NUTRITIONAL COGNITIVE NEUROSCIENCE RESEARCH AT THE CROSSROADS OF NUTRITION, PSYCHOLOGY, AND NEUROSCIENCE

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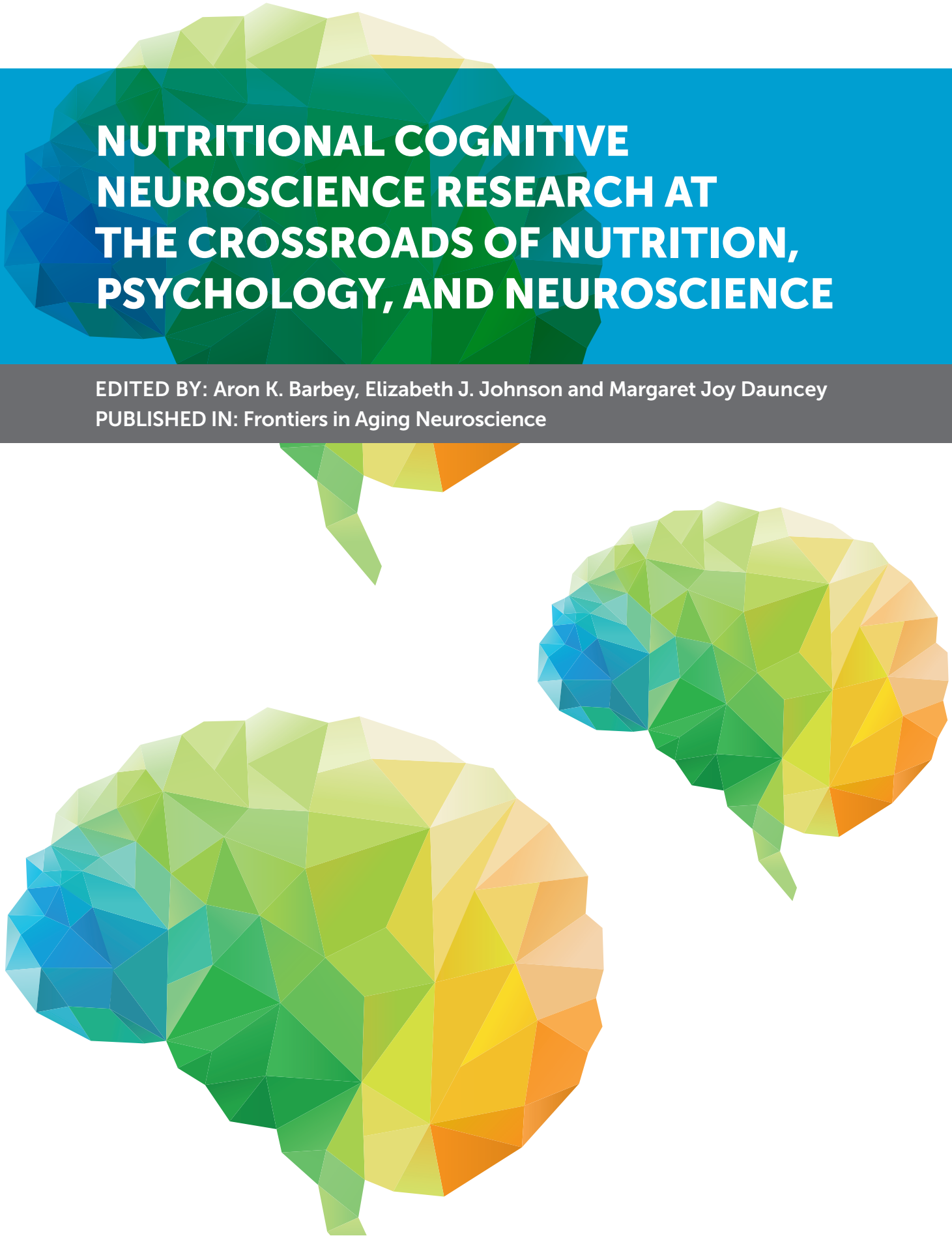

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NUTRITIONAL COGNITIVE NEUROSCIENCE RESEARCH AT THE CROSSROADS OF NUTRITION, PSYCHOLOGY, AND NEUROSCIENCE

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ORIGINAL RESEARCH
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Anti-inflammatory Effects of Homotaurine in Patients With Amnestic Mild Cognitive Impairment

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Alzheimer's disease (AD) is a fatal dementing neurodegenerative disease, currently lacking an efficacious disease-modifying therapy. In the last years, there has been some interest in the use of homotaurine as a potential therapeutic compound for AD, but more work is still needed to prove its efficacy as disease modifier in dementia. Since inflammation is believed to play a key role in AD development, we sought to investigate here the *in vivo* homotaurine effect on inflammatory response in patients at the earliest stages of AD, i.e., suffering from amnestic mild cognitive impairment (aMCI). Thus, the present study aims to evaluate the effects of homotaurine supplementation on cytokine serum levels and memory performances in MCI patients. Neuropsychological, clinical and cytokine assessment was performed at baseline (T0) and after 1 year (T12) of homotaurine supplementation in 20 patients categorized as carriers (n = 9) or no carriers (n = 11) of the ε4 allele of the apolipoprotein E (APOE) gene, the strongest genetic risk factor for AD. The serum levels of the pro-inflammatory mediators Interleukin (IL) 1β, Tumor necrosis factor-alpha (TNFα), IL-6 and IL-18, contextually with the anti-inflammatory molecules IL-18 binding protein (IL-18BP) and Transforming growth factor-beta (TGFβ), were analyzed to explore significant differences in the inflammatory status between T0 and T12 in the two APOE variant carrier groups. No significant differences over time were observed in patients as for most cytokines, except for IL-18. Following homotaurine supplementation, patients carrying the APOEε4 allele showed a significant decrease in IL-18 (both in its total and IL-18BP unbound forms), in turn associated with improved short-term episodic memory performance as measured by the recency effect of the Rey 15-word list learning test immediate recall. Thus, homotaurine supplementation in individuals with aMCI may have a positive consequence on episodic memory loss due, at least in part, to homotaurine anti-inflammatory effects. This study strongly suggests that future research should focus on exploring the mechanisms by which homotaurine controls brain inflammation during AD progression.

Keywords: tramiprosate, amnesic MCI, Alzheimer, APOE, inflammation, cytokines, interleukin-18

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive deterioration in cognition, functional ability and behavior, but its underlying causes are to date unclear and a disease-modifying cure is still unavailable. Dysfunction in the clearance of Amyloid β-protein (Aβ) and neuroinflammation are two functionally linked pathological aspects of key importance in the development and progression of the disease (Heppner et al., 2015).

Homotaurine is an aminosulfonate compound naturally found in red algae, which has been demonstrated to have neuroprotective effects in rats systemically administered with kainic acid (Fariello et al., 1982) or following ischemic stroke (Wu et al., 2014). As a possible therapeutic agent for AD treatment, homotaurine reduces Aβ levels in CSF of patients with mild to-moderate disease (Aisen et al., 2006), slows brain atrophy (Gauthier et al., 2009) and exerts positive effect on cognitive impairment (Saumier et al., 2009). In a phase III AD clinical trial, homotaurine does not satisfy pre-fixed primary outcomes failing to demonstrate changes in cognitive function compared to placebo, but this result is possibly confounded by high statistical variability of data and is paralleled by *post hoc* analyses in a subgroup of patients, revealing some protective effects on hippocampal volume loss (Aisen et al., 2011). Thus, although safe and well tolerated, homotaurine is not authorized as a new AD drug, but it is currently used as a nutraceutical for memory protection and its use in treatment of cognitive decline symptoms is still considered promising. According with its potential favorable effects, we recently demonstrated that homotaurine supplementation has a positive consequence on hippocampus atrophy and short-term episodic memory loss in individuals at the earliest clinical state of AD, namely subjects suffering from amnestic mild cognitive impairment (aMCI; Spalletta et al., 2016). Regardless the favorable disease-modifying activities of homotaurine, its therapeutic efficacy and mechanism of action have yet to be fully elucidated.

Intriguingly, the protective activity of homotaurine appears to be especially evident in AD patients carrying the apolipoprotein E (APOE) ε4 alleles (Caltagirone et al., 2012), suggesting that its effects might be influenced by APOE ε4 genotype, the most powerful genetic risk factor of AD. Since APOE proteins appear to modulate Aβ clearance (Kim et al., 2009) and from *in vitro* and preclinical studies homotaurine reduces soluble levels of Aβ, inhibits its aggregation and decreases its toxic effects on neurons (Gervais et al., 2007), it is tempting to speculate that homotaurine may act, at least in part, in an APOE-dependent way. Furthermore, since Aβ clearance defect might also be both cause and consequence of the chronically activated neuroinflammatory pathways, which in turn concur to cause neuronal death, we addressed this study to evaluate the ability of homotaurine supplementation in modulating the inflammatory response in treated aMCI patients. In fact, several studies indicate that cerebral Aβ deposits elicit a chronic, disseminated inflammatory response producing neurodegeneration in AD (Akiyama et al., 2000) and, more recently, a skewed immune response both in brain and periphery has been blamed for a defective Aβ clearance leading to AD development (Heneka et al., 2015; Marsh et al., 2016; Ransohoff, 2016). In this regard, accumulation of reactive (and possibly functionally flawed) microglia in damaged brain regions and increased cerebral/peripheral expression of pro-inflammatory cytokines have been broadly described in AD patients. Of note, in response to a peripheral inflammatory stimulus, pro-inflammatory cytokine production is higher with APOE ε4 genotype, compared to the other APOE allele, and recent observations suggest a role for APOE in modulating Aβ-induced neuroinflammation (Tai et al., 2015), supporting the relevance of APOE genotype-specific homotaurine therapeutic potential.

By a mechanistic point of view, Aβ may trigger an innate response through the activation of NALP3 inflammasome (Halle et al., 2008), a multi-protein innate immune pathogen-sensing complex, which is essential for the release of specific inflammatory mediators, determining the cytokine milieu in the brain. Indeed, the two inflammasome-dependent molecules Interleukin (IL)-1β and IL-18 have been shown to be crucial regulators in AD pathology (Shaftel et al., 2008; Bossù et al., 2010). These two cytokines may exert their inflammatory action at both brain and peripheral level. While circulating IL-1β is generally low in normal conditions, with serum concentration often below level of detection and very small visible effects in the periphery, serum IL-18 is constitutively present in high amounts but is regulated by a highly specific natural inhibitor, named IL-18 binding protein (IL-18BP).

Therefore, with the aim to evaluate the potential immunomodulating effects of homotaurine in the serum of APOE genotyped aMCI patients, in this study we measured a panel of pro- and anti-inflammatory cytokines, including other than IL-1β, Tumor necrosis factor-alpha (TNFα), IL-6 and Transforming growth factor-beta (TGFβ), also IL-18BP and IL-18. The latter was evaluated in both its forms, i.e., the total form, including the cytokine bound and unbound to its inhibitor, and the free, unbound and biologically active form. Because of previous evidence that the compound is effective on patients carrying the APOEε4 allele, we hypothesized that homotaurine has an effect on memory deficit and immunomodulation in this subgroup only.

MATERIALS AND METHODS

Subjects

Twenty subjects with aMCI were included in this study. Only patients without significant clinical factors that promote inflammation were selected. Particular attention was given to exclude patients suffering from those diseases that are known to be associated with altered cytokine production that may be common in elderly people, such as autoimmune diseases (e.g., Crohn's disease, rheumatoid arthritis, systemic lupus erythematosus), infections and cancer. Nine of the 20 aMCI patients were carriers of the APOEε4 allele (including eight patients with ε3/ε4 and only one with ε4/ε4 APOE

genotype) and 11 were no carriers (including 10 patients with ε3/ε3 and 1 with ε2/ε3 APOE genotype).

A trained senior research psychiatrist (GS) made the diagnosis of aMCI and two trained post-doc neuropsychologists made the cognitive assessment. All the clinical evaluation and blood sampling were performed at the baseline and after 12 months of supplementation with homotaurine. Medical and psychiatric histories were obtained from each subject, and they all underwent a series of standard clinical examinations, including physical, neurological and mental status examinations, neurocognitive tests, and brain magnetic resonance imaging. No patient had taken antidementia drugs lifetime, or psychotropic drugs (i.e., antidepressants, benzodiazepines and antipsychotics) in the previous 12 months.

Inclusion criteria for aMCI were: (1) diagnostic evidence of amnestic MCI consistent with Petersen guidelines (Petersen et al., 1997) and (2) a Mini Mental State Examination (MMSE) score ≥23. In particular, for the diagnosis of aMCI was required impaired performance on at least one memory test in association or not with impaired performance in at least one additional cognitive domain (i.e., praxis, attention, language and executive functions) in the absence of functional impairment. Exclusion criteria were: (1) major medical illnesses and autoimmune-inflammatory diseases; (2) co morbidity of primary psychiatric or neurological disorders and any other significant mental or neurological disorder; (3) clinically important infection within the last 30 days (e.g., chronic persistent or acute infection, such as bronchitis or urinary tract infection); (4) implant of carotid or coronary stent or other major surgical interventions; (5) use of anti-inflammatory drugs within the last 60 days (e.g., corticosteroids or nonsteroidal anti-inflammatory drugs). (6) MRI evidence of focal parenchymal abnormalities or neoplasm. The included aMCI patients underwent the first diagnostic assessment for memory problems in the Santa Lucia Foundation outpatient memory clinic in Rome and were treated with homotaurine tablets, 50 mg, QD for 2 weeks and BID for the next year. Informed written consent was obtained from all subjects or, when necessary, from their proxies in accordance with the Declaration of Helsinki. The protocol was approved by the "Fondazione Santa Lucia Ethics committee." Demographic and clinical characteristics of subjects included in the study are summarized in **Table 1**.

Neuropsychological and Functional Assessment

The MMSE was administered to obtain a global index of cognitive impairment at the baseline and the Mental Deterioration Battery (MDB; Carlesimo et al., 1996) to measure performance in specific cognitive domains at baseline and the 1-year follow-up. In particular, the MDB was administered to make a comprehensive cognitive examination at the diagnostic level.

In order to measure episodic memory performance, Rey's 15-word list learning test (RLT) was administered. In this task participants are given a list of 15 unrelated words that are repeated in five different trials and asked to recall them in any order immediately afterwards (immediate recall (I-RLT); score range: 0–75). After a 15-min interval, during which non-verbal tasks are given, the patient is asked to recall, without list repetition, as many words as possible in any order (delayed recall (D-RLT); score range: 0–15). The task allows characterizing patterns of performance relative to the position of the items in the word list, because recall accuracy varies as a function of the item's position in the study list, i.e., it is greater for words at the beginning (primacy effect) and the end (recency effect) of the list compared to the mid-list (intermediate) items (Murdock, 1962). Special scoring of word-list recall data for serial position has been suggested to improve discrimination of normal aging from dementia. Specifically, the number of correctly recalled words in the early list positions (from word 1 to word 5), the intermediate list positions (from word 6 to word 9), and the number of correctly recalled recency items (from word 10 to word 15) was calculated. The latter effect is the most efficient measure of the short-term component of episodic memory as recency items are stored in a short-term phonological buffer and probably still present in working memory when recall is solicited (Vallar and Papagno, 1986).

Functional impairment was evaluated by assessing instrumental activities of daily living (IADL; Lawton and Brody, 1969). The IADL include abilities that allow a person to live independently (e.g., food preparation, housekeeping and laundry, managing financial matters, shopping and using a telephone). When the ability is fully or at least partially preserved a score of 1 is assigned; when the ability has been lost a score of 0 is assigned. Thus, a total score ranging from 0 (total dependance) to 8 (5 for men) for IADL (total independance) is obtained.

TABLE 1 | Sociodemographic and clinical characteristics at the baseline in amnestic mild cognitive impairment (aMCI) patients treated with homotaurine carriers and no carriers of the apolipoprotein E (APOE) ε4 allele.

	Individuals				
	NO ε4	ε4			
Characteristics	***n* = 11; Mean ± SD**	***n* = 9; Mean ± SD**	**T or chi-square**	***df***	***p***
Age	71.14 ± 6.01	73.36 ± 7.46	−0.926	31	0.362
Educational level	9.77 ± 3.70	11.64 ± 4.30	−1.293	31	0.206
Gender male (*n*, %)	6 (55%)	5 (55%)	0	1	>0.999
MMSE score (baseline)	26.95 ± 2.08	26.82 ± 2.04	0.179	31	0.859
MMSE score (follow-up)	26.36 ± 2.4	25.98 ± 2.43	1.567	31	0.1279
IADL	6.82 ± 1.68	7.54 ± 1.92	−1.119	31	0.272

SD, standard deviation; MMSE, Mini-Mental State Examination; IADL, instrumental activities of daily living.

Blood, APOE Genotyping and Cytokine Measurement

Blood from included aMCI subjects, who had been fasting for approximately 10 h, was drawn in the morning, both at the baseline (T0) and at 1-year follow-up from homotaurine supplementation (T12).

Genomic DNA was purified from whole blood drawn at baseline and the APOE genotyping assessed by real-time polymerase chain reaction (PCR) using the LightCycler ApoE Mutation Detection Kit (Roche Diagnostics).

Serum samples were obtained at both T0 and T12 time points by centrifugation of clotted blood and aliquots were stored at −80°C until cytokine assays. All serum cytokines were measured by commercially available enzyme-linked immunosorbent assay (ELISA), in accordance with the manufacturer's instructions. Specifically, IL-1β, TNFα and IL-6 were evaluated by high sensitivity ELISA kits named Human Quantikine HS (R&D Systems, Minneapolis, MN, USA), with detection limits equivalent to 0.125, 0.5 and 0.156 pg/ml, respectively. TGFβ was measured by Human TGFβ1 cytoset kit (Invitrogen) and the detection limit of the assay was 31 pg/ml. IL-18 levels, corresponding to the total amount of the cytokine, i.e., both its forms: bound and not bound to its natural inhibitor IL-18BP, were determined using coating antibody (clone 125-2H), detecting antibody (clone 159-12B) and standard human recombinant IL-18 (MBL, Nagoya, Japan). IL-18BP levels were measured by an ELISA kit specifically recognizing IL-18BPa, the prevalent isoform in humans (R&D Systems, Minneapolis, MN, USA). Detection limit for both IL-18 and IL-18BP assays was 12.5 pg/mL. Concentrations of free IL-18, namely bioactive IL-18 unbound to its inhibitor, resulted from calculation based on the law of mass action, considering 1:1 stoichiometry in the complex of IL-18 and IL-18BP and a dissociation constant (Kd) of 0.4 nM, as elsewhere reported in more detail (Migliorini et al., 2010).

Statistical Analysis

StatView (SAS, San Francisco, CA, USA) and GraphPad (Prism Version 4, San Diego, CA, USA) software were used for statistical analyses. Because the data do not follow normal distribution (as showed in **Figure 1**), non-parametric statistical tests were used. In particular, the differences between continuous variables at baseline and after 12 months of homotaurine supplementation were evaluated using Wilcoxon Signed Ranks test. Kendall Rank Correlation assessed relationship between IL-18 values and memory (i.e., I-RLT primacy and recency effects) performances. *P*-values less than 0.05 were considered statistically significant.

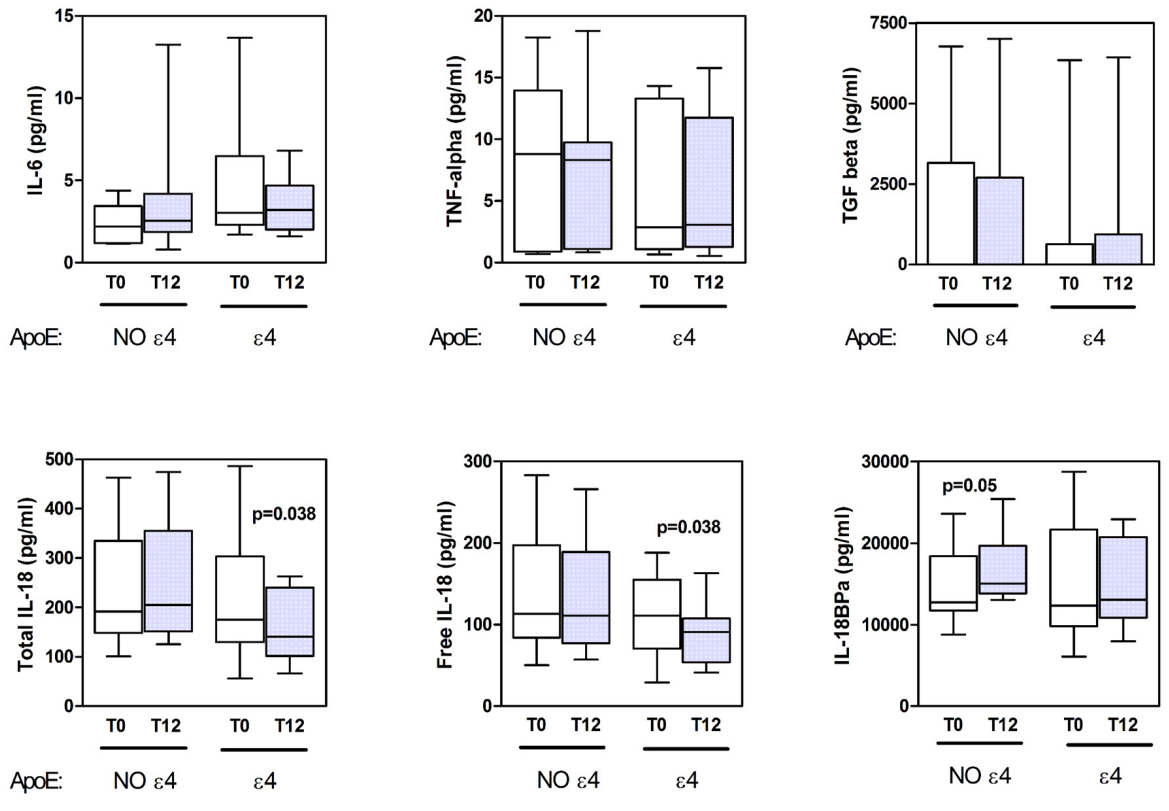

FIGURE 1 | Cytokine serum levels. The concentration of serum cytokines in amnestic mild cognitive impairment (aMCI) subjects genotyped as apolipoprotein E (Apo)Eε4 no carriers (NO ε4) and ApoEε4 carriers (ε4) at the baseline (T0) and after 12 months (T12) from homotaurine supplementation are reported in the respective box-plot displaying the distribution of data and median values, as indicated.

TABLE 2 | Primacy, Intermediate and Recency effects in aMCI-APOE ε4 carriers and no carriers at the baseline and the 1-year follow-up.

Episodic memory effects	Baseline		T0 vs. T0		1-Year		Wilcoxon	Delta (T1-T0)		Effect of group on delta
	NO ε4 (*n* = 11) (Mean ± SD)	ε4 (*n* = 9) (Mean ± SD)	*t*; *p*		NO ε4 (*n* = 11) (Mean ± SD)	ε4 (*n* = 9) (Mean ± SD)	*F*; *p*	NO ε4 (*n* = 11) (Mean ± SD)	ε4 (*n* = 9) (Mean ± SD)	U; *p*
Primacy effect (I-RWLLT)	8.41 ± 3.25	10.73 ± 3.85	−1.82; 0.08		8.50 ± 4.86	7.91 ± 3.59	3.23; 0.08	0.09 ± 4.35	−2.81 ± 4.44	168; 0.07
Intermediate effect (I-RWLLT)	3.64 ± 2.42	4.45 ± 2.98	−0.85; 0.40		4.45 ± 3.11	4.64 ± 2.98	0.50; 0.49	0.82 ± 2.59	0.18 ± 2.18	133; 0.65
Recency effect (I-RWLLT)	10.04 ± 3.26	11.18 ± 3.43	−0.93; 0.36		9.04 ± 3.48	13.18 ± 3.89	5.01; 0.03	−1 ± 3.59	2 ± 3.71	181.5; 0.02

SD, standard deviation; I-RWLLT, Rey 15-word list learning test immediate recall.

RESULTS

Reduced Serum IL-18 Levels After Homotaurine Supplementation in MCI Patients Carrying the APOE ε4 Allele

The cytokine serum levels of aMCI subjects, as evaluated at the baseline and after 12 months from homotaurine supplementation, are illustrated in **Figure 1**. Given the potential impact of ApoE genotype on inflammatory status, patients were categorized as APOE ε4 carriers ($n = 9$) and no APOE ε4 carriers ($n = 11$) and cytokine levels were evaluated accordingly, both at baseline and 1-year follow-up.

