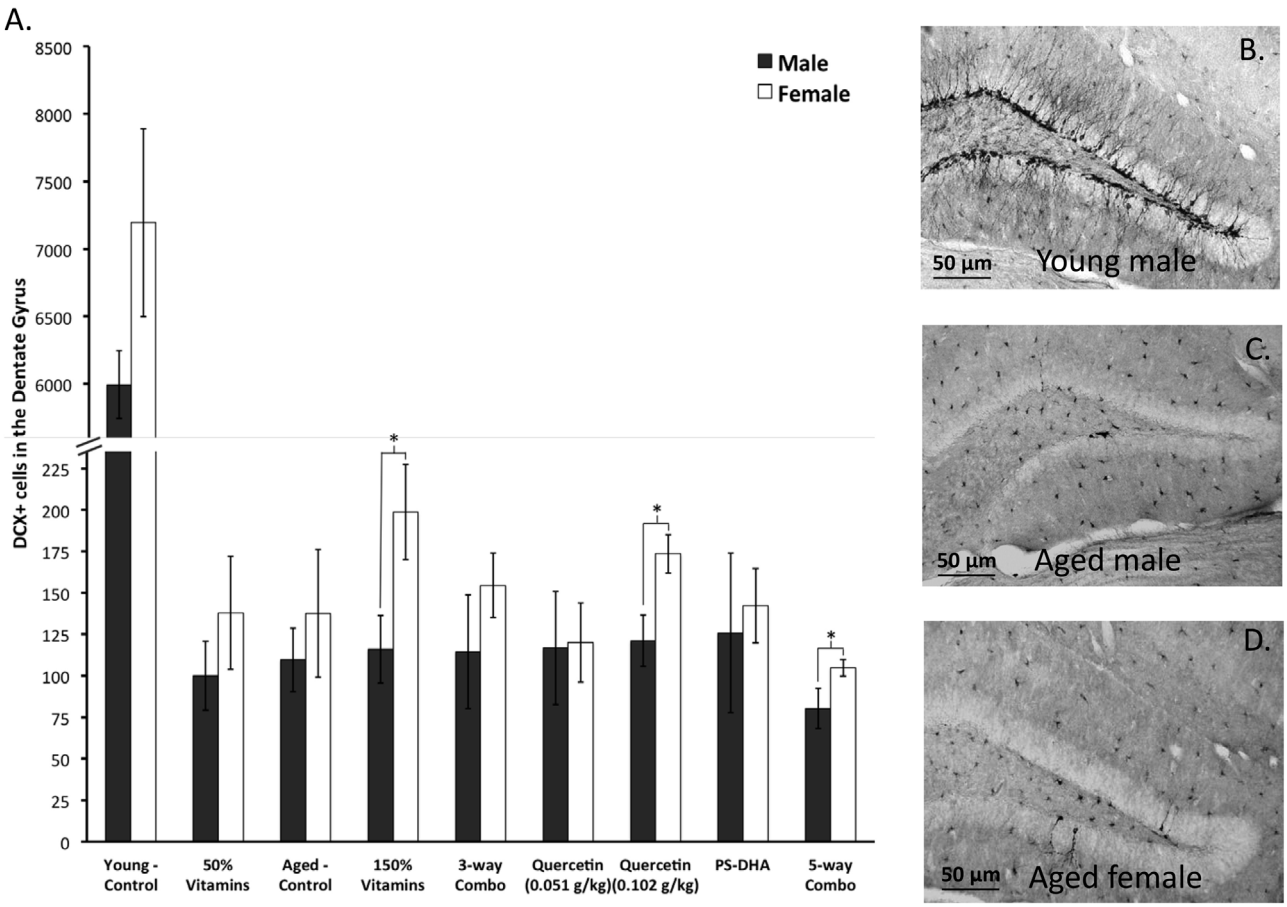

Fig. 4. Adult hippocampal neurogenesis. (A) Average total number of cells in the granule layer of the dentate gyrus expressing doublecortin (DCX) marker of immature neurons. As expected young mice displayed orders of magnitude greater number of DCX^+ cells than aged mice, and females slightly greater numbers than males, but no differences between diets were detected. Standard error bars shown (n = 19 or 20 per diet group). *indicates significant sex difference ($p < 0.05$). Photographs of representative DCX-stained sections through the dentate gyrus from a B) young adult male mouse, (C) an aged male mouse fed the 5-way Combo, (D) an aged female mouse fed the 5-way Combo.

probably a compensatory mechanism for defective mitochondria bearing mutated mtDNA during the aging process [112,114,115]. Taken together, although our data suggest that the dietary impact on mtDNA content in the hippocampus did not involve mitochondrial biogenesis, the age-related increase in mtDNA may be due to oxidative stresses during a life span, and the combination of antioxidant compounds (*i.e.*, α-tocopherol, DHA and quercetin) in the 5-way Combo contributed to diminishing this aging effect on mtDNA quantity.