Regardless of some apparent fluctuations, especially about IL-6, there were not significant changes between baseline and follow-up levels regarding IL-6, TNFα and TGFβ, both in ε4 carriers and no carriers patients (**Figure 1** upper panels).

Notably, among all cytokines tested, only IL-18 levels resulted significantly different between before and after homotaurine supplementation. In fact, aMCI patients carrying the APOE ε4 genotype showed reduced levels of both total and free (biologically active) IL-18 after 12 months of homotaurine supplementation, as compared to baseline (**Figure 1**, lower left and lower central panels, respectively). This decrease was not paralleled by changes in the IL-18BP levels. At variance, patients not carrying the APOE ε4 allele exhibited a slight increase of the IL-18 inhibitor following the homotaurine supplementation (**Figure 1**, lower right panel), with values approaching the statistical significance.

The levels of IL-1β were always below the sensitivity of our assay and therefore undetectable in all patients (not shown).

Correlation Between Serum IL-18 Levels and Memory Performance Scores (Recency Effect) in MCI Patients

Values of Primacy and recency effects of the I-RLT are reported in **Table 2**. Delta scores (considered as follow-up values minus baseline values) were calculated for the primacy and recency effects of the I-RLT and for the IL-18 cytokine. The Kendall Rank correlation analysis indicated a significant relationship (Tau = −0.395; Tied Z-value = −2.507; $p = 0.012$) between delta values of total IL-18 levels and recency effect score. In addition, results approached the significance (Tau = −0.300; Tied Z-value −1.915; $p = 0.055$) for relationship between delta values of free IL-18 levels and recency effect score. In both cases, decreased cytokine level over the supplementation therapy was correlated with increased memory performance (**Figure 2**).

DISCUSSION

The main outcome of this study reveals that in aMCI patients, who carry the APOE ε4 allele, a 12-month supplementation with homotaurine is specifically associated with a decrease in the serum levels of the pro-inflammatory cytokine IL-18, in turn related to improved episodic memory performances. This finding corroborates previous data showing that the

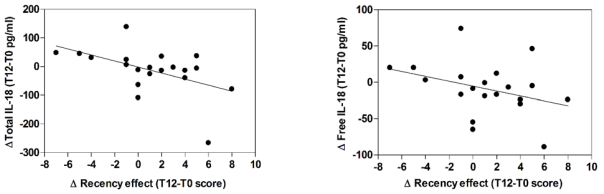

FIGURE 2 | Correlation between serum IL-18 levels and memory performance scores. The relationship between T12-T0 delta values of Total (right panel) or Free (left panel) IL-18 levels and recency effect score is reported in the respective scatterplot, as indicated. Lines represent linear regression.

Aβ aggregation-preventing compound homotaurine exerts protective effects on AD in conditions of APOE ε4 genotype (Caltagirone et al., 2012) and in the early stages of the disease, as holding a positive effect on hippocampus atrophy and short-term episodic memory loss in amnestic MCI patients (Spalletta et al., 2016). Of importance, the present work offers novel evidence on homotaurine anti-inflammatory potential in the very precocious clinical phases of aMCI.

Indeed, neuroinflammation is involved early in the pathogenesis of AD neurodegeneration by taking part in a vicious cycle of Aβ deposition, neuronal death and cognitive decline (Eikelenboom et al., 2010; Heppner et al., 2015). Pro-inflammatory cytokines, including those released into the brain as well as in the periphery, like IL-18, are able to modulate the activation of resident microglia and participate in neuronal degeneration through different mechanisms. An increase in circulating pro-inflammatory factors, including IL-18, has been often observed in patients, as compared to healthy controls (Yaffe et al., 2003; Öztürk et al., 2007; Swardfager et al., 2010; Trollor et al., 2010; Brosseron et al., 2014; Saleem et al., 2015). However, several reports have described controversial results on cytokine blood levels in AD or MCI, mainly because of high inter-individual variability, clinical confounders and differences in assay procedures. Thus, if on the one hand, these observations confirm the potential of circulating inflammatory mediators as index of disease, on the other hand they encourage performing further longitudinal studies that may clarify the issue. In fact, despite the fact we did not observe any difference of serum cytokine levels between baseline and 12 months supplementation with homotaurine in our whole group of aMCI, we found the intriguing result when we split the group based on APOE genotype, according with our hypothesis. Such result confirms the need to limit heterogeneity among patients to identify potential cytokine markers of disease.

Noteworthy, the only over time change in aMCI subgroups is IL-18, making this cytokine an inflammatory mediator of special interest not only in association with homotaurine supplementation but, possibly, in the overall MCI context. In addition, since IL-18 herein exemplifies an inflammasome-dependent cytokine (unfortunately serum levels of IL-1β were undetectable), our results suggest that the activation of the inflammasome complex may play an important role in homotaurine action and conceivably in neurodegenerative dementia progression, as indeed recently reported (Saresella et al., 2016).

Furthermore, the observed homotaurine supplementation-dependent reduction of IL-18 concerned the two circulating forms of IL-18: both total and free, IL-18BP-unbound cytokine, with no effects on the inhibitory molecule IL-18BP. Differently, a trend towards IL-18BP increase was observed in treated MCI not carrying the APOE ε4 allele, suggesting that homotaurine plays a different regulation of IL-18, depending on the APOE genotype. The latter is a quite intriguing result, which strongly encourage driving additional research efforts to the comprehension of the interconnected molecular pathways linking APOE and neuroinflammation to the increased risk to develop AD, as well as to the potential effects played by homotaurine in this context. In fact, as previously reported, ApoE isoforms affect inflammatory processes (Vitek et al., 2009), with APOE ε4 carriers having lower brain levels of ApoE protein, enhanced neuroinflammation, and greater accumulation of Aβ. Hence, the AD-linked inflammatory events may be influenced by the function of ApoE proteins, giving rise to an interesting interpretation that Aβ-dependent inflammation may participate in neurodegenerative processes involving ApoE-mediated mechanisms (Tai et al., 2015). In particular, since Aβ-dependent triggering of inflammation involves the activation of both the transcription factor Nuclear Factor kappa B (NF-kB) and NLRP3 inflammasome (Halle et al., 2008; Vallabhapurapu and Karin, 2009), which together lead to IL-18 production, it is possible that homotaurine, by lowering Aβ levels can also negatively modulate Aβ-dependent NF-kB signaling, inflammasome activation and, eventually, IL-18 release.

The second achievement of this study points to an association between the homotaurine-dependent decrease of serum IL-18 and the improvement of short-term episodic memory performances. Although it remains unclear whether IL-18 plays a role (either primary or secondary) in neurodegeneration-associated cognitive decline, the observed decrease of serum IL-18 after homotaurine supplementation and its significant correlation with clinical parameters sustain the pathogenic importance of IL-18 in AD. Our result complements a whole body of literature describing that IL-18 might inhibit the cellular mechanisms underlying learning and memory (Curran and O'Connor, 2001) and participate in different ways to neuronal damage (Alboni et al., 2010), proposing that this pro-inflammatory cytokine and its related molecular pathways are of some relevance in AD pathogenesis (Bossù et al., 2010).

The present piece of work suggests that homotaurine supplementation in aMCI individuals carrying APOE ε4 has a positive consequence on episodic memory loss due, at least in part, to homotaurine anti-inflammatory effects targeting IL-18. While some studies highlighted the potential of homotaurine and its derivatives to benefit patients with MCI (Martorana et al., 2014; Spalletta et al., 2016), and to modulate inflammation in mouse models (Sternberg et al., 2012), this is the first evidence ever provided that homotaurine supplementation holds anti-inflammatory properties associated with memory improvement in patients with cognitive impairment.

This finding sheds a new light on the therapeutic potential of homotaurine, sustaining its beneficial disease-modifying effects, encouraging further confirmatory studies for treating genetically-defined populations with moderate AD (Abushakra et al., 2017), and also inspiring future research aimed at exploring the mechanisms by which this compound might control brain inflammation during progression of neurodegenerative dementias.

AUTHOR CONTRIBUTIONS

PB and GS planned and supervisioned the study. FS, AC, ES and AM designed and performed experiments. NB, FA, MO, CC, WG and GS coordinated and performed clinical evaluations. PB, GS, FS, AC, FA and MO analyzed the data. PB and GS wrote the article. All authors made substantial contributions to the conception of the work and its interpretation of data. All authors critically revised the manuscript and approved its final version.

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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Vitamin K Deficiency Induced by Warfarin Is Associated With Cognitive and Behavioral Perturbations, and Alterations in Brain Sphingolipids in Rats

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Initially discovered for its role in blood coagulation, there is now convincing evidence that vitamin K (VK) has important actions in the nervous system. In brain, VK is present in the form of menaquinone-4 (MK-4), a byproduct of the main dietary source, phylloquinone. It contributes to the biological activation of various proteins (i.e., Gas6), and participates in the synthesis of sphingolipids, a class of lipids widely present in brain cell membranes with important cell signaling functions. In a previous study, we reported that lifetime consumption of a low VK diet resulted in mild cognitive impairment in aged rats, a finding associated with an alteration of the sphingolipid profile. To confirm the role of VK as it relates to sphingolipids, cognition, and behavior outside the context of aging, we conducted a study of acute VK deficiency using a pharmacological model of VK deficiency in brain. In this procedure, rats (8 weeks) are maintained on a ratio of warfarin (a VK antagonist) to VK whereby coagulation is maintained while inducing VK deficiency in extrahepatic tissues. After 10 weeks of treatment, rats who were subjected to the warfarin plus phylloquinone protocol (WVK) exhibited longer latencies in the Morris water maze test as well as lower locomotor activity and exploratory behavior in the open field test, when compared to control rats. The WVK treatment resulted in a dramatic decrease in MK-4 level in all brain regions despite the presence of high local concentrations of phylloquinone, which suggests an inhibition of the biosynthetic MK-4 pathway in the presence of warfarin. Additionally, WVK treatment affected sphingolipid concentrations in key brain regions, notably those of the ganglioside family. Finally, brain MK-4 was correlated with performances in the open field test. This study confirms the modulatory role of VK in cognition and behavior and the implication of sphingolipids, notably those of the ganglioside family.

Keywords: vitamin K, menaquinone-4, nutritional deficiency, brain, cognition, sphingolipids, rat model

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Abbreviations: C, control group; HPTLC, high-performance thin-layer chromatography; MK-4, menaquinone-4; VK, vitamin K; WVK, warfarin plus phylloquinone treatment.

INTRODUCTION

The preservation of cognitive abilities and mobility is of primary importance to older people as cognitive decline and physical frailty result in loss of independence and decreased quality of life. Lifestyle factors, notably nutrition, are increasingly being confirmed as powerful modulators of cognition and physical functions in older age (Landi et al., 2015; Tucker, 2016; Vauzour et al., 2017). Historically discovered for its role in blood coagulation, vitamin K (VK) has recently emerged as an important nutrient for brain function. Vitamin K occurs naturally in two forms, phylloquinone which originates from plants, is the main dietary source, while the menaquinones, which are of bacterial origin, constitute a family of compounds with unsaturated isoprenyl side chains of various lengths (Ferland, 2012a; Shearer and Newman, 2014). One of the menaquinones, menaquinone-4 (MK-4), is not a common product of bacterial synthesis but is synthesized from phylloquinone (Nakagawa et al., 2010). Under normal conditions, VK in brain is overwhelmingly in the form of MK-4 (Thijssen and Drittij-Reijnders, 1994) where it has been shown to represent more than 98% of total VK in brains of Sprague-Dawley rats (Carrié et al., 2004, 2011).

In brain, VK is involved in sphingolipid metabolism, a group of complex lipids highly enriched in the nervous system where they are major components of cell membranes. Major sphingolipids in the central nervous system include ceramides, sphingomyelin, cerebrosides, sulfatides, and gangliosides (Olsen and Færgeman, 2017). Ceramide constitutes the basal building block for the more complex sphingolipids which are generated by attaching various head groups in the C1 position of ceramide. Sphingomyelin, cerebrosides, and sulfatides are particularly present in oligodendrocytes and myelin (white matter), whereas gangliosides are major components of neuronal membranes (gray matter; Posse de Chaves and Sipione, 2010). Ganglioside biosynthesis occurs by sequential glycosylation reactions *via* two major pathways, designated the "a-pathway" and the "b-pathway" (Giussani et al., 2014; Schnaar, 2016). A simplified scheme of sphingolipid and ganglioside pathways is presented in **Figure 1**.

Initially appreciated for their structural role, sphingolipids are now viewed as key players of important cellular events such as neuronal cell proliferation, differentiation and senescence, synaptic transmission, neuronal–glial interaction, and myelin stability (Bartke and Hannun, 2009; Schengrund, 2015; Olsen and Færgeman, 2017). Furthermore, research conducted in recent years have linked alterations in sphingolipid metabolism to the aging process (Cutler et al., 2004; Ledesma et al., 2012), neurodegenerative disorders such as Alzheimer and Parkinson diseases (Han, 2010; van Echten-Deckert and Walter, 2012; Ariga, 2014; Gualtierotti et al., 2017), psychiatric disorders, and emotional behaviors (Schneider et al., 2017). Ceramides generated by the hydrolysis of sphingomyelin by the sphingomyelinase enzymes, have been shown to accumulate during aging (Cutler et al., 2004; Costantini et al., 2005; Babenko and Semenova, 2010) and to be increased by up to threefolds in the brains of Alzheimer's disease patients when compared with age-matched controls (Han et al., 2002; Cutler et al., 2004). Work conducted in recent years have also provided strong evidence for an implication of the ceramide–sphingomyelin pathway in behavioral extinction (Huston et al., 2016), and in emotional behaviors and psychiatric disorders (Schneider et al., 2017). In a series of studies, the acid sphingomyelinase–ceramide pathway was shown to play an important role in genetically (Kornhuber et al., 2014; Müller et al., 2017) and stress-induced depression (Gulbins et al., 2013; Oliveira et al., 2016).

In addition to their implication in sphingolipid metabolism, the K vitamers act as cofactors in a carboxylation reaction that results in the posttranslational modification of the glutamic acids contained in precursor proteins, the best known of which are those involved in hemostasis (Suttie, 2009). However, the VK-dependent proteins Gas6 and protein S are present in brain where they are known to possess cell signaling actions in neurons and the glia, and antithrombotic activity (reviewed in Ferland, 2012b). Activation of the VK-dependent proteins involves a series of reactions where the VK oxidoreductase enzyme supports the recycling of the K vitamers. In extrahepatic tissues including brain, this enzyme is inhibited by 4-hydroxycoumarin derivatives, such as warfarin. However, in liver where coagulation factors are produced, a coumarin insensitive NAD(P)H-dependent quinone reductase enzyme operates at high tissue concentrations of VK. By maintaining a specific ratio of warfarin to phylloquinone, it is hence possible to induce VK deficiencies in extrahepatic tissues while maintaining the hemostatic functions (Wallin et al., 1978; Tie and Stafford, 2016; **Figure 2**).

In a previous study, we reported that lifetime consumption of a low VK diet results in mild cognitive impairment in aged rats (i.e., 20 months), a finding associated with an alteration of the sphingolipid profile (Carrié et al., 2011). Vitamin K status is influenced by age (Huber et al., 1999; Ferland et al., 2016a) and sphingolipid metabolism is altered during aging. In light of this and to confirm the role of VK as it relates to sphingolipids, cognition, and behavior outside the context of aging, we conducted a study of acute VK deficiency by subjecting rats to the WVK protocol.

MATERIALS AND METHODS

Animals

All experimental procedures were approved by the Animal Care Committee of the Université de Montréal according to the guidelines of the Canadian Council on Animal Care. Male Wistar rats (age 8 weeks; initial body weights 175–250 g) were obtained from Charles Rivers, Canada. Rats were housed two per cage, in a room maintained at 22°C with a 12-h light/dark cycle. Rats were kept in the same housing conditions and rat facility throughout the experimental period. Rats had free access to water and food.

Warfarin-Induced Vitamin K Deficiency Protocol

The warfarin-induced VK deficiency protocol used in the present study was that developed by Price et al. (1982) as modified by Essalihi et al. (2003). Specifically, rats from the experimental

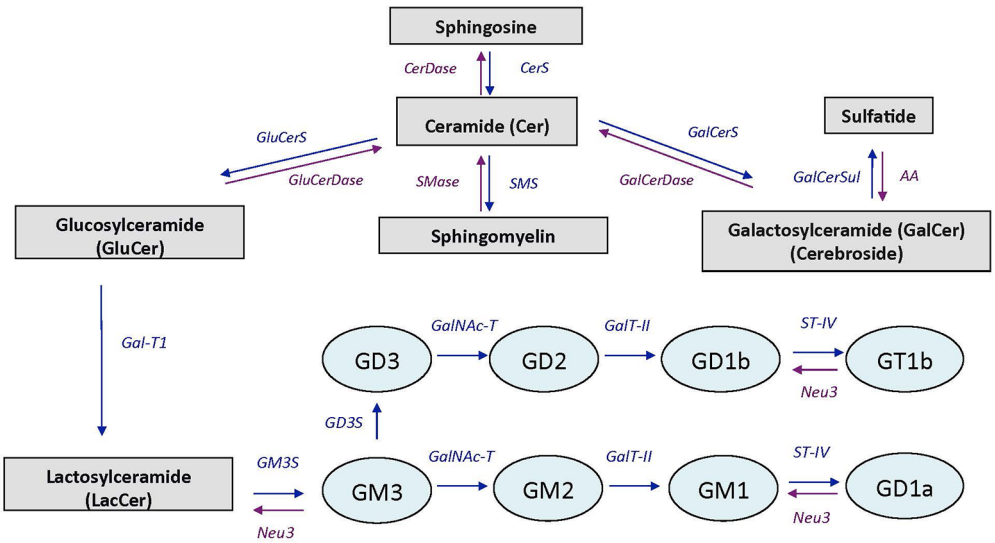

FIGURE 1 | Simplified scheme of sphingolipid and ganglioside pathways. CerDase, ceramidase; CerS, ceramide synthase; GluCerS, glucosylceramide synthase; GluCerDase, glucosylceramidase; SMase, shingomyelinase; SMS, sphingomyelin synthase; GalCerDase, galactosylceramidase; GalCerS, galactosylceramide synthase; GalCerSul, galactoceramide sulfotransferase; AA, arylsufatase A; Gal-T1, -β-1,3-galactosyltransferase; GM3S, GM3 synthase (ST3 β-galactoside-α-2,3-sialyltransferase-5); GD3S, GD3 synthase (ST8 α-*N*-acetyl-neuraminide-α-2-8-sialyltransferase); GalNAc-T, β-1-4-*N*-acetyl- galactosaminyltransferase-1; GalT-II, galactosyltransferase; ST-IV, ST6 (α-*N*-acetyl-neuraminyl-2,3-β-galactosyl-1,3)-*N*-acetylgalactosaminide-α-2,6-siasyltransferase; Neu3, sialidase 3. Adapted from Giussani et al. (2014).

group (WVK) were treated with 14 mg/kg/day W in their drinking water and subcutaneous phylloquinone (85 mg/kg/day) injections, three times per week for 10 successive weeks. Phylloquinone injections were started 1 week before warfarin administration and warfarin dosing was adjusted three times per week by checking the drunken volume. Control (C) rats were treated with normal water and injected with saline three times per week for 10 successive weeks. Both groups were fed an AIN-93-based diet containing 750-μg phylloquinone/kg diet. The health of the rats was monitored daily throughout the experimental period and included assessment of general appearance and behavior, i.e., quality of fur/grooming, presence of epistaxis or other bleeding, posture, mobility in the cage, response to external stimuli, etc. Food intake and body weights were recorded once and twice per week, respectively. Blood clotting capacity, i.e., prothrombin time of animals was monitored once a week using blood samples from the tail vein using a Coagucheck device (Roche, Canada).

Behavioral Testing

At the end of the treatment period, rats from both groups were subjected to the Morris water maze (Morris, 1984), the open field (Kelley, 1993), and the elevated plus maze (Pellow et al., 1985), these tests assessing spatial learning and memory, locomotor activity and exploration, and anxiety, respectively.

Morris Water Maze

Morris water maze test consisted of a large, circular, metal pool (diameter: 150 cm) filled approximately half-way (30 cm) with 22°C water. A fixed invisible platform (10 cm^2) which was submerged below the water surface (∼2 cm) was placed in the center of one of the four quadrants of the pool. Several objects or images (e.g., circles, squares, and triangles) were hung on the walls of the room in which the test was conducted, so the rats could use them as visual stimuli for navigating in the maze. Each day of the trial, rats were released in the water in one of the four quadrants randomly. Testing was conducted daily over five consecutive days, each rat being given three trials per day with an inter-trial interval of 20 min. Once the rat located the platform, it was allowed to remain on it for ∼30 s. If a rat did not find the platform after 120 s of swimming, it was gently put on it by the experimenter. Learning performance was based on the mean of three daily trials.

Probe trial

On day 6, memory retention was further assessed by removing the platform from the pool and allowing the rats to swim freely during 30 s. The pool was divided into the same four quadrants as for the learning condition and the percent time spent in each quadrant was computed. Rats were allowed two trials.

Cue test

Immediately after the probe trial, a cue test was conducted to ensure that poor performances were not due to visual deficits. In this test, rats had to find the platform that had been rendered visible by lowering the pool water level (2 cm below the top of the platform). Rats were allowed two trials. Latencies to find the platform, swim speed, and time in each quadrant were monitored with a camera mounted above the pool and recorded with a DVD recorder. Latencies to find the platform, swim speed and time spent in each quadrant were analyzed using the TopScan 2.0 system (Clever Systems Inc., Reston, VA, United States).

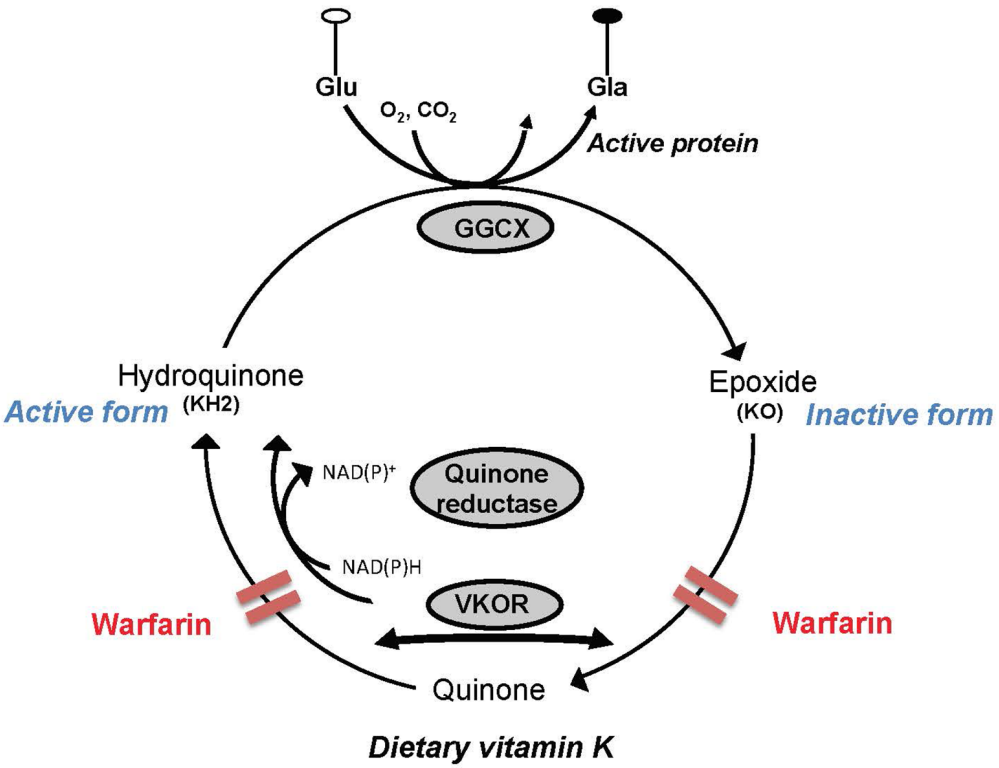

FIGURE 2 | Simplified depiction of the vitamin K (VK) cycle. VK acts as a cofactor for the γ-glutamyl carboxylase enzyme (GGCX) involved in the posttranslational synthesis of gamma-carboxyglutamic acid (Gla) from glutamic acid (Glu) residues contained in VK precursor proteins. In the course of the catalytic sequence, hydroquinone (active form) is oxidized to VK 2,3-epoxide (KO), which in turn is recycled to the quinone and hydroquinone forms by the VK oxidoreductase (VKOR). Activity of VKOR is inhibited by 4-hydroxycoumarin derivatives such as warfarin. In the liver where coagulation factors are produced, a coumarin insensitive NAD(P)H-dependent quinone reductase enzyme operates at high tissue concentrations of VK and can therefore support carboxylation of the hepatic blood coagulation factors in the presence of coumarins.