We also investigated the neuro-immune status of the hippocampus following dietary supplementation because chronic low-grade inflammation is known to contribute to cognitive aging [74,116,117], and PS-DHA, α-tocopherol, quercetin, and folate, in addition to vitamins C, B6, and B12 in our diets, were hypothesized to impact immune signaling. Our data confirm a profile of low-grade neuro-inflammation in the aged relative to young mice in parallel with cognitive impairment [118–121], but the diet which significantly enhanced cognitive performance in our study, the 5-way Combo, had little impact on the neuro-immune markers. The only difference we observed was increased hippocampal expression of IL-10 in aged mice that received the micronutrient combination (5-way Combo) compared to levels in young control mice, and not observed in aged mice fed the Control diet. Our findings are consistent with observations from a recent study suggesting that flavonoid-rich food can directly induce the expression of IL-10 [122]. An intervention with flavonoid-rich dark chocolate in middle-aged men experiencing acute psychosocial stress, raised expression of IL-10 in peripheral circulation; and the level of this expression correlated with the content of the flavonoid epicatechin in the dark chocolate [122]. IL-10 is an anti-inflammatory cytokine that represses the expression of pro-inflammatory cytokines by activating the IL-10 receptor on microphages [123] and on non-macrophage cells [124], eventually triggering intracellular transcription factors that suppress genes that induce inflammatory cytokines [125]. Specific mechanisms by which flavonoids directly influence the expression of IL-10 have recently been elucidated by experiments that used flavocoxoid, a medical food product made from plant-derived flavonoids, in the treatment of a mouse model of experimental autoimmune encephalomyelitis. These mechanisms include the promotion of the inflammatory resolving macrophage phenotype M2, reduction of the expression of the immune-reactive MHCII/costimulatory molecules, and the inhibition of the differentiation of the encephalitogenic Th1/Th17 cells [126]. All of these mechanisms demonstrate the inflammation-resolving influence of flavonoids on cellular immune processes. Our data suggest that similar mechanisms may be partially at play in our model, with the caveat that these mechanisms were observed for the diseased condition, whereas our study used a normal, healthy aging mouse model. Exploration of these mechanisms in the aged hippocampus after dietary interventions, presents an opportunity for future investigation.

In addition to quercetin, PS-DHA may have contributed to the increased IL-10 expression in the 5-way Combo. Experiments in Δ6-desaturase (D6D) KO mice, which lack the rate-limiting enzyme

that initiates the metabolism of the dietary essential n-6 and n-3 PUFAs, have demonstrated induced expression of IL-10 by immune mononuclear cells concomitant to a decrease in IL-6, IL-1β and IFNγ, when fed a diet supplemented only with menhaden fish oil, which is rich in EPA/DHA fatty acids [127]. DHA can also protect cells from inflammation by enhancing the function of M2 macrophages and inducing the generation of regulatory T cells, as shown in adult mice [128]. Taken together, we suggest combining quercetin and PS-DHA increased the anti-inflammatory actions of IL-10 in the hippocampus, which contributed to the enhanced learning and memory that we observed in the aged mice.

The pro-cognitive effects of the combination in the 5-way Combo was not accompanied by increased numbers of immature neurons, markers of adult hippocampal neurogenesis, in the aged mice. We had hypothesized that micronutrient combination might increase neurogenesis *via* anti-oxidant and anti-inflammatory processes [129–131], but such was not the case. Taken together, our results suggest the combination (5-way Combo) enhances cognitive performance *via* mechanisms, which differ (in at least some aspects) from those affected by exercise, specifically not involving increased hippocampal neurogenesis [132,133].

In summary, we discovered an enriched diet that includes multiple micronutrients is effective at improving cognitive function in an aged mouse model. Collectively results from our studies suggest that a diet that includes a combination of neuroprotective compounds can be significantly more beneficial than a single-compound dietary supplementation, to cognitive function in aged mice. These effects could be the result of synergistic actions on cellular mechanisms that promote neuroimmune protection, and reduce effects of aging on mtDNA content, as shown in the hippocampus. The combined micronutrients likely also improved the absorption, bioavailability and actions of neuroprotective compounds in the brain. At least some of these added micronutrients could simply have filled gaps due to age, and thus made the body better able to respond to beneficial stimuli. Even though cognitive performance was improved by a wholesome dietary intervention in late adulthood, it was not completely rejuvenated. It is possible that we have not yet identified all of the nutrient gaps existing due to aging in this model, since we tried to design these interventions around some known or suspected insufficiencies in older humans. It is also possible that a multi-nutrient dietary regimen, if applied earlier in life (perhaps around middle age) could be more effective at retarding age-related effects on cellular function by promoting neuroimmune protection and improving energetic status in the aged hippocampus. This hypothesis warrants investigation. Identification of specific molecular mechanisms responsive to dietary interventions is challenging, but a growing literature suggests that it will be at least part of a future strategy for cognition enhancement and/or as neuroprotective therapy during brain pathologies and normal aging.

Acknowledgements

We wish to thank the Center for Nutrition, Learning and Memory and Abbott Nutrition for funding and the UIUC Microanalysis laboratory (http://scs.illinois.edu/microanalysis/) with assistance with the elemental analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2016.11.019.

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