Open Field

The open field consisted of a black wooden arena measuring 60 cm × 50 cm × 50 cm with the floor divided in 25 cm × 10 cm squares. After 30 min of adaptation to the room, rats were placed individually into the open field and allowed to explore it freely for 5 min. Locomotor activity was based on total distance moved (cm) and total number of square crossed, while exploratory behavior was assessed by the animals' % time spent in center squares and number of center crossings. Motor activity and exploratory components were recorded using a HVS 2020 tracker over 1 day, and analyzed by the Field 2020's software (HVS Image).

Elevated Plus Maze

Anxiety-like behavior was measured using the elevated plus maze, a test that relies on the rodent's innate fear of open spaces and height. The elevated plus maze consisted of a gray wooden cross with four arms (90 cm × 8 cm) that was elevated 70 cm from the floor. Two opposite arms were open, while the other two were enclosed by side end walls (10-cm high). After 30 min of adaptation to the room, rats were placed in the middle of the intersection of the four arms facing an open arm and their behavior was recorded for 5 min. During this period, the total time spent in the open arms, number of open arm entries, and the total number of arm entries were measured. The test was conducted once.

Biochemical Analyses

After completion of the behavioral tests, rats were anesthetized with pentobarbital and bled from the abdominal aorta. The brains were gently removed on ice and dissected into midbrain, prefrontal cortex, hippocampus, striatum, and sensorimotor cortex. The brain regions were frozen in liquid nitrogen and stored at −80°C until assessments.

Vitamin K Analysis

Phylloquinone and MK-4 were quantified by reverse-phase HPLC as previously described (Carrié et al., 2011). Briefly, tissue samples were pulverized in anhydrous Na_2SO_4 and extracted with acetone containing an internal standard [2-methyl-3-(3,7,11,15,19-pentamethyl-2-eicosenyl)-1,4 naphthalenedione; GL Synthesis, Inc.]. Dried extracts were then reextracted with a mixture of hexane and water before being further purified by solid phase extraction on silica gel columns (J. T. Baker). Quantitative analysis of phylloquinone and MK-4 was performed by reverse-phase HPLC using a C-18 reverse phase column and

fluorescence detection. The calibration standard consisted of a mixture of phylloquinone, MK-4, and 2-methyl-3-(3,7,11,15,19-pentamethyl-2-eicosenyl)-1,4-naphthalenedione at 2 ng in 50 μl. The percent recovery for the samples was calculated from the internal standard and found to be 85–90%.

Sphingolipid Analyses

Sphingolipids which included ceramides, sphingomyelin, cerebrosides, sulfatides, and gangliosides, were assessed in the various brain regions as described previously (Carrié et al., 2011). Briefly, lipids were extracted from the brain regions using chloroform:methanol (2:1, v:v) and partitioned according to the method of Folch et al. (1957). Gangliosides were eluted according to the method of Williams and McCluer (1980), and were measured by quantification of free sialic acids according to Jourdian et al. (1971). Ceramides, cerebrosides, sulfatides, and sphingomyelin were loaded onto LC-NH2 columns (Supelco) and eluted sequentially. The sulfatide fraction was further applied to a C-18 silica column. Each fraction was evaporated and suspended in chloroform:methanol (2:1, v:v). Ceramides, cerebrosides, and sphingomyelin were quantified by determination of sphingosine with fluorescamine according to the method of Naoi et al. (1974) and sulfatides with azure A according to the method of Kean (1968).

Ganglioside Analyses

Gangliosides subtypes were analyzed by high-performance thin-layer chromatography (HPTLC) using 20 cm × 10 cm silica gel 60 HPTLC plates (Merck, Darmstadt, Germany). Purified gangliosides standards (GT1b, GD1a, GM1, and GD1b) were purchased from Matreya LLC. Each ganglioside standard mixtures were spotted in duplicate on each plate. The HPTLC plates were prewashed with chloroform to eliminate contamination that could affect gangliosides mobility (Ravindranath et al., 2004). After a brief drying period, plates were placed in a chamber containing 200 ml of developing solvent [chloroform/methanol/0.25% aqueous $CaCl_2$ (60/35/7.5 v/v/v); Ardail et al., 2003] which had equilibrated for at least 2 h (Ledeen and Yu, 1982). Plates were run for 60 min, air dried, and sprayed with Resorcinol reagent (10 ml of 2% resorcinol in water, 40 ml concentrated HCl and 0.250 ml of 0.1-M copper sulfate; Svennerholm, 1957). Plates were then placed face down on a clean glass cover plate in an oven at 120°C until appearance of the bands (about 20 min; Hauser et al., 2004). The percent distribution of the individual gangliosides was determined by scanning the HPTLC plates and computing the bands using Image J software (version 1.42, National Institutes of Health, Bethesda, MD, United States). Individual bands from each lane were identified by comparison with a standard mixture.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism (version 6.01). All data were expressed as means ± SEM. Body weight and Morris water maze test were assessed using a mixed, between-within ANOVA model, with WVK treatment as the between-subject effect and time as the repeated measure. Phylloquinone, MK-4, total VK, MK-4/total VK, and sphingolipids were assessed for regional differences by one-way ANOVA followed by Bonferroni or Tukey's *post hoc* tests. Group difference (C vs. WVK) in Probe trial, Cue test, swimming speed, latencies on individual days of the Morris water maze, open field, and elevated plus maze; prothrombin time; phylloquinone, MK-4, MK-4/total VK, sphingolipids, and ganglioside subtypes within a brain region, were analyzed by Student's *t*-test. Pearson's correlation test was performed to estimate the linear relationship between MK-4 and behavioral performances. *p*-Values <0.05 were considered to be statistically significant.

RESULTS

Body Weight and Health of Animals

Body weights increased in both C and WVK groups during the 10-week experimental period although the WVK-treated rats gained significantly less weight compared to controls [$F_{(1,200)} = 102.0$; $p < 0.0001$]. Specifically, mean weights of WVK rats were ∼10% [(C) 418.5 ± 5.9 g vs. (WVK) 380.4 ± 7.1 g] and ∼14% [(C) 556.1 ± 12.0 g vs. (WVK) 477.5 ± 12.4 g] lower at weeks 5 and 10 of treatment, respectively ($p < 0.05$). These differences between C and WVK groups are comparable to those reported by other teams having used this model (Price et al., 1982; Essalihi et al., 2003) and are likely due to difference in food intake. Mean food intake for the entire study was 9% lower for the WVK (21.84 ± 0.41 g/day) compared to the control group (24.06 ± 0.49 g/day; $p < 0.05$). General appearance of animals (i.e., grooming) and behavior (i.e., mobility, response to external reflex, etc.) remained normal throughout the study. Importantly, no animal showed signs of bleeding although mean prothrombin times for the study were slightly higher in the WVK than C group [(C) 1.1 s vs. (WVK) 2.6 s; $p < 0.01$].

Behavioral Testing

Morris Water Maze

Time to find the hidden platform decreased across the successive training days in both groups [$F_{(4,82)} = 34.6$; $p < 0.0001$], suggesting that learning occurred across trials (**Figure 3A**). However, there was a significant interaction effect suggesting that learning across days differed between groups [$F_{(4,82)} = 2.7$; $p < 0.05$]. Specifically, rats from the WVK group presented significantly longer latencies compared to those of C group on day 2 ($p < 0.05$). Probe trial indicated that both groups had a preference for the quadrant in which the platform was located during the learning trials but the percent time spent in the target quadrant was not statistically different between groups ($p = 0.45$; **Figure 3B**). Finally, performance on the Cue test ($p = 0.46$) and animals' swim speed ($p = 0.62$) were similar confirming that visual acuity and mobility in the pool were not affected by the WVK treatment (**Figure 3C**).

Open Field

Locomotor activity and exploratory behavior were affected in the experimental group. Specifically, distance moved [$F_{(12,7)} = 1.26$; $t_{(19)} = 2.67$, $p = 0.015$] and total number of squares crossed [$F_{(12,7)} = 1.42$; $t_{(19)} = 2.76$, $p = 0.012$] were significantly

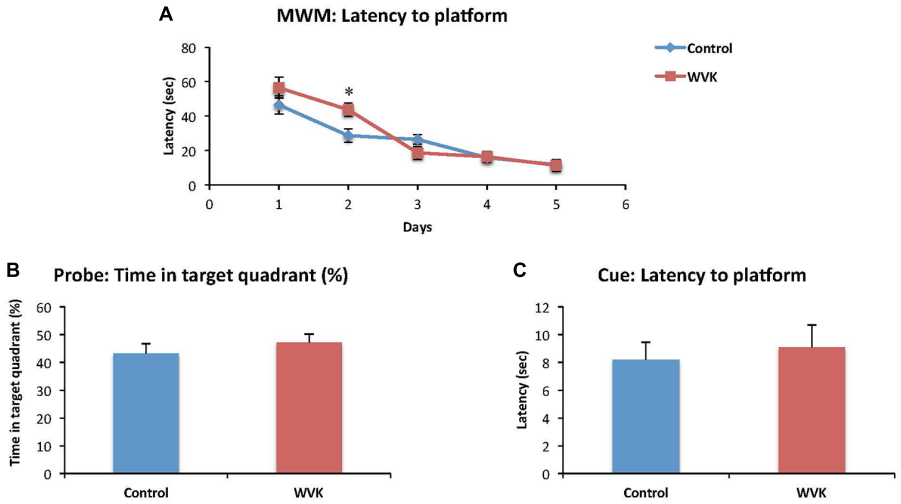

FIGURE 3 | Effect of WVK treatment on spatial learning, memory retention, and cue test in the Morris water maze. Figures show **(A)** the mean latency (s) to reach the submerged platform over the 5-day test; **(B)** the percentage (%) of time spent in the target quadrant with platform removed; **(C)** the mean latency (s) to reach the visible platform. Values are mean ± SEM, n = 8–13 rats per group. Data were analyzed by **(A)** repeated-measures ANOVA, followed by Bonferroni *post hoc* test or **(B,C)** Student's unpaired t-test. Statistically different between C and WVK groups, $^*p < 0.05$.

reduced compared to control rats (**Figures 4A,B**). Similarly, WVK rats presented lower exploratory behavior compared to their control counterparts with lower percent time spent in the center squares [$F_{(12,7)} = 1.58$; $t_{(19)} = 4.69$, $p = 0.002$] and number of center square crossings [$F_{(12,7)} = 1.33$; $t_{(19)} = 3.87$, $p = 0.001$; **Figures 4C,D**].

Elevated Plus Maze

Anxiety as assessed with this paradigm was not affected by the WVK treatment. Time spent in the open arms [$F_{(12,7)} = 1.31$; $t_{(19)} = 0.94$, $p = 0.35$], percent time spent in open arms/total time [$F_{(12,7)} = 1.34$; $t_{(19)} = 0.55$, $p = 0.59$], and percent open arm entries/total entries [$F_{(12,7)} = 0.27$; $t_{(19)} = 1.33$, $p = 0.20$] did not differ significantly between groups (data no shown).

Vitamin K Status

In rats from the C group, MK-4 was by far the principal K vitamer in brain, representing ∼85% of total VK. In WVK rats, phylloquinone and total VK were significantly higher than in C rats ($p < 0.05$); however, MK-4 concentrations were 25–40% those of controls depending on the brain region (**Figure 5A**) and represented ∼20% of total VK ($p < 0.01$; **Figure 5B**). Furthermore, in C rats, MK-4 was unevenly distributed in the brain regions [$F_{(4,23)} = 4.45$; $p < 0.01$], higher concentrations being observed in prefrontal cortex, midbrain, and sensorimotor cortex. Interestingly, these regional differences totally disappeared in brains of WVK rats, concentrations of MK-4 being comparable across all brain regions [$F_{(4,34)} = 0.79$; $p = 0.54$; cf. figure legend for individual K vitamer statistics].

Sphingolipid Status

Concentrations of each class of sphingolipids generally varied across brain regions. In both groups of rats, highest concentration of sphingomyelin (C: $F_{(4,30)} = 50.69$, $p < 0.0001$; WVK: $F_{(4,29)} = 8.76$, $p < 0.0001$), cerebrosides (C: $F_{(4,30)} = 149.3$, $p < 0.0001$; WVK: $F_{(4,29)} = 89.33$, $p < 0.0001$), and sulfatides (C: $F_{(4,30)} = 107.8$, $p < 0.0001$; WVK: $F_{(4,29)} = 75.12$, $p < 0.0001$) were observed in the midbrain ($p < 0.05$). By contrast, the midbrain contained the least amounts of ceramides whereas the other regions showed statistically comparable concentrations (C: $F_{(4,25)} = 8.95$, $p < 0.0001$; WVK: $F_{(4,24)} = 5.19$, $p < 0.001$). In C rats, gangliosides varied significantly across brain regions with the striatum differing from all other regions and midbrain differing from striatum, hippocampus, and sensorimotor cortex [$F_{(4,30)} = 13.8$, $p < 0.0001$]. By contrast, regional gangliosides differences were largely mitigated in the WVK group with only the midbrain differing from prefrontal cortex and striatum, the other regions being statistically similar [$F_{(4,30)} = 6.6$, $p < 0.05$]. Furthermore, the WVK treatment was associated with decreased concentrations of ceramides in prefrontal cortex (↓20%, $p < 0.05$) and midbrain (↓13%, $p < 0.05$), decreased sphingomyelin in midbrain (↓39%, $p < 0.01$), and increased concentrations of gangliosides (↑25%) and sulfatides (↑20%) in the prefrontal cortex ($p < 0.05$; **Figure 6**).

HPTLC Ganglioside Fractions

In both C and WVK groups, gangliosides subtypes GD1b, GD1a, GT1b, and GM1 were differentially distributed across brain regions. Specifically, GD1a was present in highest concentrations in prefrontal cortex, striatum, and hippocampus followed by sensorimotor cortex (C: $F_{(4,30)} = 87.36$, $p < 0.0001$; WVK: $F_{(4,30)} = 95.03$, $p < 0.0001$) while significantly higher levels of GD1b (C: $F_{(4,30)} = 15.77$, $p < 0.0001$; WVK: $F_{(4,30)} = 8.21$, $p < 0.0001$) and GT1b (C: $F_{(4,30)} = 60.77$, $p < 0.0001$; WVK: $F_{(4,30)} = 11.64$, $p < 0.0001$) were observed in the midbrain. In the C group, GM1 was present in highest concentrations in the striatum and midbrain [$F_{(4,30)} = 10.83$, $p < 0.0001$], while in

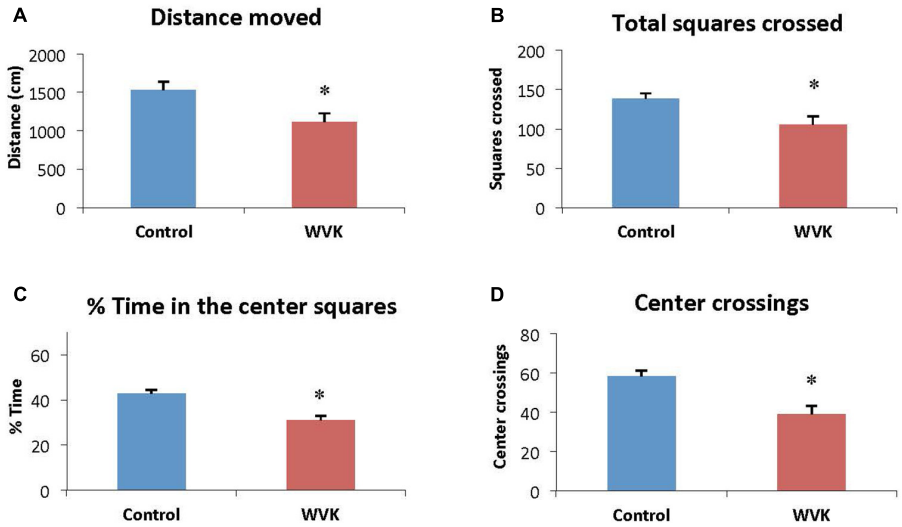

FIGURE 4 | Effect of WVK treatment on locomotor activity and exploratory behavior in the open field. Figures show **(A)** the total distance moved; **(B)** the total number of squares crossed; **(C)** the mean % time spent in center squares; **(D)** the total number of center crossings. Values are mean ± SEM, n = 8–13 rats per group. Data were analyzed by Student's unpaired t-test. Statistically different between C and WVK groups, $^{*}p < 0.05$.

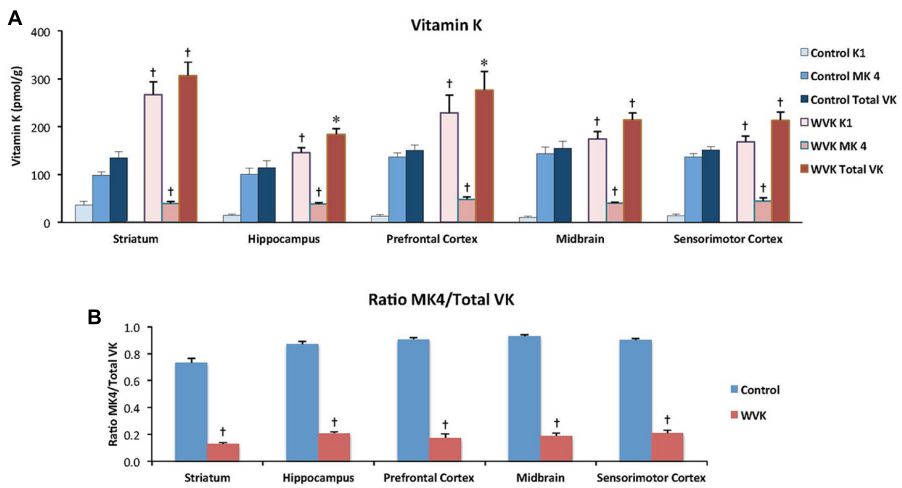

FIGURE 5 | Effect of WVK treatment on VK status in different brain regions in C and WVK groups. Figures show **(A)** phylloquinone (K_1), menaquinone-4 (MK-4), and total VK concentration in striatum, hippocampus, prefrontal cortex, midbrain, and sensorimotor cortex; **(B)** the ratio of MK-4/total VK in these brain regions. Values are mean ± SEM, n = 6–7 rats per group. Differences between brain regions were analyzed for individual vitamer within C and WVK groups by one-way ANOVA. [Control gp. K_1: $F_{(4,23)} = 5.687$, $p < 0.002$, MK-4: $F_{(4,23)} = 4.45$, $p < 0.01$, total VK: $F_{(4,23)} = 1.81$, $p = 0.16$, MK-4/total VK: $F_{(4,23)} = 11.90$, $p < 0.001$; WVK gp. K_1: $F_{(4,34)} = 4.77$, $p < 0.01$, MK-4: $F_{(4,34)} = 0.79$, $p = 0.54$, total VK: $F_{(4,34)} = 4.38$, $p < 0.01$, MK-4/total VK: $F_{(4,34)} = 2.99$, $p < 0.05$]. Group differences within a given brain region were analyzed for individual vitamer by Student's unpaired t-test. Statistically different from control group, $^{*}p < 0.05$, $^{\dagger}p < 0.01$.

WVK rats, GM1 were highest in midbrain compared to other regions [$F_{(4,30)} = 9.43$, $p < 0.0001$; data not shown]. Importantly, WVK treatment resulted in a significant reduction in GD1a in the hippocampus ($p < 0.05$), and in significant increases in GT1b in the striatum and prefrontal cortex ($p < 0.05$; **Figure 7**).

Relationship Between Brain MK-4 and Locomotion and Exploratory Behavior

The specific relationships between brain MK-4 and locomotor activity and exploratory behavior as assessed in the open field test were determined. Specifically, correlations were established considering individual brain MK-4 (and MK4/total VK) and total squares crossed (**Figures 8A,B**), and % time in center squares (**Figures 8C,D**). As illustrated in the figures, both brain MK-4 and ratio MK4/total VK were strongly correlated with behavior, higher brain MK-4 and MK4/total VK ratios being associated with greater locomotor activity and exploratory behavior. In all cases, the poorer performances were observed in the WVK group. Additional positive correlations were also observed for locomotor activity (MK4/total VK vs. distance moved, $r^2 = 0.216$, $p < 0.05$)

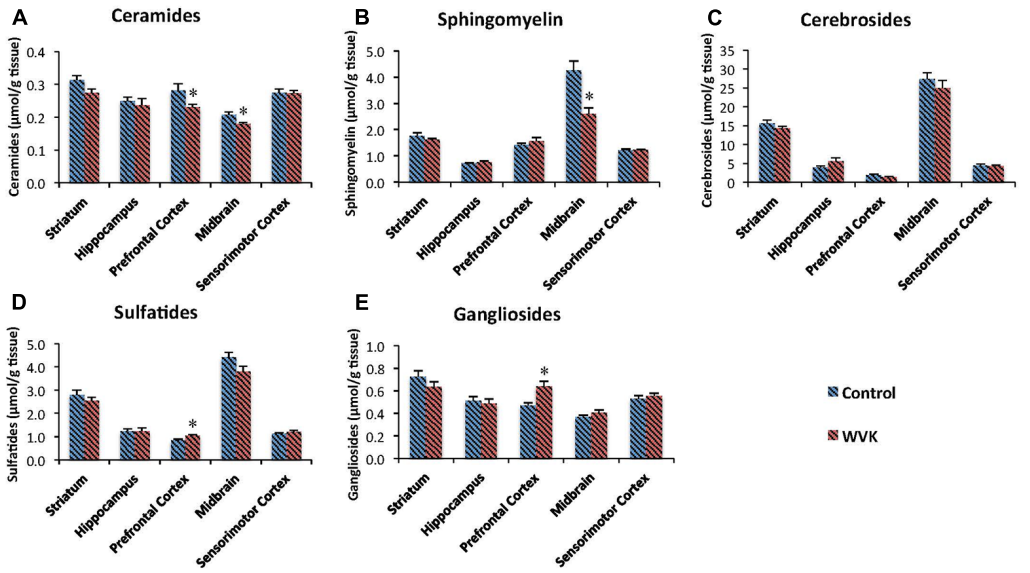

FIGURE 6 | Effect of WVK treatment on sphingolipid profile in different brain regions in C and WVK groups. Figures show **(A)** ceramides; **(B)** sphingomyelin; **(C)** cerebrosides; **(D)** sulfatides; **(E)** gangliosides in striatum, hippocampus, prefrontal cortex, midbrain, and sensorimotor cortex. Values are mean ± SEM, n = 6–7 rats per group. Differences between brain regions were analyzed within C and WVK groups by one-way ANOVA, followed by Tuckey's *post hoc* test. [Sphingomyelin C: $F_{(4,30)} = 50.69$, $p < 0.0001$; WVK: $F_{(4,29)} = 8.76$, $p < 0.0001$. Cerebrosides C: $F_{(4,30)} = 149.3$, $p < 0.0001$; WVK: $F_{(4,29)} = 89.33$, $p < 0.0001$. Sulfatides C: $F_{(4,30)} = 107.8$, $p < 0.0001$; WVK: $F_{(4,29)} = 75.12$, $p < 0.0001$. Ceramide C: $F_{(4,25)} = 8.95$, $p < 0.0001$; WVK: $F_{(4,24)} = 5.19$, $p < 0.001$. Gangliosides C: $F_{(4,30)} = 13.8$, $p < 0.0001$; WVK: $F_{(4,30)} = 6.6$, $p < 0.05$]. Group differences within a given brain region were analyzed by Student's unpaired t-test. Statistically different from control group, $^*p < 0.05$.

and exploratory behavior (MK4 vs. center crossings, $r^2 = 0.263$, $p < 0.05$; MK4/total VK vs. center crossings, $r^2 = 0.393$, $p < 0.01$), rats from the WVK group performing again, less well.

DISCUSSION

In the present study, we provide new evidence of the detrimental effect of low VK status in brain on spatial learning ability, locomotor activity, and exploratory behavior, a phenotype associated with decreased cerebral MK-4 concentrations and altered sphingolipid pattern in key brain regions, notably of the ganglioside subtypes.

Cognitive and Behavior Functions

When subjected to the Morris water maze test, rats from the WVK group showed higher latencies on day 2 which suggests a slower rate of spatial learning acquisition (**Figure 3A**). This result was neither confounded by motor dysfunction in the pool nor by differences in visual acuity as swim speed and performances on cue test were not different between groups. A similar finding of slower learning acquisition was also observed in aged animals (20 months), rats having been fed a low phylloquinone diet throughout their lives showing longer latencies on days 2 and 5 on the Morris water maze test (Carrié et al., 2011). Noteworthy, in this study, the low VK diet had no impact on cognition at 6 and 12 months suggesting that it was the chronic exposure to the low phylloquinone diet that lead to the detrimental effect on cognition. Interestingly, in a recent report by our group involving cognitively healthy older individuals who were administered an episodic memory task, those with lower VK status (sub-optimal status but not deficient) needed more exposure time to the words to be memorized than participants with higher VK status (Presse et al., 2013). Taken together and acknowledging that the impact of VK on cognition is mild and subtle, results from this and our previous studies advocate for a specific role for VK in the memory consolidation process.

In the present study, the WVK treatment was also associated with lower locomotor activity and exploratory behavior (**Figure 4**). These results are in agreement with those from an older study in which short-term warfarin treatment was associated with a significant shift from more exploratory to less exploratory behavior in male Sprague-Dawley rats (Cocchetto et al., 1985). By contrast, locomotion and exploratory behavior were not affected in our aged rats who had consumed a low phylloquinone diet since weaning (Carrié et al., 2011). This discrepancy could be due to various factors including different experimental design, i.e., pharmacological vs. nutritional approach. Another factor could be the sex of the animals. In the present study, male rats were investigated, whereas our aging study was conducted in female rats who are known to be more resistant to VK deficiency (Metta et al., 1959; Gustafsson et al., 1962; Ferland et al., 2016a).

VK Status

Strong evidence has been provided in recent years supporting the bioconversion of phylloquinone into MK-4, a reaction catalyzed by the UbiA prenyltransferase domain-containing 1 enzyme

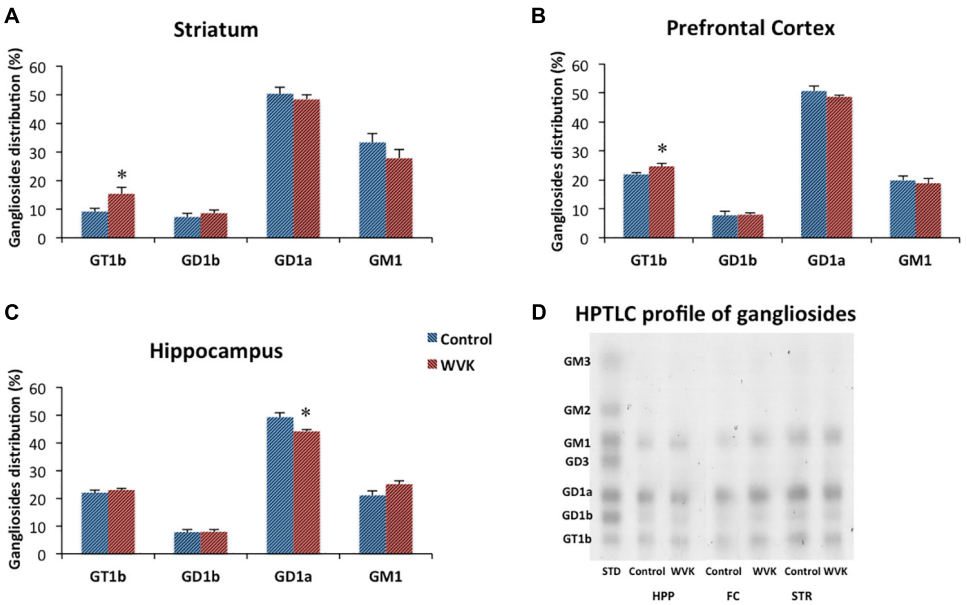

FIGURE 7 | Effect of WVK treatment on ganglioside subtypes in specific brain regions. Figures show gangliosides in **(A)** striatum; **(B)** prefrontal cortex; **(C)** hippocampus; **(D)** ganglioside HPTLC gel. Values are mean ± SEM, n = 6–7 rats per group. Group differences within a given brain region were analyzed by Student's unpaired t-test. Statistically different from control group, $^*p < 0.05$.

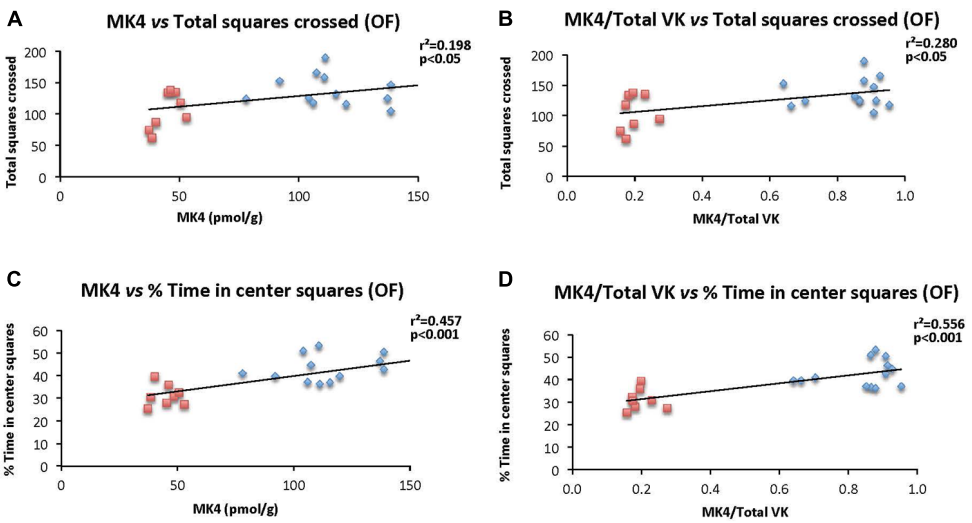

FIGURE 8 | Relationship between brain MK-4 and locomotor activity and exploratory behavior. Figures show relationships of **(A)** MK-4 vs. total squares crossed; **(B)** MK-4/total VK vs. total squares crossed; **(C)** MK-4 vs. % time in central squares (OF); **(D)** MK-4/total VK vs. % time in central squares (OF). Data were analyzed by Pearson correlation test, n = 14–21 rats per correlation. Statistically significant, $p < 0.05$.

(Nakagawa et al., 2010) which involves the menadione form of VK as an intermediate (Hirota et al., 2013). In the present study, MK-4 was by far the principal K vitamer in brains of C rats, representing ∼85% of total VK (**Figure 5**), a result in line with previous reports (Thijssen and Drittij-Reijnders, 1994; Thijssen et al., 1996; Carrié et al., 2011). By contrast, MK-4 concentrations in brains of WVK rats represented no more than 20% of total VK. In a study by Thijssen et al. (1996) administration of 1 mg/kg (s.c.) of warfarin to rats fed standard amounts of phylloquinone and/or menadione was associated with MK-4 concentrations that were ∼20% those of control animals. In an older report, significant decreases in MK-4 concentrations were observed in kidneys and hearts of rats treated with the coumarin drug, Dicoumarol (Taggart and Matschiner, 1969). The fact that, in the present study, brain MK-4 concentrations remained low in WVK rats despite an excess of phylloquinone in

brain suggests an alteration of the MK-4 biosynthetic pathway in the presence of warfarin. The partial loss of MK-4 brain regional differences in the WVK group also supports this notion. Future studies should include assessment of the UbiA prenyltransferase domain-containing 1 enzyme to determine whether its activity is inhibited by warfarin *in vivo*.

The highly increased phylloquinone concentration in brains of WVK rats suggests that phylloquinone can reach this organ. Previous studies from our groups (Carrié et al., 2004; Ferland et al., 2016a) and others (Thijssen and Drittij-Reijnders, 1994; Thijssen et al., 1996) have shown phylloquinone concentrations to increase in brain with phylloquinone intake, and intravenous supplementation (Ronden et al., 1998). To date, details of the phylloquinone transport into CSF and brain are not known. Unlike other fat-soluble vitamins, VK has no known carrier protein. In the circulation, phylloquinone is principally carried in triacylglycerol-rich lipoproteins (>50%) with each of the LDL and HDL fractions accounting for ∼15% of the circulating vitamer (Lamon-Fava et al., 1998). Noteworthy, the scavenger receptor class B type 1 has been shown to promote the uptake of HDL-associated vitamin E (i.e., a-tocopherol) in porcine brain capillary endothelial cells, suggesting this receptor could, in part, be responsible for the vitamin E transport across the blood brain barrier (Goti et al., 2001). Alpha-tocopherol and phylloquinone have similar molecular weights and are both associated with the lipoprotein fractions (Kayden and Traber, 1993; Lamon-Fava et al., 1998). Whether phylloquinone enters the brain through a route similar to that of a-tocopherol remains to be determined.

Finally, it should be emphasized that brain MK-4 was found to be strongly correlated with behavior, higher concentrations being associated with increased locomotor activity and exploratory behavior (**Figure 8**). Such correlations have not, to our knowledge, been reported before, and provide additional support for the modulatory role of MK-4 in behavior.

Sphingolipid Analyses

Sphingolipids are pivotal constituents of the plasma membranes and are important for proper brain functions. In the present study, sphingolipid profile was altered as a function of warfarin treatment and depletion of brain MK-4. While gangliosides varied significantly across brain regions with the striatum containing the highest amounts, regional differences were largely mitigated in the WVK group. Sphingolipid profiles similar to those in the C group were previously observed in 6- and 20-month-old Sprague-Dawley rats who had been fed various levels of phylloquinone since weaning (Carrié et al., 2004, 2011). Furthermore, WVK treatment modified the sphingolipid content of the midbrain and prefrontal cortex. Specifically, sphingomyelin was found to be particularly decreased in the midbrain whereas gangliosides were increased in the prefrontal cortex. Ceramides and sulfatides were, respectively, decreased and increased in the prefrontal cortex. Sulfatides and cerebrosides are involved in the process of oligodendrocyte differentiation (Jana et al., 2009) and contribute to the maintenance of myelin and axon structures of the central nervous system (Schmitt et al., 2015). Ceramides, which can be generated from *de novo* synthesis or from hydrolysis of sphingomyelin through the action of sphingomyelinases (**Figure 1**), are intracellular messenger with potent cell signaling actions. Studies conducted *in vitro* and *in vivo* suggest important roles for sphingomyelin-ceramide signaling in the regulation of cell proliferation, differentiation and survival (Cutler and Mattson, 2001; Ledesma et al., 2012). In a series of studies, the acid sphingomyelinase–ceramide pathway was shown to play an important role in genetically induced depression (Kornhuber et al., 2014; Müller et al., 2017), mice over-expressing acid sphingomyelinase activity and increased ceramide concentrations showing decreased neurogenesis, neuronal maturation/survival and depression-like behavior (Gulbins et al., 2013). Evidence was also reported for a role of ceramide in stress-induced depression, various unavoidable stressors being associated with enhanced acid sphingomyelinase activity and/or ceramide levels in the brain (Gulbins et al., 2013; Oliveira et al., 2016). The fact that animals from the WVK group were not found to be more anxious than controls on the elevated plus maze test are in line with what appears to be a downregulation of the sphingomyelin–ceramide pathway in this WVK model.

Gangliosides are major components of cell membranes where they participate in key neuronal functions such as axon outgrowth/regeneration, nerve cell excitability, and myelin stability (Schengrund, 2015; Schnaar, 2016). In the present study, WVK treatment resulted in a significant reduction in GD1a in the hippocampus ($p < 0.05$), and in significant increases in GT1b in the striatum and frontal cortex ($p < 0.05$; **Figure 7**). In addition to their general functions, ganglioside subtypes have been involved in specific conditions and physiological processes. Ganglioside GD1a has notably been shown to be reduced in frontal gray matter and white matter in patients with Rett syndrome, a neurodevelopmental disorder associated with autism-like behavior and behavioral disturbances (Lekman et al., 1991). In a genetic mouse model of the disease, GD1a concentrations were found to be decreased by 15% in the cerebrum and brainstem (Seyfried et al., 2009). Ganglioside GD1a has also been involved in anti-inflammatory processes (Wang et al., 2008; Nikolaeva et al., 2015). In a recent study, GD1a inhibited *Escherichia coli* lipopolysaccharide-induced inflammation in murine RAW264.7 macrophages by suppressing phosphorylation of mitogen-activating protein kinases and nuclear translocation of NF-κB family members through the Toll-like receptor 4 signaling pathway (Wang et al., 2015). The specific neuroprotective role of GD1a was further evidenced in the study by Bernardo et al. (2009) where increased brain levels of GD1a, through inhibition of GD3S and synthesis of gangliosides from the b-series, was shown to mitigate Aβ-associated neurotoxicity and rescue spatial-memory impairment in the double-transgenic (APP/PSEN1) mouse model of Alzheimer's disease. By contrast, ganglioside GT1b has been associated with neurotoxicity and pro-inflammatory conditions. In a series of experiments, GT1b was shown to activate microglia and induce the production of inflammatory mediators such as IL-1β, iNOS, TNF-α, and COX-2 (Pyo et al., 1999). Microglial activation was subsequently shown to be mediated by protein kinase C and NADPH oxidase (Min et al., 2004). Ganglioside GT1b has also been found to be neurotoxic to dopaminergic neurons *in vitro* (Chung et al., 2001)

through a modulation of the Akt/GSK-3/Tau signaling pathway (Chung et al., 2010). *In vivo*, injection of GT1b into the substantia nigra resulted in the death of nigral neurons, a finding associated with activation of microglia (Ryu et al., 2002).

In light of their biological actions, the observed results for GT1b in the striatum, frontal cortex, and hippocampus could have contributed to the poor performance of the WVK-treated rats in the Morris water maze and open field as these regions are directly linked to the tasks associated with these tests. Although it is well established that hippocampus supports the spatial processing demands of the water maze (D'Hooge and De Deyn, 2001), studies have recently highlighted significant implications for striatum and prefrontal cortex in the Morris water maze paradigm (Woolley et al., 2013). As for striatum, it has long been known to play important roles in motor behavior and locomotor activity, two components associated with the open field test (David et al., 2005; Dang et al., 2006).

Limitations of this study include the fact that except for the ganglioside family, total measures of sphingolipids were obtained, possibly missing on the implications of specific sphingolipids sub-species. When investigated in the context of behavioral extinction, only specific ceramide species were found to be affected (Huston et al., 2016). Another limitation of the study concerns the fact that activity of the enzymes involved in sphingolipid metabolism were not assessed. In future studies, sphingolipid sub-species should be quantitated using mass spectrometry technology and their respective metabolic enzymes be determined. Finally, animals from the WVK group had lower food intake than controls suggesting that results could have been influenced by overall lower intake of energy, and/or specific nutrients other than VK.

The present study aimed to gain mechanistic insight on the impact of targeted brain VK depletion on cognition as it relates to sphingolipid metabolism outside the context of aging. This was achieved through the concurrent administration of large amounts of warfarin to deplete the brain and large doses of phylloquinone to maintain coagulation. The amount of warfarin involved in this experimental paradigm does not compare in any way to the doses used in the clinical setting where VK antagonists such as warfarin are prescribed for the prevention of thromboembolic diseases; the primary end-point of VK antagonists being to suppress the coagulation cascade (Ansell et al., 2008). In recent years, our group has conducted a number of studies in elderly patients undergoing anticoagulation therapy with VK antagonists and while use of these drugs was found to be independently associated with lower cognitive function in cross-sectional analyses, results did not suggest cumulative long-term detrimental effects (Annweiler et al., 2015; Ferland et al., 2016b). Hence, whether VK antagonists alter brain function through their impact on sphingolipid metabolism or other mechanisms, or benefit cognition through their antithrombotic effects, remains a topic of current investigation.

CONCLUSION

In conclusion, this study provides further evidence that targeted depletion of MK-4 in brain is associated with cognitive impairment, lower locomotor activity, and exploratory behavior, and with an alteration of sphingolipids in key brain regions, notably those of the ganglioside family. Results also suggest that, *in vivo*, phylloquinone in brain is not bio-transformed into MK-4 in the presence of W. Future studies should focus on the risk–benefit balance of VKA treatment as it relates to cognition and behavior. Finally, results from this study confirm the role of VK in brain and underline the importance of commonly consumed diets in providing this nutrient in adequate amounts.

AUTHOR CONTRIBUTIONS

GF designed the study. ST-N performed the experiments. BO and JR supervised the work. GF and ST-N analyzed the data and wrote the manuscript.

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Brain Distribution and Modulation of Neuronal Excitability by Indicaxanthin From *Opuntia Ficus Indica* Administered at Nutritionally-Relevant Amounts

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Several studies have recently investigated the role of nutraceuticals in complex pathophysiological processes such as oxidative damages, inflammatory conditions and excitotoxicity. In this regard, the effects of nutraceuticals on basic functions of neuronal cells, such as excitability, are still poorly investigated. For this reason, the possible modulation of neuronal excitability by phytochemicals (PhC) could represent an interesting field of research given that excitotoxicity phenomena are involved in neurodegenerative alterations leading, for example, to Alzheimer's disease. The present study was focused on indicaxanthin from *Opuntia ficus indica*, a bioactive betalain pigment, with a proven antioxidant and anti-inflammatory potential, previously found to cross blood-brain barrier (BBB) and to modulate the bioelectric activity of hippocampal neurons. On this basis, we aimed at detecting the specific brain areas where indicaxanthin localizes after oral administration at dietary-achievable amounts and highlighting eventual local effects on the excitability of single neuronal units. HPLC analysis of brain tissue 1 h after ingestion of 2 μmol/kg indicaxanthin indicated that the phytochemical accumulates in cortex, hippocampus, diencephalon, brainstem and cerebellum, but not in the striato-pallidal complex. Then, electrophysiological recordings, applying the microiontophoretic technique, were carried out with different amounts of indicaxanthin (0.34, 0.17, 0.085 ng/neuron) to assess whether indicaxanthin influenced the neuronal firing rate. The data showed that the bioelectric activity of neurons belonging to different brain areas was modulated after local injection of indicaxanthin, mainly with dose-related responses. A predominating inhibitory effect was observed, suggesting a possible novel beneficial effect of indicaxanthin in reducing cell excitability. These findings can constitute a new rationale for exploring biological mechanisms through which PhC could modulate neuronal function with a relapse on complex cognitive brain process and related neurodegenerative conditions.

Keywords: indicaxanthin, nutraceuticals, electrophysiology, microiontophoresis, excitability, neuroprotection, brain localization

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INTRODUCTION

In the recent years a growing bulk of studies expanded knowledge about the positive impact of dietary phytochemicals (PhC), or nutraceuticals, on complex physiological processes and brain aging. Many of the potential health benefits from compounds extracted by edible vegetables are based on their biological activities and the possible modulation of cellular responses. Among the numerous effects exerted by PhC, cumulative evidence has highlighted their antioxidant and anti-inflammatory properties (Ghanim et al., 2010; Obrenovich et al., 2011). Given that inflammation and oxidative stress have been reported to sustain neurological alterations leading to neurodegeneration and cognitive decline (Lassmann, 2011), the use of nutraceuticals was suggested for therapeutic approaches. In this context, specific dietary regimens including plant-derived compounds have been suggested to enhance cognitive performances and reduce neurodegenerative impairments (Spencer, 2008, 2009; Rendeiro et al., 2012; Meng et al., 2015; Almeida et al., 2016), with a remarkable significance for age-related conditions (Joseph et al., 2005; Youdim and Joseph, 2001). In the brain, excitotoxicity represents a further risk for cell survival with possible involvement in neurodegeneration (Pallo et al., 2016; Wang and Reddy, 2017). Noticeably, sound evidence highlighted the influence of PhC on cell excitability and their positive implications on cognitive functions in aging (Williams et al., 2004). Notwithstanding, only a few researches have been provided on the modulation of neuronal excitability by nutraceuticals in order to unveil new cellular targets of PhC and related beneficial employments. The present study is focused on indicaxanthin, a yellow betalain pigment contained in cactus pear fruit (*Opuntia ficus indica*). This plant, distributed in Mexico, arid regions of America, Africa, Australia and Mediterranean area (de Wit et al., 2010; Albano et al., 2015) has recently emerged as a potential source of valuable components for human health and food industry (Feugang et al., 2006; Kim et al., 2012; Heinrich et al., 2014; Sánchez et al., 2014; Alencar et al., 2018). Indicaxanthin shares many of the favorable effects of PhC, as described in previous scientific reports (Butera et al., 2002). Indeed, indicaxanthin has proven its antioxidant potential both by preventing lipid peroxidation and reducing reactive oxygen/nitrogen species (Allegra et al., 2005, 2007; Tesoriere et al., 2006), but also by interfering with specific redox-dependent signaling pathways in experimental models of innate immunity *in vitro* (Allegra et al., 2014a; Tesoriere et al., 2015). Relevantly and in line with its redox-modulating effects, the pigment has also been shown to exert significant anti-inflammatory effects *in vivo* (Allegra et al., 2014b). All the effects exerted by indicaxanthin gain a more interesting value in the light of its remarkable bioavailability *in vivo* (Tesoriere et al., 2004). More recently a multidisciplinary approach has demonstrated that indicaxanthin orally administered at dietary-relevant doses is able to cross blood-brain barrier (BBB) and accumulate within the rat brain; beyond this, the intriguing ability of this phytochemical to modulate the bioelectric activity of hippocampal neurons for the first time has emerged (Allegra et al., 2015). In this light, the current research aimed to detect a brain distribution pattern of indicaxanthin, after oral administration of nutritionally-relevant amounts, by HPLC assay. Afterwards, microiontophoretic recordings were carried out to explore a possible influence of the indicaxanthin on the bioelectric activity of single neuronal units in specific brain areas.

Therefore, the present study is addressed to shed new light on the direct modulation of membrane excitability by edible PhC. In particular, this could unveil the nutritional importance of indicaxanthin and its influence on a pivotal aspect of neuron physiology, with a possible impact on the conditions of altered cell excitability that often underlie neuronal damages and, ultimately, cognitive dysfunctions.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (Morini, Milan, Italy) weighing 220−280 g were used in all experiments. Animals had access to food and water *ad libitum*. The light cycle was automatically controlled (on at 07:00, off at 19:00) and the room temperature was thermostatically regulated at 22° ± 1°C.

All procedures were performed in strict accordance with the European directive 2010/63/EU and the institutional guidelines, authorized by the Italian Ministry of Health (authorization no. 258-95-A) and approved by the Committee for the Protection and Use of Animals of the University of Palermo. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Quantitative Assessment of Indicaxanthin Concentration in Selected Brain Areas

Reagents

Unless stated otherwise, all reagents were from Sigma-Aldrich (Milan, Italy) and of the highest grade commercially available.

Extraction and Purification of Indicaxanthin From Cactus Pear Fruits

Indicaxanthin was isolated from cactus pear (*Opuntia ficus-indica*) fruits (yellow cultivar). Briefly, the phytochemical was separated from a methanol extract of the pulp by liquid chromatography on Sephadex G-25 as previously reported (Allegra et al., 2015). Fractions containing the pigment were submitted to cryodesiccation, purified as described (Allegra et al., 2015), quantified by HPLC as below reported, and suspended in PBS for the experiments.

Surgical Procedure

Rats (n = 4) received intragastric administration of indicaxanthin (2 μmol/kg) or saline 0.9%. According to previous pharmacokinetic study of indicaxanthin maximal brain levels, animals were sacrificed after 1 h with pentobarbital and perfused with normal saline to remove any compounds still circulating in the blood, then brains were removed. Cerebral sections, including cortex, hippocampus, striato-pallidal complex, diencephalon (thalamic and subthalamic regions), brainstem and cerebellum, were dissected and harvested following

standardized coordinates for chromatographic quantification as previously reported (Paxinos and Watson, 1998; Chan et al., 2010; Zhang et al., 2015). In particular, discrete brain tissues were maintained on a dry ice bed, weighed, wrapped in aluminum foil to protect against light and stored at −20°C until sample analysis.

Quantification of Indicaxanthin in Brain Samples

The amount of indicaxanthin within brain samples was evaluated as reported below. With regard to each cerebral section, tissues were carefully washed with saline, pooled and homogenized in PBS. Indicaxanthin was extracted from samples with chloroform: methanol, 2:1 by volume (1 g of tissue with 3 volumes of extraction mixture). The methanol phase from all samples at each time point was dried under nitrogen, re-suspended in 1% acetic acid in water, analyzed on a Varian Microsorb C-18 column (4.6 Å ∼250 mm; Varian, Palo Alto, CA, USA), and eluted with a 20-min linear gradient elution from solvent A (1% acetic acid in water) to 20% solvent B (1% acetic acid in acetonitrile) with a flow rate of 1.5 mL/min. Spectrophotometric revelation was at 482 nm. Under these conditions, indicaxanthin eluted after 8.15 min and was quantified by reference to standard curves constructed with 0.2−10 ng of purified compounds and by relating its amount to the peak area.

Electrophysiological Assessment of Indicaxanthin Effect in Different Brain Areas

Surgical Procedures

Adult male Wistar rats (n = 8) were anesthetized with urethane (Sigma Chemical Co., St. Louis, MO, USA) at the dose of 1.2 g/kg i.p. and positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). Body temperature was maintained at 37–38°C using a heating pad. The skull was exposed and holes were drilled. Coordinates were determined using a stereotaxic atlas, anterior (A) from interaural line, lateral (L) from midline, ventral (V) to cortical surface (Paxinos and Watson, 1998): cortex (A: 4.7–10.4, L: 1–5, V: 1–2.5), hippocampus (A: 4.8–5.8, L: 1–4, V: 2–3.5), striatum (A: 7.4–10.7, L: 1.5–5, H: 3–8), globus pallidus (A: 7–8.2, L: 2.6–4, H: 5–7), thalamus (A: 5.2–7.4, L: 2–4, V: 4–6.5) and subthalamic nucleus (A: 4.7–5.4, L: 2–3.2, H: 6.8–8).

Electrophysiological Recordings

A seven-barrel glass micro-electrode was used for both recording and drug ejection, as previously reported (Carletti et al., 2009). The center recording barrel (1.1−2.0 MΩ resistance) was filled with 2 M NaCl with 1% Fast Green (Sigma), one side barrel was filled with 2 M NaCl 0.9% buffered saline solution (for automatic current balancing), and the others were filled respectively with 12 μM, 6 μM and 3 μM indicaxanthin at pH 7.4, prepared by dilution 1:1000 of stock solution of indicaxanthin 12 mM, 6 mM and 3 mM, respectively. Negative currents of 8−10 nA were applied to the barrels (20−70 MΩ) to retain drugs (Neurophore BH-2 System, Harvard Apparatus, Hamden, CT, USA). To concentrate the drug at the pipette tip, at the beginning of each track, maximal ejection currents were applied to each barrel for 30 min. Under these conditions a spontaneous discharge was evident for all recorded cells (**Figure 1**). Amplification, filtering and acquisition of recordings was performed for off-line analysis as previously described (Carletti et al., 2016). Neuronal activity was continuously displayed and updated online every 5 s via a rate-meter histogram with a counter window on the computer screen to detect any variations of neuronal firing rate. All computer operations were performed using the SciWorks package, version 5.0 (Datawave Technologies, Loveland, CO, USA). Neuronal units were not taken into consideration if marked changes in amplitude or configuration of the spike were observed, or if there was early death of the cell in the course of recording. Baseline activity of each neuron was recorded for 3−5 min before acute vehicle or drug administration (**Figure 1**).

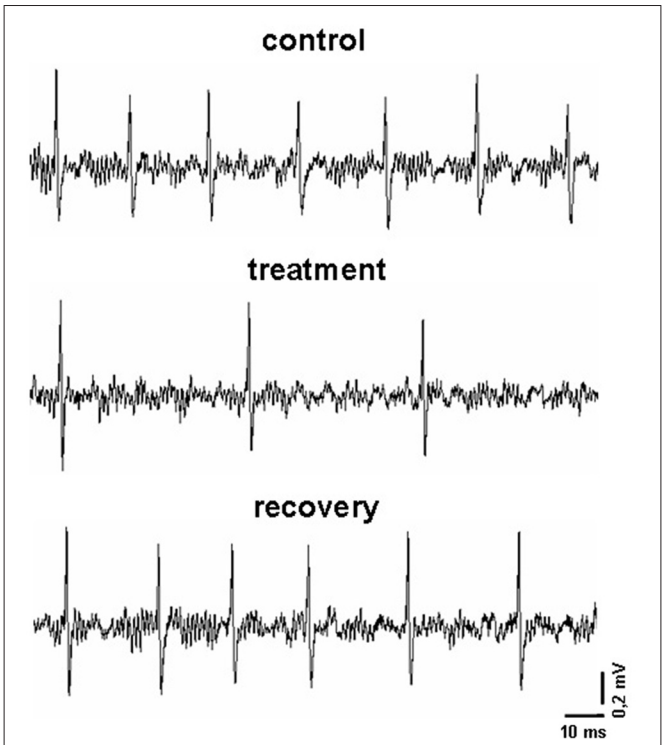

FIGURE 1 | Representative recordings obtained in an experimental session. In particular, it is displayed the firing pattern of a pallidal neuron before, during and after the microiontophoretic treatment with indicaxanthin (0.17 ng/neuron).

Indicaxanthin Treatment

As previously described (Allegra et al., 2015), pilot recordings with the maximal concentration of 12 μM indicaxanthin (below reported) were carried out at increasing ejection currents to identify the minimal current exerting an effect on all responsive neurons in each brain area of investigation. For this purpose, neurons were tested with 90 s pulses of indicaxanthin at 40, 60 and 80 nA ejection currents, with a 90 s interpulse interval to prevent any influence of the previous pulse on the following one. The minimal current effective on all responsive neurons resulted to be 60 nA. After this step, all recorded neurons

underwent the iontophoretic administration of indicaxanthin at different amounts for 5 min with ejection current of 60 nA to correlate eventual modifications of neuronal response with the amount of the drug. Once the application of indicaxanthin was terminated, recording continued until the spontaneous firing rate recovered to baseline (**Figure 1**). Control tests, applying the same ejection currents used for the treatments, were performed with vehicle solutions and no influence on the neuronal firing were found. It is worth to highlight that ejection times and currents, in our microiontophoretic approach, result in a total amount of indicaxanthin interacting with single neurons in a nanogram range. Indeed, indicaxanthin released from a barrel in 5 min at 60 nA was equivalent to 0.1 μl, corresponding approximatively to 0.34 ng (for 12 μM), 0.17 ng (for 6 μM), and 0.085 ng (for 3 μM). Apart from brain localization, microiontophoretic recordings were carried out in telencepahalic and diencephalic regions (cortex, hippocampus, striatum, globus pallidus, thalamus and subthalamic nucleus), involved in cognitive processes and mostly investigated by our group in previous electrophysiological researches (Sardo et al., 2003, 2006, 2009, 2012; Carletti et al., 2012, 2017; Plescia et al., 2014). Hindbrain areas were not taken in consideration for recordings because of stereotaxic limitations. Lastly, at the end of each experiment, the recording site was marked with Fast Green through the electrode, using a 50 mA ejection current for 15 min. After transcardial perfusion with saline followed by 10% buffered formalin, brains were removed and cryoprotected in 30% sucrose/PBS. Coronal frozen sections were cut at 50 mm and stained with cresyl violet for histological verification and reconstruction of recording sites (Supplementary Figure S1). Images of sections were acquired using a Leica DFC camera attached to a stereomicroscope (Leica Microsystems Imaging solutions Ltd., Cambridge, UK).

Microiontophoretic Data Analysis

Neuronal firing rate was off-line analyzed before, during and after drug administration for each recorded unit. Individual rate-meter histograms (5 s bin width) were analyzed by means of a nonparametric Mann−Whitney *U* test to detect any statistically significant treatment-related change in neuronal firing. To analyze the effects induced by treatments, neurons were considered responsive if changes were significant (probability level $P < 0.05$) for at least six consecutive bins for indicaxanthin, the first of which was respectively labeled as the onset of a response. The intensity of the effect on neuronal firing (changes in the discharge with respect to baseline, considered as 1 min recording before treatment) was expressed as percentage magnitude (hereafter, magnitude), rather than absolute values, in order to normalize the responses of neurons with different baseline discharge frequencies. The latency of the effect was considered as the time from the appearance of the effect, and the duration as its timespan. Comparisons of the effects of different amounts of indicaxanthin on the mean magnitude of discharge frequencies, latency and duration were performed by means of a Mann–Whitney *U* test. For all statistical tests used, the null hypothesis was rejected at a *P* lower than 0.05. If not otherwise indicated, all results are expressed as mean ± SD.

RESULTS

Indicaxanthin Selectively Distributes Within Brain Areas

Distribution of indicaxanthin in rat brain ($n = 4$) was investigated by assessing the amount of the pigment accumulated in selected brain areas (i.e., cortex, hippocampus, striato-pallidal complex, diencephalon, cerebellum and brainstem) per mg of fresh tissue, 1 h after oral administration of 2 μmol/Kg. Interestingly, indicaxanthin accumulated in all examined areas with the exception of striato-pallidal complex (**Figure 2**) and no known metabolite was recovered in brain samples (not shown). In particular, indicaxanthin reached a maximum and minimum amount of the pigment in cerebellum and cortex (0.09 ± 0.002) and (0.05 ± 0.002) ng/mg of fresh tissue, respectively (**Figure 2**).

Microiontophoretic Recordings

Cortex

Cortical neurons (n = 13 cells) were administered with indicaxanthin ejected at 60 nA for 5 min at 0.34, 0.17 and 0.085 ng/neuron (**Figure 3A**). Significant inhibition of firing rate was recorded after indicaxanthin administration at 0.34 ng/neuron in 9 cells (69.23%, $P < 0.05$) with a mean magnitude of −41.89 ± 9.59%, a mean latency of 28.89 ± 23.29 s and a mean duration of 246.56 ± 58.41 s (**Figure 3B**). Administration of indicaxanthin at 0.17 ng/neuron reduced neuronal discharge in 7 cells (53.85%, $P < 0.05$) showing a mean magnitude of −36.38 ± 15.06%, a mean latency of 66.43 ± 79.83 s and a mean duration of 75.00 ± 75.17 s (**Figure 3B**). No effects were observed for treatment with indicaxanthin at 0.085 ng/neuron. Between-treatment analysis showed that only the duration of the effect was significantly higher for indicaxanthin at 0.34 with respect to 0.17 ng/neuron

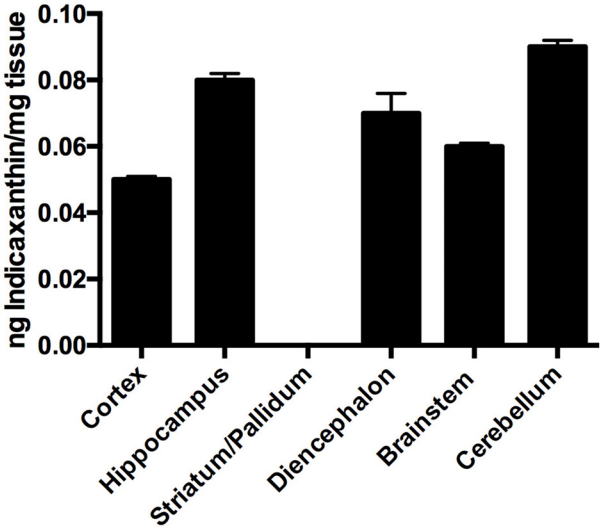

FIGURE 2 | Distribution of indicaxanthin in selected rat brain areas 60 min after ingestion of 2 μmol/kg, expressed as ng/mg tissue. Values are indicated as means ± SEM.

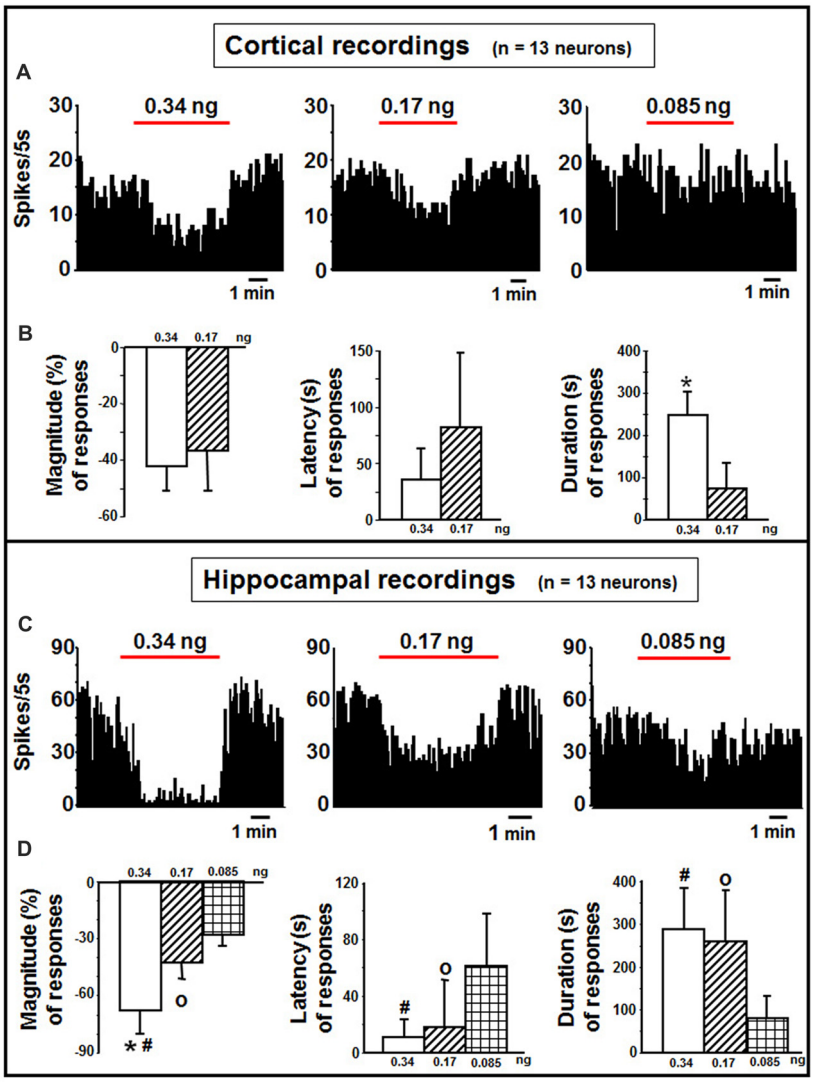

FIGURE 3 | Cortical and hippocampal recordings. **(A)** Representative rate-meter histogram (bin width 5 s) showing the firing rate of a cortical neuron following indicaxanthin administration at various amounts (0.34, 0.17, 0.085 ng/neuron) for 5 min. **(B)** Bar histogram for magnitude, latency and duration showing the significant effect of indicaxanthin at different amounts (0.34, 0.17 ng/neuron) on cortical neuronal responses (n = 13). Each bar represents mean values (± standard deviation; *statistically significant effect of indicaxanthin 0.34 vs. 0.17 ng/neuron). (Statistical significance for $P < 0.05$). **(C)** Representative rate-meter histogram (bin width 5 s) showing the firing rate of a hippocampal neuron following indicaxanthin administration at various amounts (0.34, 0.17, 0.085 ng/neuron) for 5 min. **(D)** Bar histogram for magnitude, latency and duration showing the significant effect of indicaxanthin at different amounts (0.34, 0.17, 0.085 ng/neuron on hippocampal neuronal responses (n = 13). Each bar represents mean values (± standard deviation; *statistically significant effect of indicaxanthin 0.34 vs. 0.17 ng/neuron; #statistically significant effect of indicaxanthin 0.34 vs. 0.085 ng/neuron; °statistically significant effect of 0.17 vs. 0.085 ng/neuron). Statistical significance for $P < 0.05$.

($Z = -3.07$, $P = 0.0021$), while magnitude and latency were not modified between different amounts of the pigment (**Figure 3B**).

Hippocampus

Neurons belonging to the hippocampus (n = 13 cells) were treated with indicaxanthin ejected at 60 nA for 5 min at 0.34, 0.17 and 0.085 ng/neuron (**Figure 3C**). Indicaxanthin at 0.34 ng/neuron induced significant inhibition in firing rate in 10 cells (76.92%, $P < 0.05$) with a mean magnitude of −67.85 ± 12.57%, a mean latency of 12.00 ± 13.17 s and a mean duration of 287.50 ± 98.86 s (**Figure 3D**). Administration of 0.17 ng/neuron indicaxanthin at 60 nA caused inhibition of neuronal discharge in 8 cells (61.54%, $P < 0.05$) showing a mean magnitude of −42.82 ± 8.85%, a mean latency of 20.63 ± 32.78 s and a mean duration of 261.25 ± 118.98 s (**Figure 3D**). Lastly, indicaxanthin at 0.085 ng/neuron reduced firing rate of 6 neurons (46.15%, $P < 0.05$) revealing a mean magnitude of −27.78 ± 7.82%, a mean latency of 60.83 ± 38.39 s and a mean duration of 79.17 ± 54.90 s (**Figure 3D**). Between-treatment comparisons revealed that the effect of indicaxanthin was markedly dose-dependent. In detail, considering magnitude values, the inhibition by indicaxanthin at 0.34 was higher than

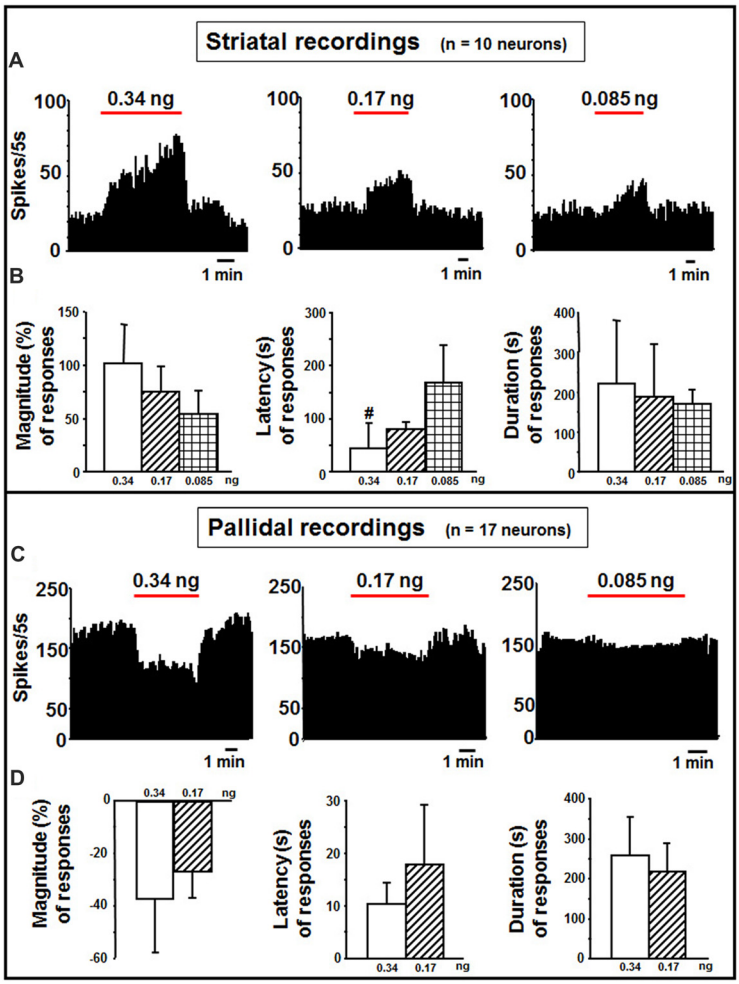

FIGURE 4 | Striatal and pallidal recordings. **(A)** Representative rate-meter histogram (bin width 5 s) showing the firing rate of a striatal neuron following indicaxanthin administration at various amounts (0.34, 0.17, 0.085 ng/neuron) for 5 min. **(B)** Bar histogram for magnitude, latency and duration showing the significant effect of indicaxanthin at different amounts (0.34, 0.17, 0.085 ng/neuron) on striatal neuronal responses (n = 10). Each bar represents mean values (± standard deviation; #statistically significant effect of indicaxanthin 0.34 vs. 0.084 ng/neuron). Statistical significance for $P < 0.05$. **(C)** Representative rate-meter histogram (bin width 5 s) showing the firing rate of a pallidal neuron following indicaxanthin administration at various amounts (0.34, 0.17, 0.085 ng/neuron) for 5 min. **(D)** Bar histogram for magnitude, latency and duration showing the significant effect of indicaxanthin at different amounts (0.34, 0.17, 0.085 ng/neuron) on pallidal neuronal responses (n = 17). Each bar represents mean values (± standard deviation). Statistical significance for $P < 0.05$.

the effect of indicaxanthin at both 0.17 ($Z = -3.199$, $P = 0.0014$) and 0.085 ng/neuron ($Z = -3.254$, $P = 0.0011$), and a significant reduction was observed comparing effect at 0.17 with that at 0.085 ng/neuron ($Z = -2.453$, $P = 0.0142$). The latency of the effect was reduced comparing 0.34–0.085 ng/neuron ($Z = -2.82$, $P = 0.0048$) and 0.17–0.085 ng/neuron ($Z = -2.19$, $P = 0.0282$). Lastly, with regard to the duration of the effect, the inhibition was enhanced at 0.34 and at 0.17, respectively, vs. 0.085 ng/neuron ($Z = -3.037$, $P = 0.0024$ and $Z = -2.84$, $P = 0.0045$; **Figure 3D**).

Striatum

In the striatum, indicaxanthin at 0.34, 0.17 and 0.085 ng/neuron was injected at 60 nA for 5 min to 10 neurons (**Figure 4A**). The highest amount caused excitation in 5 cells (50%, $P < 0.05$), whilst the other amounts in 3 cells (30%, $P < 0.05$). The effect of indicaxanthin at 0.34 ng/neuron presented a mean magnitude of 104.62 ± 32.30%, a latency of 44.00 ± 45.88 s and a duration of 223.00 ± 159.24 s (**Figure 4B**). Excitation by indicaxanthin at 0.17 ng/neuron showed a mean magnitude of 75.74 ± 18.02%, a latency of 80.00 ± 39.05 s, and a duration of 188.33 ± 136.50 s (**Figure 4B**). Lastly, indicaxanthin at 0.085 ng/neuron produced an excitation characterized by a mean magnitude of 53.43 ± 24.34%, a latency of 166.67 ± 70.06 s, and a duration of 170.00 ± 39.69 s (**Figure 4B**). Between-treatment analysis on the different concentrations of indicaxanthin revealed that the latency of the effect was significantly shorter at 0.34 vs. at 0.085 ng/neuron ($Z = -2.23$, $P = 0.0253$; **Figure 4B**).

Globus Pallidus

Pallidal neurons (n = 17 cells) was administered with indicaxanthin with current of 60 nA for 5 min at 0.34, 0.17 and 0.085 ng/neuron (**Figure 4C**). In 15 cells (88.24%, $P < 0.05$)

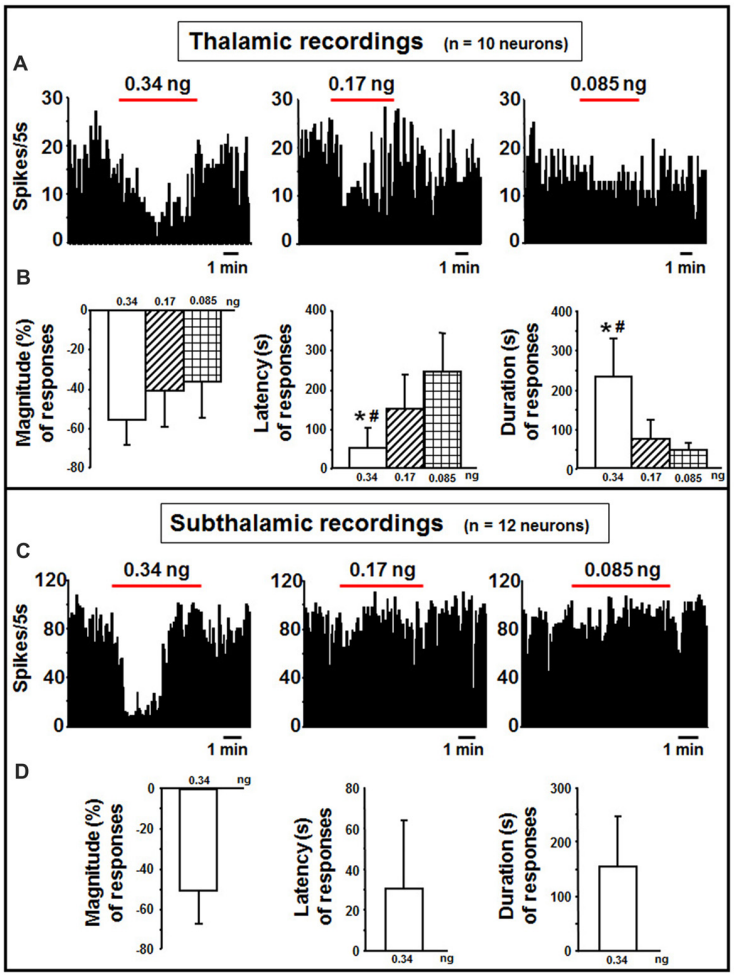

FIGURE 5 | Thalamic and subthalamic recordings. **(A)** Representative rate-meter histogram (bin width 5 s) showing the firing rate of a thalamic neuron following indicaxanthin administration at various amounts (0.34, 0.17, 0.085 ng/neuron) for 5 min. **(B)** Bar histogram for magnitude, latency and duration showing the significant effect of indicaxanthin at different amounts (0.34, 0.17, 0.085 ng/neuron) on thalamic neuronal responses (n = 10). Each bar represents mean values (± standard deviation; *statistically significant effect of indicaxanthin 0.34 vs. 0.17 ng/neuron; #statistically significant effect of indicaxanthin 0.34 vs. 0.085 ng/neuron). Statistical significance for $P < 0.05$. **(C)** Representative rate-meter histogram (bin width 5 s) showing the firing rate of a subthalamic neuron following indicaxanthin administration at various amounts (0.34, 0.17, 0.085 ng/neuron) for 5 min. **(D)** Bar histogram for magnitude, latency and duration showing the significant effect of indicaxanthin at 0.34 ng/neuron on subthalamic neuronal responses (n = 12). Each bar represents mean values (± standard deviation). Statistical significance for $P < 0.05$.

a reduction of firing was observed after administration of indicaxanthin at 0.34 ng/neuron. Mean magnitude of firing was of −36.81 ± 20.94%, mean latency 10.33 ± 3.99 s and mean duration 258.33 ± 95.71 s (**Figure 4D**). Inhibition of neuronal discharge was found in 9 neurons (52, 94%, $P < 0.05$), treated with indicaxanthin at 0.17 ng/neuron with a mean magnitude of −26.55 ± 10.38%, mean latency 17.78 ± 11.49 s and mean duration 217.22 ± 67.37 s (**Figure 4D**). No effects were observed with indicaxanthin at 0.085 ng/neuron. Statistical analysis between concentrations revealed that discharge parameters were not modified comparing 0.34 with 0.17 ng/neuron.

Thalamus

In the thalamus, indicaxanthin was administered at 0.34, 0.17 and 0.085 ng/neuron at 60 nA current for 5 min in 10 neurons (**Figure 5A**). After indicaxanthin administration at 0.34 ng/neuron, 9 neurons (90%, $P < 0.05$) exhibited inhibition of firing rate with a mean magnitude of −55.46 ± 12.58%, a mean latency of 56.11 ± 61.84 s and a mean duration of 231.67 ± 97.60 s (**Figure 5B**). Furthermore, administration of indicaxanthin at 0.17 ng/neuron induced reduction of neuronal discharge in 5 cells (50%, $P < 0.05$) with a mean magnitude of −38.99 ± 19.83%, a mean latency of 151.00 ± 85.32 s and a mean duration of 74.00 ± 50.17 s (**Figure 5B**). Lastly, the administration of indicaxanthin at 0.085 ng/neuron reduced discharge in 3 neurons (30%, $P < 0.05$) with a mean magnitude of −35.75 ± 18.40%, latency of 243.33 ± 97.51 s and duration of 45.00 ± 25.98 s (**Figure 5B**). As for between-treatment analysis, the latency of the effect was reduced at 0.34 vs. 0.17 ($Z = -2.067$, $P = 0.0388$)

and 3 mM ($Z = -2.126$, $P = 0.0335$). Also, the duration was enhanced at 0.34 compared to 0.17 ($Z = -2.733$, $P = 0.0063$) and to 0.085 ng/neuron ($Z = -2.496$, $P = 0.0126$; **Figure 5B**).

Subthalamic Nucleus

Subthalamic neurons ($n = 12$) were treated with indicaxanthin at 0.34, 0.17 and 0.05 ng/neuron at 60 nA currents for 5 min (**Figure 5C**). Eleven of them were inhibited by the highest concentration (91.6%, $P < 0.05$). The effect showed a mean magnitude of $-50.15 \pm 16.52\%$, a latency of 33.45 ± 33.43 s, and a duration of 154.55 ± 92.10 s (**Figure 5D**). The administration of lower amounts did not influence discharge activity.

DISCUSSION

Biodistribution and neurophysiological data from this study fall within the remit of intense research on PhC and human health, focusing on the interaction of indicaxanthin with central nervous system. Within the immense pharmacological cornucopia of nutraceuticals, only few of them (curcumin, resveratrol, tea polyphenols) have been reported to cross the BBB and accumulate in different brain areas exerting neuroprotective effects (Milbury and Kalt, 2010; Vingtdeux et al., 2010; Pandareesh et al., 2015). In the present research, we demonstrate for the first time that indicaxanthin, a betalain pigment able to cross the BBB (Allegra et al., 2015), when administered at nutritionally-relevant amounts accumulates within different brain areas and remains unmodified at levels comparable or even greater than those showed by other PhC (Vingtdeux et al., 2010; Vanmierlo et al., 2012). Moreover, while some PhC distribute within the brain homogeneously (Milbury and Kalt, 2010), we here show that indicaxanthin gains a specific access to selected brain areas. This phytochemical was indeed found in different amount in cortex, hippocampus, diencephalon, brainstem, cerebellum, but not in the striato-pallidal complex. While indicaxanthin peculiar ability to cross BBB may be due to its amphiphilicity, high bioavailability and affinity to lipid membranes (Turco Liveri et al., 2009), a more complex scenario maybe envisaged to explain the selective accumulation of the pigment in some brain areas and the exclusion from others such as the striato-pallidal complex. Anatomically speaking, the discrepancy could be ascribed to the peculiar structural features of this subcortical region belonging to the basal ganglia; in particular, the presence of different fiber bundles, encapsulating the striatum and pallidum, could not allow indicaxanthin accumulation as easily as in the rest of the brain (Parent and Hazrati, 1995). The chemical structure of indicaxanthin could also account for its absence in some brain areas unlike, for example, the anthocyanins contained in the blueberry, capable to reach the striatum after oral consumption (Andres-Lacueva et al., 2005). Surely enough, concentrations of indicaxanthin necessary to enter in distinct brain structures can vary on the basis of the mechanism employed for the passage, for instance membrane transporters or endocytosis, and this could also be responsible for the lack of indicaxanthin accumulation both in the striatum and pallidum. This aspect surely deserves further studies focusing on the interaction of indicaxanthin with the different biological barriers lying in the brain.

TABLE 1 | Effects on neuronal firing rate in different brain structures after microiontophoretic administration of three amounts of indicaxanthin.

	0.34 ng	0.17 ng	0.085 ng
Cortex	–	–	○
Hippocampus	–	–	–
Striatum	+	+	+
Globus pallidus	–	–	○
Thalamus	–	–	–
Subthalamic Nucleus	–	○	○

Amounts (ng) refer to the indicaxanthin ejected per neuron by microinthophoresis in 5 min at 60 nA. Neurons belonging to each structure displayed either excitatory or inhibitory effects. Excitatory, inhibitory and null responses are indicated with (+), (−), (○), respectively.

Considering the permeability of indicaxanthin to BBB and its distribution, we extensively investigated the neuromodulatory potential of indicaxanthin. To this purpose, electrophysiological recordings were conducted in the brain areas mainly involved in forebrain cognitive functions, including striato-pallidal complex; single neuronal unit recordings associated with microiontophoresis represent an adequate and direct method to examine the electrophysiological outcomes of exogenous substances with unknown properties. Furthermore, administrations of indicaxanthin at three different amounts were carried out to trace a dose-response profile (**Table 1**). In detail, during cortical recordings, the evaluation of cell firing revealed that discharge frequency was inhibited by indicaxanthin at 0.34 and 0.17, but not at 0.085 ng/neuron. The inhibition induced by indicaxanthin at 0.34 appeared more intense than that at 0.17/ng neuron and characterized by an increased magnitude of effect accompanied by a lower latency and a greater duration of the effect, though statistical significance between effects at different amount of the molecule was observed only for the effect duration. This ability of indicaxanthin to modulate neuronal excitability emerges more clearly in the hippocampus, where the phytochemical always resulted effective. Indeed, the different amounts of indicaxanthin administered induced a dose-dependent significant inhibition of cell firing, especially on the magnitude of the effect. This was confirmed by the related changes in latency and duration of the effect. These data suggest that hippocampal neurons are more responsive to indicaxanthin with respect to cortical ones. In the striatum, we observed an opposite outcome since indicaxanthin determined the excitation of firing rate with an intensity related to the amount applied. In particular, the greater magnitude of the effect was associated to shorter latency and longer duration of the response, although statistical significance came out only for the latency between 0.34 vs. 0.085 ng/neuron. In the globus pallidus, belonging to basal ganglia circuit as well as striatum, indicaxanthin has instead induced the inhibition of neuronal firing, but only after the injection at 0.34 vs. 0.085 ng/neuron, that did not statistically differ between them. As for recordings performed in the thalamus, it

was evidenced a marked reduction of neuronal excitability. Amount-related changes in magnitude were found and were confirmed by significant differences between doses in latency and duration. Finally, the subthalamic nucleus was pointed out as the areas that was less responsive to treatments since indicaxanthin was effective only at 0.34 ng/neuron in reducing cell firing.

This evidence sustains a prevalent inhibitory effect of indicaxanthin on discharge activity of neurons in rat brain (**Table 1**). As for the exclusive excitatory effect observed in the striatum, in order to explain this different result, it should be noted that all the other structures examined are mainly based on glutamatergic or GABAergic transmission, while in the striatum a relevant dopaminergic influence integrate glutamatergic input and contribute to the neuronal excitability (Parent and Hazrati, 1995; Michaelis, 1998). Therefore, the modulatory action of indicaxanthin occurred in a peculiar synaptic environment, more finely regulated, possibly underlying the increase in discharge frequency. Interestingly, in our previous researches the striatum already showed its particularity, given that the microiontophoretic administration of nitric oxide donors, likely intervening in glutamatergic and GABAergic transmission, induced excitation in all basal ganglia structures, except for the striatum where inhibition was observed (Sardo et al., 2003, 2006, 2009, 2011; Carletti et al., 2009, 2012).

Taking into account the areas inhibited, indicaxanthin action could be framed within the context of neurotransmission systems exploiting glutamate and GABA, as already mentioned. In this view, the inhibition of neuronal activity could be due to the reduction of glutamate neurotransmission or the enhancement of GABA transmission. Although our data do not allow to assert the presence of a direct interaction with these systems, our previous article demonstrated the potential affinity of indicaxanthin to subunit N2A of NMDA glutamate receptor (NMDAR), that is pivotal for NMDAR function but is also involved in alteration linked to aging neurodegeneration (Gardoni et al., 2006; Allegra et al., 2015). Moreover, the ability of indicaxanthin to reduce glutamate-mediated neuronal excitability in the hippocampus was previously outlined (Allegra et al., 2015). In support of this interpretation, other researches propose the interaction between PhC and glutamate receptors and suggest, for example, that a diet enriched with blueberry ameliorated hippocampal learning process by preventing dysfunctions in the glutamatergic transmission (Williams et al., 2004; Coultrap et al., 2008). High expression of NMDAR subunit N2A in both the cortex and hippocampus suggests that this could be a potential site of action for indicaxanthin in these regions. Furthermore, the ubiquitous expression of this subunit in the entire adult brain could explain the widespread and reproducible effects of indicaxanthin. On the other hand, the implication of an interplay between indicaxanthin with GABA system should be also considered. In this regard, an enhancement of GABA-currents induced by the flavone hispidulin, exerting anticonvulsive outcomes, was evidenced in an animal model of seizure (Kavvadias et al., 2004). Furthermore, the finding that flavonoids produced anxiolytic effect by virtue of an affinity for benzodiazepine site on GABA receptor (Medina et al., 1998) suggests a fine addressed action of PhC in modulating neuronal excitability. However, the possible interaction of indicaxanthin with other channels determining the excitability state of neurons cannot be ruled out and deserves further studies.

In the light of the present results, a noticeable direct neuromodulatory action for the indicaxanthin emerges. This influence on the cell excitability could have implications in some cellular dysfunctions induced by alterations of membrane mechanisms. Indeed, increased excitation could bring to excitotoxicity, especially in cortical areas, that triggers damaging and neurodegenerative disorders, such as Alzheimer disease, above all, but also epilepsy (Lipton and Rosenberg, 1994; Danysz and Parsons, 2003; Dong et al., 2009; Mehta et al., 2013). Since many beneficial neuroprotective effects of nutraceuticals are almost exclusively ascribed to their anti-inflammatory and antioxidant properties, the findings of a direct influence of indicaxanthin on the bioelectric activity of neurons shed new light on the possible mechanisms lending to dietary vegetables their favorable and healthy features. In particular, considering the prevalent inhibitory effect of indicaxanthin, the potential brake to excitatory transmission could constitute a further factor underpinning the benefits for maintenance of cell function. In this framework, inhibitory action of indicaxanthin in cortical and hippocampal areas, involved in cognitive functions and often affected by neurodegenerative deficits, represents an intriguing perspective for the use of this and, possibly, other phyto-nutrients in the implementation of anti-neurodegenerative strategies. Indeed, several researches already reported the positive effects of diets enriched with PhC on the brain function by improving telencephalic cognitive processes (Spencer, 2008). For example, in aged rats, the chronic supplementation with flavonoids from gingko biloba increased hippocampal LTP via the reduction of neuronal excitability (Williams et al., 2004). The altered excitability underlying age-related amyloid pathologies could also lead to epileptogenesis and some dietary plant-derived nutrients already revealed anticonvulsant effects (Kavvadias et al., 2004; Nassiri-Asl et al., 2008; Gurevicius et al., 2013; Diniz et al., 2015; Aggleton et al., 2016), also in aberrant cortico-thalamic hyperexcited connections (Gurevicius et al., 2013). Since this detrimental link between neurodegeneration and altered neuronal excitability could constitute one of the factors concurring to brain diseases, this study opens novel therapeutic perspectives and prompts future investigations about mechanism of action of indicaxanthin in the neuronal environment. Together with the neuroprotective action of nutraceuticals due to their antioxidant and anti-inflammatory properties, a further cellular target at synaptic level was individuated with a view to deepen knowledge on beneficial effects of phytonutrients on neuronal function.

AUTHOR CONTRIBUTIONS

GG, FC, MA and AA conducted the experiments. GG, FC, MA and PS performed the data analyses. FC, MA, PS, GF, LT and MAL designed the experiments. FC designed and directed

the project. GG, FC and MA wrote the manuscript with input from all the authors. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2018.00133/full#supplementary-material

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Glycerophospholipid Supplementation as a Potential Intervention for Supporting Cerebral Structure in Older Adults

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Modifying nutritional intake through supplementation may be efficacious for altering the trajectory of cerebral structural decline evident with increasing age. To date, there have been a number of clinical trials in older adults whereby chronic supplementation with B vitamins, omega-3 fatty acids, or resveratrol, has been observed to either slow the rate of decline or repair cerebral tissue. There is also some evidence from animal studies indicating that supplementation with glycerophospholipids (GPL) may benefit cerebral structure, though these effects have not yet been investigated in adult humans. Despite this paucity of research, there are a number of factors predicting poorer cerebral structure in older humans, which GPL supplementation appears to beneficially modify or protect against. These include elevated concentrations of homocysteine, unbalanced activity of reactive oxygen species both increasing the risk of oxidative stress, increased concentrations of pro-inflammatory messengers, as well as poorer cardio- and cerebrovascular function. As such, it is hypothesized that GPL supplementation will support cerebral structure in older adults. These cerebral effects may influence cognitive function. The current review aims to provide a theoretical basis for future clinical trials investigating the effects of GPL supplementation on cerebral structural integrity in older adults.

Keywords: glycerophospholipid, supplementation, intervention, cerebral structure, older adults

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INTRODUCTION

Cerebral structure has been observed to decline with increasing age. Age-related reductions in cerebral structural integrity are evident at both the macro- and microstructural levels (e.g., reduced whole and regional volumes, elevated cortical thinning, increased severity of white matter lesions, and decreased integrity of microscopic white matter pathways), and may begin as early as young adulthood (Resnick et al., 2003; Allen et al., 2005; Fotenos et al., 2005; Walhovd et al., 2005; Bendlin et al., 2010; Hsu et al., 2010; Westlye et al., 2010; Sala et al., 2012; Taki et al., 2013a). There is also a growing literature purporting reduced cerebral structural integrity as a significant predictor of cognitive functioning apparent in older age (Davis et al., 2009; Bendlin et al., 2010; Lockhart et al., 2012; Arvanitakis et al., 2016).

Although all adults demonstrate at least some deterioration in cerebral structure (and cognitive function) with age, the trajectory of decline is not fixed. While some adults appear to demonstrate cerebral decline consistent with "normal aging," others have conditions such as (in order of worsening dysfunction) "age-associated memory impairment" (AAMI), "mild cognitive impairment" (MCI), and Alzheimer's dementia (AD), all of which are characterized by more severe degradation of cerebral structure, as well as cognitive impairment (Anstey and Maller, 2003; Hänggi et al., 2011; Smith et al., 2011; Bosch et al., 2012; Maillard et al., 2012; Wang et al., 2014; Zheng et al., 2014). These conditions are not inevitable features of increasing age, and it may be possible to reduce their incidence within the population though administration of interventions capable of supporting cerebral structure in older adults.

One factor believed to influence cerebral structural integrity with age is nutritional intake (Scholey, 2018). Previous cross-sectional and longitudinal work has indicated that greater ingestion and bioavailability of B vitamins such as B_6, B_{12}, and folate (Erickson et al., 2008; Vogiatzoglou et al., 2008; Tangney et al., 2011; Hsu et al., 2015; Hooshmand et al., 2016; Köbe et al., 2016) or omega-3 polyunsaturated fatty acids (ω3-PUFA; Samieri et al., 2012; Tan et al., 2012; Titova et al., 2013; Pottala et al., 2014; Gu et al., 2016) predicts greater cerebral macro- and micro-structural integrity in older adults. Moreover, data from available clinical trials indicates that chronic supplementation with these nutrients, but also others such as resveratrol, may either enhance cerebral structural integrity, or improve the trajectory of cerebral decline over time (Smith et al., 2010; Douaud et al., 2013; Witte et al., 2013; Jerneren et al., 2015; Köbe et al., 2017; Zhang et al., 2017). It is possible that these structural effects, at least partly, underpin the benefits to cognitive performance in older adults following supplementation (Durga et al., 2007; Yurko-Mauro et al., 2010; de Jager et al., 2012; Witte et al., 2013).

The consumption of phospholipids (PL) and in particular the glycerophospholipids (GPL), may also benefit cerebral structure and subsequently cognitive function in older adults. The GPL species phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) are abundant in mammalian cell membranes, and there is growing evidence that provision of these GPL (particularly PC and PS) can improve cognitive function in animals via oral supplementation (Zanotti et al., 1989; Furushiro et al., 1997; Lim and Suzuki, 2000; Suzuki et al., 2001; Kataoka-kato et al., 2005; Yaguchi et al., 2009, 2010; Lee et al., 2010; Babenko and Semenova, 2011; Nagata et al., 2011; Park et al., 2013; Zhang et al., 2015; Qu et al., 2016; Wen et al., 2016a,b) or intraperitoneal/intracerebral injection (Drago et al., 1981; Zanotti et al., 1984; Corwin et al., 1985; Sakai et al., 1996; Blokland et al., 1999; Claro et al., 1999, 2006; Suzuki et al., 2000). Similar results are also evident following oral supplementation in older humans with varying levels of cognitive function (i.e., normal cognitive function with subjective memory complaints, age-related "cognitive dysfunction," AAMI, MCI, or dementia). The details and findings of these trials are summarized in **Tables 1–3**.

While the evidence for GPL supplementation benefiting cognitive function is relatively robust, there is comparatively less work investigating their effects on cerebral structure. In a number studies with middle-aged and older rodents, GPL supplementation was found to improve hippocampal cell morphology (Nunzi et al., 1987; Crespo et al., 2004; Qu et al., 2016), as well as elevate cellular proliferation and survival within the dentate gyrus (Maragno et al., 2015). Likewise, 7 months oral supplementation with PE (1,2-dilinoleoyl-*sn*-glycero-3-phosphoethanolamine) has been observed to reduce age-related hippocampal neuron death in senescence accelerated SAMP8 rodents (Yaguchi et al., 2010). However, to date there have been no such studies with older humans.

Interestingly, GPL supplementation may potentially modify a number of factors predicting compromised cerebral structural in older humans. These include elevated homocysteine (HcY) concentrations, unbalanced activity of reactive oxygen species (ROS) activity and increased oxidative stress (OxS), higher levels of pro-inflammatory messengers, as well as poorer cardio- and cerebrovascular functioning. By modifying these factors, GPL supplementation may support cerebral structural integrity, and subsequently cognitive function, in older adults.

In this review, an introduction to GPL—their chemical structure, how they are synthesized *in vivo* and from what foods they are available from—will be presented. Following this, there will be an overview of each of the aforementioned factors, how they relate to cerebral structure (e.g., whole and regional cerebral volume, cortical thinning, severity of white matter lesions, and the integrity of microscopic white matter pathways) as well as cognitive function. This will be complemented with an overview of the available studies investigating the extent to which GPL supplementation modifies, or protects against, these risk factors. Overall, it is anticipated that GPL supplementation, particularly species containing choline and/or ω3-PUFA, may beneficially modify risk factors predicting cerebral structural decline, thereby supporting cerebral structure and subsequently cognitive function in older adults.

Abbreviations: AAMI, Age-associated memory impairment; MCI, Mild cognitive impairment; AD, Alzheimer's dementia; PL, Phospholipid; GPL, Glycerophospholipid; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PS, Phosphatidylserine; CDP-choline, Cytidine disphosphocholine; PEMT, Phosphatidylethanolamine *N*-methyltransferase; PSS1, Phosphatidylserine synthase-1; PSS2, Phosphatidylserine synthase-2; PSD, Phosphatidylserine decarboxylase; PUFA, Polyunsaturated fatty acid; ω3/ω6, Omega 3 or 6; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid; ALA, α-Linolenic acid; AA, Arachidonic acid; HcY, Homocysteine; NFT, Neurofibrillary tangles; OxS, Oxidative stress; ROS, Reactive oxygen species; SOD, Superoxide dismutase; GSH, Glutathione; GPx, Glutathione peroxidase; MDA, Malondialdehyde; NrF2, NF-E2 related factor 2; NADPH, Nicotinamide adenine dinucleotide phosphate; Nox, NADPH oxidase; IL, Interleukin; TNF-α, Tumor necrosis factor alpha; NF-kB, Nuclear factor kappa B; COX, Cyclooxygenase; LOX, Lipoxygenase; BP, Blood pressure; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; AS, Arterial stiffness; BBB, Blood-brain barrier.

PHOSPHOLIPIDS

What Are Phospholipids?

The term "phospholipid" (PL) may be used to describe any lipid (fatty acid) with a phosphoric acid residue (Hanahan, 1997). There are two major classes of PL—glycerophospholipids

TABLE 1 | GPL supplementation and cognitive function in older adults with subjective memory complaints (trials listed in reverse chronological order) and by subject type.

Trial	Design	Subjects	*N*	Age (years)	Treatment	Time	Outcomes
More et al., 2014 (sub-study 1)	R, DB, PC, PG	SMC	72	60–80	SB-PS (300 mg/d) + PA (240 mg/d)	3 mo	No treatment effects on WMS performance when comparing baseline low performers. Significant differences in WMS performance between groups favoring treatment in baseline higher performers
Vakhapova et al., 2014	OLE	SMC	122	72.4 ± 8.3 (naïve); 72.1 ± 7.9 (continuers)	100 mg PS/d (as well as 26 mg DHA + EPA)	15 w	Significantly improved sustained attention and memory recognition performance in the PS-DHA naïve participants (who did not receive PS in the earlier clinical trial)
Richter et al., 2013	OL	SMC	26	50–90 (74.6 ± 1.7)	300 mg/d SB-PS	12 w	Significantly improved memory performance, executive functioning and mental flexibility
Kato-Kataoka et al., 2010	R, DB, PC, PG	SMC	73	T: 59.6 ± 1.0 (high dose) T: 59.1 ± 1.1 (low dose) P: 59.6 ± 1.1	100mg/d SB-PS or 300 mg/d SB-PS	6 mo	Memory performance significantly increased from baseline for all groups. Improved performance on HDS-R test in high dose treatment participants who has poor performance at baseline vs. placebo. Improved delayed verbal memory recall in participant receiving high or low dose treatments vs. placebo
Richter et al., 2010	OL	SMC	8	69.3 ± 3.2	300 mg PS/d (as well as 37.5 mg DHA + EPA)	6 w	Significantly improved delayed verbal recall following treatment
Vakhapova et al., 2010	R, DB, PC, PG	SMC	157	T: 72.9 ± 8.20 P: 73.01 ± 8.28	300 mg/d PS (as well as 79 mg DHA + EPA)	15 w	Significantly improved immediate verbal recall. A trend toward reduced time for completing RCF was observed but failed to reach significance ($p = 0.079$)
Jorissen et al., 2001	R, DB, PC, PG	AAMI	120	T: 65.8 ± 1.1 (high dose) T: 65.3 ± 0.9 (low dose) P: 64.6 ± 0.9	300 mg/d SB-PS; 600 mg/d SB-PS	12 w (+3 w placebo washout)	No treatment effects were observed for any measure of cognitive function after treatment or washout
Schreiber et al., 2000	OL	AAMI	15	65–78 (71.3 ± 3.58)	300 mg PB-PS	12 w	Significantly improved memory performance
Crook et al., 1991	R, DB, PC, PG	AAMI	149	T: 62.61 ± 6.31 P: 64.88 ± 6.81	300 mg/d BC-PS	12 w	Significantly improved performance on Name/Face Acquisition and delayed recall, face recognition, as well as telephone number recall and misplaced objects recall

Design—R, Randomized; DB, Double Blind; PC, Placebo Controlled; PG, Parallel Groups; OL, Open Label; OLE, Open Label Extension; Subjects-SMC, Subjective Memory Complainers; AAMI, Age-Associated Memory Impairment; Age—T, Treatment Group; P, Placebo Group; Treatment—SB, Soybean; PB, Plant Based; BC, Bovine Cortex; PS, Phosphatidylserine; PA, Phosphatidic Acid; DHA, Docosahexaenoic Acid; EPA, Eicosapentaenoic Acid; Time—d, Day; w, Weeks; mo, Months; Outcomes—WMS, Weschler Memory Scale; HDS-R, Hasegawa's Dementia Scare Revised; RCF, Rey Complex Figure.

TABLE 2 | GPL supplementation and cognitive function in older adults with age-related "cognitive deterioration" (trials listed in reverse chronological order).

Trial	Design	Subjects	N	Age (years)	Treatment	Time	Outcomes
Nagata et al., 2011	OL, PG	CD	310	59–95 (76 ± 1.2)	100 mg/d DLPhtCho; 90 mg/d POPhtCho; Combined 50 mg/d DLPhtCho + 45 mg/d POPhtCho	5 mo	MMSE scores were markedly increased following all treatments. MMSE score increase was significantly greater following dual supplementation compared to single supplementation.
Cenacchi et al., 1993	R, DB, PC, PG	CD	494	T: 77.8 ± 5.6 P: 77.3 ± 6.3	300 mg/d BC-PS	6 mo	Significantly improved verbal memory performance following 3 and 6 months treatment compared to placebo.
Allegro et al., 1987	OL	CD	(30)	72.4 ± 4.8	300 mg PS	60 d	Significantly improved verbal and working memory performance over 60 day's treatment. Increased memory function compared to baseline 30 days post-treatment, though most scores reduced to below 60-day treatment scores.
Caffarra and Santamaria, 1987	OL	CD	30	69.2 ± 5.6	300 mg PS	60 d	Significantly improved verbal memory (acquisition and recall), immediate semantic memory performance as well as attention/concentration.
Villardita et al., 1987	R, DB, PC, PG	CD	170	55–80 (65.7 ± 7.5)	300 mg/d BC-PS	90 d	Significantly improved attention/vigilance, verbal and working memory performance, as well as immediate and delayed semantic memory compared to placebo.

Design—R, Randomized; DB, Double Blind; PC, Placebo Controlled; PG, Parallel Groups; OL, Open Label; Subjects—CD, Cognitive deterioration; Age—T, Treatment Group; P, Placebo Group; Treatment—BC, Bovine Cortex; DLPhtCho, 1,2-dilynoleoyl-sn-glycero-3-phosphocholine; POPhtCho, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; PS, Phosphatidylserine; Time—d, Days; mo, Months.

(GPL) and sphingolipids, both being essential components of cellular membranes, including those forming cerebral tissue. This review will focus on GPL, specifically PC, PE, and PS as these are the most common GPL species within mammalian cell membranes (Castro-Gómez et al., 2015), but also the most frequently examined GPL species in relation to neurocognitive health, or risk factors pertaining to neurocognitive health in older adults.

Glycerol forms the backbone of the GPL molecule and it contains three hydroxyl groups (sn-1, sn-2, and sn-3). A phosphate group distinguishing the overall species of GPL is attached to the glycerol backbone at sn-3 (Ridgway, 2016). The attached phosphate may be choline, ethanolamine, or serine, thereby forming PC, PE, or PS, respectively (Ridgway, 2016). Meanwhile, fatty acids are attached to glycerol at sn-1 and sn-2. Quite often sn-1 is occupied by a saturated fatty acid, while sn-2 is occupied by an unsaturated fatty acid, with polyunsaturated fatty acids being especially prevalent in GPL composing cerebral tissue membranes (Castro-Gómez et al., 2015). The combination of different phosphate groups and fatty acids gives rise to over 1,000 different subspecies of GPL (Vance, 2008). The structure of GPL species evident within mammalian cell membranes (PC, PE, PS as well as phosphatidylinositol) is presented in **Figure 1**.

The phosphate group at sn-3 forms the hydrophilic head of the GPL molecule, whereas the fatty acids attached at sn-1 and sn-2 form the hydrophobic tail (Castro-Gómez et al., 2015). The presence of a hydrophilic head and hydrophobic tail results in the formation bi-lipid layers when GPL are suspended together in aqueous solutions (Cooper and Hausman, 2007). This proclivity toward forming bi-lipid layers allows GPL (together with sphingolipids and proteins) to form the cell bi-lipid membrane, though different species of GPL appear to be differentially concentrated within membrane layers. PC is primarily located within the outer membrane layer (sometimes termed the outer leaflet) alongside sphingomyelin. Conversely, PE is mostly concentrated within the inner membrane layer (the inner leaflet), with PS being exclusively located within the inner leaflet (Devaux and Zachowski, 1994; Castro-Gómez et al., 2015).

Biosynthesis of GPL

GPL such as PC, PE, and PS may be synthesized *in vivo* via a number of different pathways. Some of these pathways involve *de novo* synthesis, whereas others involve remodeling of pre-existing GPL.

An essential process prior to the *de novo* synthesis of PC and PE (but also phosphatidylinositol) is the initial formation of phosphatidic acid (Kent, 1995). Once synthesized, phosphatidic acid is converted to either 1,2-diacylglycerol or cytidine diphosphate (CDP) diacylglycerol by two different enzymes—phosphatidic acid phosphatase and CDP-diacylglycerol synthase, respectively (Kent, 1995). 1,2-diacylglcerol is important for synthesis of PC and PE, whereas CDP-diacylglycerol is essential for the formation of phosphatidylinositol in mammalian cells (Kent, 1995). In mammalian cells, PS synthesis is not reliant upon phosphatidic acid synthesis. The major *de novo* and remodeling pathways associated with GPL biosynthesis in mammalian cells are outlined in **Figure 2.**

TABLE 3 | GPL supplementation and cognitive function in older adults with dementia (trials listed in reverse chronological order) and by subject type.

Trial	Design	Subjects	N	Age (years)	Treatment	Time	Outcomes
Zhang et al., 2015	R, PC, PG	AD	57	T: 74.9 ± 18.2 P: 75.3 ± 11.8	300 mg/d PS	20 w	Significantly improved memory performance (vocabulary-picture matching) following treatment
More et al., 2014 (sub-study 2)	DB, R, PC, PG	AD	96	50–90 (75.3)	300 mg/d SB-PS + 240 mg/d PA	2 mo	No further deterioration in ADL score following treatment, though further deterioration evident following placebo. Slight, though non-significant improvements in both groups, though there was a higher proportion of participants in the treatment group progressing from abnormal (≤23) to normal score range (>23) than placebo.
Heiss et al., 1994	R, OL, PG	AD	70	48–79	CT + PS (400 mg/d); CT + P* (1,200 mg/d); CT; SS	6 mo	Significantly improved orientation performance (on MMSE) after 8 and 16 weeks treatment for CT+ PS group relative to SS or CT, but not compared to CT + P*. No between group differences on MMSE at 6 months. Within group analysis indicates that no treatment effects were evident for SS or CT. CT+P* demonstrated increased orientation scores at 8 weeks and verbal fluency at 16 weeks. CT+PS demonstrated significantly higher MMSE at 8 and 16 weeks with a trend toward higher scores at 6 months. A similar trend was evident for orientation scores. Visuospatial performance also improved in the CT+PS group.
Heiss et al., 1993	OL, R, PG	AD	80	Not Specified	CT + PS (400 mg/d); CT + P* (1,200 mg/d); CT; SS	6 mo	Significantly improved MMSE score, as well as block span test (short term memory performance) only evident for CP+PS group
Amaducci, 1988	R, DB, PC, PG	AD	142	T: 62.0 ± 7.4 P: 62.2 ± 6.9	200 mg/d BC-PS	3 mo	Significantly improved BDRS score following 3 months treatment. Significant improved BDRS score, relative to baseline, was evident 3 months post-treatment.
Yaguchi et al., 2009	OL, PG	MCI, D	67	59–93 (77.1 ± 0.8)	300 mg/d POPhtCho	6 mo	Treatment effects on mean MMSE score. Mean MMSE score was increased following treatment, with no change identified in control subjects.
Granata and Di Michele, 1987	OL	D	35	61–80 (70.94 ± 5.43)	300 mg PS	60 d	Significantly improved verbal and working memory performance
Puca et al., 1987	OL	D	27	55–80 (65.5 ± 8.6)	300 mg PS	60 d	Significantly improved verbal memory performance after 60 days. Some improvements in memory performance were maintained after 30 days no treatment.

Design—R, Randomized; DB, Double Blind; PC, Placebo Controlled; PG, Parallel Groups; OL, Open Label; Subjects—MCI, Mild Cognitive Impairment; AD, Alzheimer's Dementia; D, Dementia; Time—d, Days; w, Weeks; mo, Months; Age—T, Treatment Group; P, Placebo Group; Treatment—SB, Soybean; BC, Bovine Cortex; PS, Phosphatidylserine; PA, Phosphatidic Acid; DLPhtCho, 1,2-dilynoleoyl-sn-glycero-3-phosphocholine; POPhtCho, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; CT, Cognitive Training; SS, Social Support; P, Pyritinol; Outcomes—ADL, Activities of Daily Living; MMSE, Mini-Mental State Examination; BDRS, Blessed Dementia Rating Scale.*

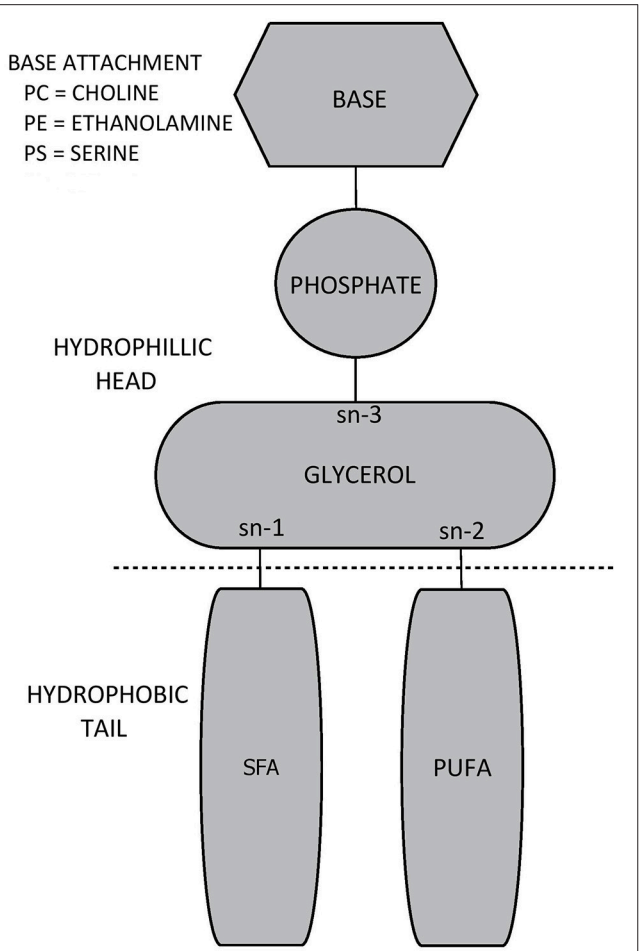

FIGURE 1 | General biochemical structure of a glycerophospholipid.

As shown in **Figure 2**, PC is produced *in vivo* via two major pathways—the cytidine disphosphocholine (CDP-choline) pathway, and the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway. The CDP-choline pathway involves *de novo* synthesis of PC whereas the PEMT pathway involves remodeling of pre-existing PE. PE is synthesized analogously to PC via the CDP-ethanolamine pathway, but also by remodeling pre-existing PS via the PS decarboxylase (PSD) pathway. In mammalian cells, PS is primarily synthesized through base exchange reactions mediated by specialized synthases. PS synthase-1 (PSS1) causes free serine to be switched for choline from PC, while PSS2 switches ethanolamine in PE with free serine, thereby formulating PS. Detailed decriptions of the specific biochemical processes involved in each of these pathways have been provided elsewhere (Kent, 1995; Vance, 2008; Vance and Tasseva, 2013).

While different pathways may produce the same general species of GPL (e.g., CDP-choline and PEMT both produce PC), the pathway from which the GPL was synthesized appears to influence the fatty acid composition of the resultant GPL. For example, PC resulting from the CDP-choline pathway typically contains saturated, monounsaturated or diunsaturated fatty acid species, whereas PC synthesized via the PEMT pathway may also contain polyunsaturated fatty acids (PUFA), such as the ω3-PUFA species DHA (Delong et al., 1999; Pynn et al., 2011). Although this may be due to a substrate preference of the PEMT pathway for PE containing ω3-PUFA (Delong et al., 1999), others (e.g., Vance, 2014) suggest that the extent to which DHA enriched PC is produced is more likely dependent upon the availability of DHA within the overall reservoir of PE (Ridgway and Vance, 1988).

Differences in fatty acid composition depending on synthesis pathways are also apparent for PE. PE produced via the CDP-ethanolamine pathway predominately contains mono- or di-unsaturated fatty acid species at sn-2. Conversely, PE derived from the PSD pathway, predominately synthesizes PE containing PUFA such as the ω6-PUFA arachidonic acid (ARA) or ω3-PUFA's such as DHA or eicosapentaenoic acid (EPA) (Bleijerveld et al., 2007). Substrate specificity is apparent during PS synthesis in mammalian cells. Data from several studies indicate that PSS1 and PSS2 preference species of PC or PE containing ω3-PUFA, particularly DHA, compared to other subspecies (Kim et al., 2004; Kimura and Kim, 2013).

Dietary Sources of GPL Containing ω3-PUFA

In addition to *in vivo* synthesis, GPL may be obtained from the diet. The estimated dietary intake of GPL is two to eight grams per day, representing one to ten percent of daily fat intake (Cohn et al., 2010). GPL are present in foods such as milk, eggs, skeletal, and organ (e.g., brain) meat from terrestrial animals (e.g., cow or pig), as well as fish (especially krill or squid). GPL may also be obtained by consuming certain seeds and legumes such as rapeseeds, sunflower seeds and soybeans (Weihrauch and Son, 1983).

The types of fatty acids contained within GPL appear to vary between food sources. The GPL derived from foods such as marine fish (e.g., krill or squid), mammalian brain (e.g., cow or pig) or eggs (particularly those from hens whose feed is fortified with ω3-PUFA) appear to contain ω3-PUFA such as DHA (Bourre et al., 1993; Bourre and Dumont, 2002; Favrelière et al., 2003; Chen and Li, 2008). In soybeans, ω3-PUFA is present in the form of α-linolenic acid (ALA) (Bourre and Dumont, 2002; Chen and Li, 2008), which may be later converted to longer chain ω3-PUFA species such as EPA and DHA (Burdge and Calder, 2005). Soybeans, but also fish, mammalian brain and eggs also provide varying levels of ω6-PUFA and other fatty acids (Bourre and Dumont, 2002; Favrelière et al., 2003; Chen and Li, 2008).

Aside from whole foods, GPL may be obtained from commercially available supplements. Importantly, the fatty acid composition of these supplemental GPL will vary depending on the food from which the GPL was extracted. GPL supplements may be consumed as capsules (e.g., krill oil), as well as powdered compounds (summarized in Küllenberg et al., 2012). Supplements in powder form are liquefiable thereby making them easier to consume. This may be particularly important for older adults especially those who experience difficulty

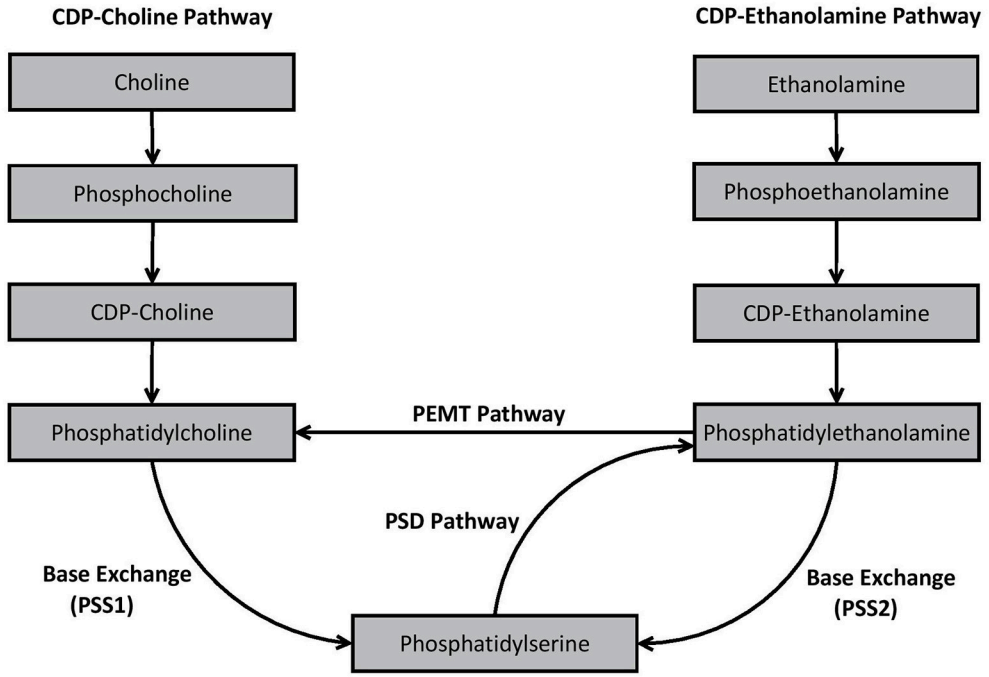

FIGURE 2 | Biosynthesis pathways of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine in mammalian cells.

swallowing (a common occurrence in the general population termed dysphagia; Aslam and Vaezi, 2013). These adults may be at an increased risk of developing nutritional insufficiency or deficiency (Sura et al., 2012).

Prior work has indicated that the bioavailability of ω3-PUFA may be increased following ingestion of whole foods or supplements containing this species of fatty acids (Popp-Snijders et al., 1986; Bourre et al., 1993; Yaqoob et al., 2000; Bourre and Dumont, 2002; Kew et al., 2004; Ulven et al., 2011; Browning et al., 2012). Other work demonstrates that the bioavailability of PUFA may be enhanced when they are consumed as GPL rather than triglycerides (Wijendran et al., 2002; Ramprasath et al., 2013). As such, GPL supplementation may be an effective method of increasing the bioavailability of nutrients such as ω3-PUFA. Given that other nutrients such as choline may also be attached to GPL, it is likely that GPL supplementation will also boost the bioavailability of these nutrients.

GPL SUPPLEMENTATION AS A MEANS OF SUPPORTING CEREBRAL STRUCTURE IN OLDER ADULTS

GPL Supplementation and Factors Predicting Cerebral Structural Integrity

The concentrations of PL within cerebral tissue appears to decline with increasing age (Soderberg et al., 1990, 1991; Svennerholm et al., 1994, 1997). As such, increasing the dietary intake of PL, especially GPL, may benefit cerebral structure integrity due their incorporation into neuronal membranes. However, supplementation with GPL may also indirectly support cerebral structure by modifying, or protecting against, a number of factors that appear to predict poorer cerebral structure in older adults. It is these potential indirect effects that form the focus of this review. Specifically, this review will discuss biochemical (HcY, ROS activity and OxS, and inflammation) and biophysical (cardio- and cerebrovascular function) risk factors associated with poorer cerebral structural integrity, but also cognitive decline, and the extent to which GPL supplementation has been observed to modify, or protect against, these factors. There will also be a discussion of the potential pathways through which GPL supplementation may facilitate these effects. Overall, it is anticipated that, GPL supplementation will benefit cerebral structural integrity and subsequently cognitive function in older adults, via modification of, or protection against, the aforementioned factors.

Homocysteine

Homocystiene and Cerebral Structure in Older Adults

HcY is a sulfur containing amino acid derived from s-adenosylhomocysteine, a by-product of methyl reactions involving *s*-adenosylmethionine (Selhub et al., 2000). A number of studies with older adults have determined that elevated HcY is a significant predictor of reduced total and regional cerebral tissue volumes (Williams et al., 2002; den Heijer et al., 2003b; Firbank et al., 2010; Narayan et al., 2011a; Rajagopalan et al., 2011; Tangney et al., 2011; Feng et al., 2013; Madsen et al., 2015), increased frequency and volume of white matter lesions or silent brain infarcts (Vermeer et al., 2002; Wright et al., 2005; Seshadri et al., 2008; Raz et al., 2012), as well as reduced

cerebral microstructural integrity (Bettcher et al., 2014; Hsu et al., 2015). Given the link between measures of cerebral macro- and microstructural integrity and cognitive functioning, it follows that HcY may also be an important predictor of cognitive function. In fact multiple studies have observed an increased risk of developing MCI and dementia in adults with elevated HcY (Launtenschlager et al., 2005; Kim et al., 2007; Whalley et al., 2014), but also poorer performance across a number of cognitive domains (Narayan et al., 2011b; Allam et al., 2013; Jochemsen et al., 2013; Parizkova et al., 2017).

While HcY concentrations may be influenced by non-modifiable factors such as genetics and age, there are also a number of modifiable factors believed to impact HcY concentrations. One of the most important modifiable factors is nutritional intake, in particular B vitamin ingestion/absorption (Joosten et al., 1996; Jacques et al., 2001; Refsum et al., 2006). Vitamins B_6, B_{12}, and folate are essential for HcY metabolism, with lower intake or bioavailability of these vitamins predicting elevated HcY concentrations (Jacques et al., 2001; Refsum et al., 2006). Supplementation with these B vitamins has been shown to lower HcY concentrations (Eussen et al., 2006; McMahon et al., 2006; Smith et al., 2010; Clarke et al., 2014; Ford and Almeida, 2014). Moreover, data from the VITACOG study indicates that chronic supplementation with vitamins B_6, B_{12} and folate is beneficial to cerebral structure, with reduced rates of total and regional cerebral atrophy due to significant reductions in HcY concentrations (Smith et al., 2010; Douaud et al., 2013; Jerneren et al., 2015). Lowering HcY through supplementation may also improve cognitive performance with several studies with older adults having observed improved memory performance and processing speed following HcY subsequent to B vitamin supplementation (Durga et al., 2007; de Jager et al., 2012). However, several systematic reviews and meta-analyses have indicated that lowering HcY through B vitamin supplementation does not improve cognitive function in older adults (Clarke et al., 2014; Ford and Almeida, 2014), though methodological weaknesses in these reviews has led to their results being criticized in more recent work (Smith and Refsum, 2016).

Overall, it would appear that lowering HcY through nutritional supplementation may benefit cerebral structural integrity in older adults. It may also be beneficial to cognitive function, though these latter effects remain controversial within the literature.

GPL Supplementation and HcY Concentrations

Choline is similar to B vitamins, in that its level of dietary intake appears to predict HcY concentrations in adults. A number of animal and plant based foods such as red meats, poultry, fish, eggs, and milk, but also broccoli, potatoes, green beans, legumes, and nuts, contain choline (Cho et al., 2006; Detopoulou et al., 2008). Following ingestion and absorption, choline may be oxidized into betaine, which functions as a methyl donor in the betaine-homocysteine-methyltransferase pathway, whereby HcY is converted to methionine in a reaction catalyzed by the enzyme betaine-homocysteine methyltransferase (Olthof et al., 2005). Importantly, increased ingestion or bioavailability of choline (or its metabolite betaine) has been observed to negatively predict HcY concentrations in adults (Schwab et al., 2002; Steenge et al., 2003; Cho et al., 2006; Chiuve et al., 2007; Atkinson et al., 2008; Detopoulou et al., 2008).

Choline is highlighted here as it forms the base attachment distinguishing PC (but also the sphingolipid known as sphingomyelin). In fact, choline represents 15% of PC molecular weight (Cheatham et al., 2012) thereby making PC an important carrier of choline that may be sourced from the diet. As such, greater ingestion of PC may be expected to negatively predict HcY concentrations. In several cross sectional studies utilizing FFQ data from the Framingham Heart Study (Cho et al., 2006) as well as the Nurses Health Studies (Chiuve et al., 2007), a number of dietary compounds containing choline were examined in relation to HcY. Cho et al. (2006), determined that estimated PC ingestion was inversely associated with HcY concentrations with similar associations being identified for other choline containing compounds including sphingomyelin, phosphocholine, and glycerophosphocholine. However, after controlling for additional factors related to HcY concentrations (e.g., B vitamin intake, caffeine, and alcohol consumption) PC was no longer associated with HcY concentrations, though other choline containing compounds remained as significant predictors. Interestingly, Chiuve et al. (2007) observed a negative association between choline from phosphocholine and glycerophosphocholine with HcY while there appeared to be a non-significant positive association with PC intake.

In perhaps the only study examining the direct effect of PC supplementation on HcY concentrations, Olthof et al. (2005) administered a daily oral dose of 34 g soybean lecithin (comprised of PC) delivering 2.6 g choline, to adult males aged 50–71 years over 2 weeks. Olthof et al. (2005) observed that HcY concentrations were 18% lower following treatment than placebo. However, it is important to note that the level of choline ingested in this study is well beyond the estimated mean daily intakes reported by both Cho et al. (2006) and Chiuve et al. (2007) (313 $\pm$ 61 and 323 mg/d, respectively). As such, it is possible that PC, due the provision of choline, will lower HcY at doses attainable through a combination of normal diet and supplementation than from diet alone.

Lowering HcY has been observed to benefit cerebral structural integrity in a number of clinical trials involving chronic B vitamin supplementation. As such, it is plausible that similar effects may be evident following chronic supplementation with PC due to the provision of choline. However, to date there has been minimal investigation of the use of PC as a means of lowering HcY. This is intriguing as choline, similar to select B vitamins, is an essential nutrient for the methylation of HcY. The relative lack of data determining the relationship between PC intake and HcY concentrations should be rectified by additional well-designed RCT's. The added utilization of advanced imaging methods in future studies will help determine how PC intake and subsequent HcY levels relate to cerebral structural integrity.

Lowering HcY and Potential Benefits to Cerebral Structure

Despite there being a paucity of work determining how PC supplementation benefits cerebral structure in aging humans, it is

plausible that PC supplementation may benefit cerebral structure by lowering HcY and alleviating the detrimental effects theorized to be associated with elevated concentrations of HcY. One effect of elevated HcY is an increased risk of hyperphosphorylated tau buildup as well as an increased presence of neurofibrillary tangles (NFT) within cerebral tissues (Luo et al., 2007; Zhang et al., 2008; Popp et al., 2009; Wei et al., 2011; Hooshmand et al., 2013; Li et al., 2014). Both of these factors have been linked to poorer cerebral macro- and microstructural integrity in adults across the spectrum of cognitive function (Whitwell et al., 2008; Polvikoski et al., 2010; Tosun et al., 2010; Glodzik et al., 2011, 2012; Marnane et al., 2016; de Souza et al., 2017; Hoy et al., 2017; Kantarci et al., 2017).

Increased HcY concentrations appears to exacerbate the production of hyperphosphorylated tau through inhibition of kinases, such as protein phosphatase 2A (PP2A), which are responsible for tau dephosphorylation (Luo et al., 2007; Zhang et al., 2008; Wei et al., 2011). However, lowering HcY concentration (through B vitamin supplementation) has been observed to alleviate PP2A inactivation thereby lowering tau hyperphosphorylation (Zhang et al., 2008; Wei et al., 2011). Further, while there have been no trials examining HcY lowering and changes to NFT load, given that NFT are largely composed of hyperphosphorylated tau proteins, lowering HcY should theoretically influence the extent to which NFT are formed and therefore evident within cerebral tissues. Although data is limited, PC supplementation, due to the provision of choline, has been observed to lower HcY concentrations. As such, it is possible that by lowering HcY, PC supplementation (administered in moderate to high doses) may facilitate reduced concentrations of hyperphosphorylated tau and subsequently NFT, thereby supporting cerebral structural integrity in the long term. These effects remain to be investigated in older adults.

In addition to increased hyperphosphorylated tau and NFT load, elevated HcY may facilitate more subtle effects potentially leading to reduced cerebral structural integrity. Elevated HcY concentrations inhibit cellular PEMT activity leading to reduced production of PC containing ω3-PUFA thereby facilitating distorted concentration ratios of PC and its precursor PE within cellular membranes (Innis et al., 2003; Miller et al., 2003; Devlin et al., 2007; Selley, 2007).

Lowered production of PC containing DHA, due to PEMT inhibition, may also partly explain the reduced concentrations of DHA observed within biological tissues when HcY levels are elevated (Innis et al., 2003; Miller et al., 2003; Li et al., 2006; Devlin et al., 2007; Selley, 2007; Rasmussen et al., 2010; Huang et al., 2013; Kume et al., 2013; Iglesia et al., 2017). The production of PC containing ω3-PUFA such as DHA by the PEMT pathway is a key process for transporting DHA from the liver into plasma and onwards to cerebral tissue (Smith and Refsum, 2016). Ultimately, lower concentrations of ω3-PUFA such as DHA within membranes (including those within cerebral tissue) may then facilitate an increased risk of OxS and elevated inflammation, subsequently leading to poorer cardio- and cerebrovascular function (elevated blood pressure, arterial stiffness, and permeability of the blood-brain barrier). These factors will be discussed in later sections of this review.

PC is a major dietary source of choline. As such increased ingestion of PC via supplementation, likely facilitates increased choline bioavailability thereby lowering HcY concentrations and subsequently alleviating the inhibitory effects of HcY upon PEMT activity. This would influence the extent to which ω3-PUFA are present in cellular membranes (including those in cerebral tissue) due to restored *in vivo* synthesis of PC comprised of ω3-PUFA such as DHA. However, depending on the source from which it as derived, the administered PC may also contain ω3-PUFA. Supplementing with PC containing ω3-PUFA such as DHA would likely facilitate increased concentrations of these PUFA within cell membranes. This would impact the extent to which factors such as elevated OxS and inflammation are apparent, thereby decreasing the likelihood of disturbed cardio- and cerebrovascular function, and subsequently the extent to which cerebral structure deteriorates over time.

Although a great deal more work is required examining how PC ingestion relates to HcY concentrations, the limited data available indicates that PC supplementation, in high enough doses, may lower HcY concentrations. By lowering HcY, PC supplementation may benefit cerebral structural integrity and potentially cognitive function in a similar way to B vitamins. *It is hypothesized that chronic supplementation of GPL containing choline (i.e., PC) will significantly reduce HcY concentrations in older adults. Based upon earlier work whereby lowering HcY through nutritional supplementation benefited cerebral structural integrity, it is anticipated that HcY lowering via PC supplementation would likewise support cerebral structural integrity and subsequently cognitive functioning in older adults.*

Oxidative Stress

Risk of Oxidative Stress and Cerebral Structure in Older Adults

A ready supply of oxygen is essential for proper cellular functioning, although a consequence of oxygen metabolism is the formation of ROS (Chiurchiù et al., 2016). ROS are essential for many vital physiological functions including the destruction of pathogens during the inflammatory immune response and the regulation of smooth muscle tone (Popa-Wagner et al., 2013). Under normal conditions, ROS activity is balanced with that of endogenous antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione (GSH), but also dietary antioxidants (Chiurchiù et al., 2016). However, unbalanced ROS activity may result in oxidative damage to lipids and proteins comprising cellular membranes thereby impacting cellular membrane structure and functioning (Popa-Wagner et al., 2013; Von Bernhardi et al., 2015).

Damage to biological tissues due to unbalanced ROS activity is termed oxidative stress (OxS). Cerebral tissue may be particularly susceptible to OxS, due to it requiring a considerable amount of oxygen so as to sustain disproportionate metabolic activity. Other factors facilitating an increased risk of OxS within cerebral tissues include a marked production of ROS through neurochemical reactions as well as deposits of iron that may promote oxidation (Popa-Wagner et al., 2013).

An additional factor contributing to cerebral tissues susceptibility to OxS, is that the PUFA within cellular membranes are highly susceptible to oxidative damage by ROS (Chan et al., 1982; Brett and Rumsby, 1994; Bongarzone et al., 1995), with increased ROS activity (without a corresponding increase in antioxidant activity) having been observed to facilitate destruction of myelin lipids and proteins (Chia et al., 1983a,b; Konat and Wiggins, 1985; Bongarzone et al., 1995). However, there appears to be an absence of data linking ROS activity or measures reflecting OxS with cerebral structural integrity, as determined using magnetic resonance imaging (MRI), in older humans. It is highly likely that oxidation of lipids and proteins within cerebral tissue membranes, at least partly contributes to those reductions in cerebral microstructural integrity identified in cognitively normal (Davis et al., 2009; Burzynska et al., 2010; Westlye et al., 2010) but also cognitively impaired older adults (i.e., MCI or AD) (Bosch et al., 2012; Wang et al., 2012; Zhang et al., 2013). Though they are not perfect measures, the diffusion tensor imaging (DTI) metrics "radial diffusivity" and "axonal diffusivity" have been suggested to reflect myelin and axonal integrity, respectively, within cerebral white matter (Song et al., 2002, 2003; Sun et al., 2006). Future work utilizing these measures may provide additional insight into how ROS activity, and subsequently OxS, contributes to declining cerebral structural integrity with age.

Due to a heightened susceptibility of cerebral tissues to OxS, it follows that measures indicative of OxS, or an elevated susceptibility to OxS due to unbalanced ROS activity (e.g., poorer concentrations of endogenous or dietary antioxidants), may be predictive of poorer cognitive function. In fact, compared to healthy adults, those with MCI or AD appear to demonstrate reduced concentrations of endogenous and diet derived antioxidants (Rinaldi et al., 2003; Padurariu et al., 2010; Mandal et al., 2015). In addition, adults with MCI or AD also demonstrate elevated OxS than cognitively normal adults, evidenced by increased concentrations of malondialdehyde (MDA), F_2 and F_4 isoprostanes (measures of ω6 and ω3-PUFA oxidation, respectively), and protein carbonyls (Mangialasche et al., 2009; Padurariu et al., 2010; Torres et al., 2011).

Conversely, greater intake of dietary antioxidants (e.g., carotenoids) are associated with greater cognitive performance in older adults (Jama et al., 1996; Berr et al., 1998; Akbaraly et al., 2007; Wengreen et al., 2007), though conflicting data is available (Crichton et al., 2013). Moreover, higher concentrations of endogenous antioxidants, such as GPx and GSH appear to predict better performance on the mini-mental state examination as well as the clinical dementia rating scale but also improved executive functioning in older adults with MCI, AD or "cognitive dysfunction" (Umur et al., 2011; Mandal et al., 2015; Revel et al., 2015).

GPL Supplementation and Oxidative Stress

Preclinical data appears to demonstrate that pre-treatment with GPL such as PS, may protect cells against the deleterious effects of elevated ROS, thereby reducing the risk of OxS. In one early study, Latorraca et al. (1993) suspended cultured human fibroblast cells in a solution containing acetaldehyde (37.5 mM) and 50 mU of xanthine-oxidase, which triggered a reaction resulting in high ROS production. Cell damage facilitated by increased ROS activity was quantified by measuring lactate dehydrogenase, with levels increasing by 40% when cells were exposed to both acetaldehyde and xanthine oxidase, compared to control (vehicle solution + acetaldehyde, but no xanthine oxidase). However, in cells pre-cultured with 13 μM PS for 4 days prior to suspension in the experimental solution, there was no significant increase in lactate dehydrogenase, suggesting that exposure to PS prevented tissue oxidative damage otherwise expected following increased ROS production.

Pre-culturing cells with GPL may facilitate decreased production of ROS, thereby lowering the risk of OxS occurring. Hashioka et al. (2007a) administered 400 ng/ml lipopolysaccharide (LPS) combined with 400 ng/ml of phorbol 12-myristate-12-acetate, to microglial cells sourced from rodents. This combination resulted in the production of the ROS and reactive nitrogen species—superoxide and nitric oxide, respectively, which together may form a powerful oxidant called peroxynitrite. However, when microglial cells were pre-cultured for 1 h with liposomes comprised of PS and PC or PC alone, both superoxide and nitric oxide production was markedly reduced. This would suggest that by lowering the production of superoxide and nitric oxide, treatment with liposomes comprised of PC and PS may facilitate reduced peroxynitrite production, lowering the likelihood of OxS. Similarly, Chaung et al. (2013) pre-cultured C6 cells (rodent glial cells modeling glioma) with 25 μM of DHA or 25 μM of PS, alone or in combination, for 24 h prior to these cells receiving electrical stimulation in order to stimulate ROS production. Across treatments, electrical stimulation of these glial cells successfully elevated ROS production, though for cells pre-cultured with either DHA or PS, ROS production was significantly lower. Moreover, the combination of DHA and PS appeared to facilitate even lower ROS production than when either treatment in isolation.

Prior work with animals indicates that supplementing with GPL containing ω3-PUFA may prevent OxS. Hiratsuka et al. (2008) fed rodents one of three experimental diets fortified with different lipid species. In the control diet lipids were issued as safflower oil (control), while experimental diets were fortified with the ω3-PUFA DHA delivered as either triglycerides or as GPL (PC, PE and SM) sourced from skipjack tuna ovaries. Rodents were fed their respective diets for a period of 5 weeks, though after the first 2 weeks' rodents received five daily injections of strepozotocin (40 mg/kg), so as to model diabetes, which subsequently caused elevated lipid peroxidation within cerebral tissue. Upon completion of the dietary intervention, no significant difference in cerebral lipid oxidation was identified between rodents within the control group or those receiving DHA as triglycerides. However, cerebral lipid oxidation was significantly lower in rodents fed DHA delivered as GPL, suggesting that supplementation with GPL containing DHA may lower the risk of OxS within cerebral tissue.

In addition to the above studies, others have determined that GPL supplementation may boost the concentrations of endogenous antioxidants. Liu et al. (2012) repeatedly administered pentylenetetrazol to induce epileptic-like seizures

in rodents. This treatment facilitated a significant increase in the concentrations of reactive nitrogen species such as nitric oxide, while also reducing concentrations of endogenous antioxidants such as SOD within cerebral and liver tissues. Following seizure inducement, Liu et al. (2012) provided rodents with experimental diets fortified with 2.5 mg/kg DHA and/or 300 mg/kg PS for a period of 36 days. Dietary fortification with either DHA or PS was observed to increase SOD concentrations within cerebral tissue, whilst the combination of these nutrients boosted SOD concentrations within the liver. Nitric oxide was reduced in cerebral tissue following consumption of a diet fortified with DHA or PS, though liver concentrations were reduced following separate or combined treatment paradigms. Likewise, Zhang et al. (2015) observed that intracranial injection of 5.0 μL of amyloid-β_{1-42} facilitated a significant decline in SOD concentrations, while consumption of a diet enriched with PS sourced from bovine cortex (thereby containing DHA among other fatty acids) increased cerebral SOD expression in a dose dependent manner. More recently, Qu et al. (2016) modeled AD in rodents via intracerebral injection of amyloid-β_{25-35}, subsequently reducing the concentrations of endogenous antioxidants such as SOD and GPx within the cortex and hippocampus. Compared to the AD control model, rodents modeling AD who received a medium (0.1 g/kg/d) or high (0.2 g/kg/d) dose of marine sourced PC (containing high levels of DHA and EPA) for 30 days demonstrated increased GPx within cortical tissue. Rodents that received a low (0.05 g/kg/d), medium or high dose of PC demonstrated elevated SOD activity within cortical tissue, although increased SOD activity within the hippocampus was only apparent following supplementation with the highest dose of PC. No treatment effects were observed within the hippocampus for GPx.

GPL Supplementation and OxS—Potential Pathways of Effect

There is data from a range of preclinical and clinical trials indicating that supplementation with GPL may reduce the risk of OxS within biological tissues, including cerebral tissue. GPL from marine sources (e.g., krill or squid), mammalian (e.g., cow or pig) brain as well as soybeans may reduce the risk of OxS through the provision of fatty acids, especially ω3-PUFA such as DHA or EPA. Although maintaining redox balance is complex and facilitated via a wide range of biological processes, discussion in this review will be limited to two potential pathways through which supplementation with GPL, due to the provision of ω3-PUFA, may modulate the activity or concentrations of ROS and endogenous antioxidants thereby lowering the risk of OxS.

The first pathway through which GPL containing ω3-PUFA may reduce the risk of OxS involves upregulation of transcription factors associated with the synthesis of endogenous antioxidants. Prior work indicates that when cells are incubated with ω3-PUFA (DHA, EPA, or ALA) there appears to be an increase in the concentrations of endogenous antioxidants such as SOD, GSH, and GPx (Di Nunzio et al., 2011, 2016; Saw et al., 2013). Increased concentrations of these endogenous antioxidants has been linked to elevated activity of NF-E2-related factor 2 (NrF2)—a factor involved in the transcription of genes coding for those antioxidants (Di Nunzio et al., 2011, 2016; Saw et al., 2013). These effects may occur in response to oxidation of ω3-PUFA such as DHA and EPA, with the byproducts resulting from oxidation of these lipids (e.g., 4-hydroxy-2E-hexenal) subsequently inhibiting factors known to decrease NrF2 nuclear translocation, such as Keap1 (Gao et al., 2007). These putative effects were examined recently by Zhang et al. (2014), who identified that the administration of fish oil (rodent models) or pretreatment with the ω3-PUFA's DHA and EPA (rodent embryonic neuronal cells *in vitro*) significantly reduced the destruction of neuronal cells following oxygen-glucose deprivation. Zhang et al. (2014) also observed that cells pretreated with DHA, exhibited greater nuclear translocation of NrF2 following oxygen glucose deprivation. Moreover, they observed that 4-hydroxy-2E-hexenal, an end product of ω3-PUFA oxidation, was a far more potent inducer of NrF2 activity than 4-hydroxy-2E-nonenal—an end product of ω6-PUFA oxidation.

As indicated earlier, supplementation with ω3-PUFA is efficacious for elevating the bioavailability of these PUFA within biological tissues (Popp-Snijders et al., 1986; Bourre et al., 1993; Yaqoob et al., 2000; Bourre and Dumont, 2002; Favrelière et al., 2003; Kew et al., 2003, 2004; Browning et al., 2012). However, delivery of ω3-PUFA as PL may be a more effective for elevating tissue concentrations of these lipids than triglycerides (Ramprasath et al., 2013). Typically, increased concentrations of ω3-PUFA within membranes occurs at the expense of ω6-PUFA such as ARA (Calder, 2015). As such, increasing the bioavailability of ω3-PUFA within biological tissues, may increase the likelihood of these species of PUFA being oxidized thereby increasing the production of 4-hydroxy-2E-hexenal, rather than 4-hydroxy-2E-nonenal (from ω6-PUFA) leading to increased NrF2 activity and production of endogenous antioxidants. This process may account for the increased concentrations of endogenous antioxidants and therefore reduced risk of OxS, following GPL administration in earlier studies.

A second pathway through which GPL containing ω3-PUFA may reduce the risk of OxS is by downregulating the activity of select enzymes that produce ROS. One particular class of enzymes associated with ROS production are the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, abbreviated as Nox (Lambeth, 2004; Bedard and Krause, 2007). Nox are membrane bound proteins which facilitate electron transfer from NADPH onto molecular O_2, thereby generating ROS, particularly superoxide. Dysregulated Nox activity may contribute to endothelial dysfunction, distorted smooth muscle growth as well as inflammation (Giordano and Visioli, 2014), and has been linked to an increased risk of MCI and AD, while also predicting Braak stage—a measure of AD pathology (Bruce-Keller et al., 2010; Ansari and Scheff, 2011). There are a number of different Nox enzymes (e.g., Nox1, Nox 2, Nox 3, Nox 4, Nox 5) which may be expressed in low to high concentrations within a wide variety of biological tissues (Lambeth, 2004; Bedard and Krause, 2007). Importantly, the activity of Nox proteins is

regulated by a number of different proteins including $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the GTPase Rac (Bedard and Krause, 2007).

Supplementation with GPL may downregulate Nox activity, subsequently lowering the risk of OxS, assuming the GPL contain ω3-PUFA. Richard et al. (2009) pre-cultured human aortic endothelial cells with DHA for 48 h, observing that DHA treatment facilitated a reduction in Nox4 expression and subsequently ROS production, which was otherwise upregulated due to exposure to angiotensin II and IL-1β. Additionally, the topical application of DHA to hairless mouse skin significantly attenuated the expression of Nox4 following exposure to ultraviolet radiation in a dose dependent manner compared to control (Rahman et al., 2011). In a more recent study by Depner et al. (2013), rodents were fed chow with a nutritional composition resembling that of the western diet regularly consumed by humans. Some of the rodents also received supplementation with ω3-PUFA's such as EPA and/or DHA, whereas controls only received olive oil. Although Depner et al. (2013) did not observe increased NrF2 expression following ingestion of any diet with added supplementation of ω3-PUFA, they did observe marked reduction in Nox regulating proteins including $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, as well as RAC1, a member of the RAC subgroup of GTPase. Based upon the findings of these earlier studies, it is possible that supplementation of GPL (containing ω3-PUFA) may reduce the risk of OxS by down regulating the activity of enzymes known to produce ROS, as well as factors that stimulate the activity of those enzymes.

Overall, it would appear that supplementation with GPL, particularly those also containing ω3-PUFA, may modify the activity of several different protein messengers leading to reduced production of ROS, but increased synthesis of endogenous antioxidants, thereby lowering the risk of OxS. *As such, it is anticipated that chronic supplementation with GPL (containing ω3-PUFA) is expected to facilitate a greater balance between the concentrations of ROS and endogenous antioxidants in older adults. This will likely manifest as lower concentrations of markers indicative of OxS. It is anticipated that in an aging sample, lower expression of OxS related markers or elevated expression of anti-oxidants (endogenous and dietary) will be associated with greater cerebral structural integrity, which in turn would be predictive of greater cognitive functioning.*

Inflammation

Inflammation and Cerebral Structure in Older Adults

Inflammation is an integral component of the immune response following injury to body tissues or infection. An important regulating influence of the inflammatory response is the production and release of cellular messengers specialized for either promoting or alleviating inflammation. Common pro-inflammatory messengers include interleukin (IL)-1β, IL-6, IL-8, IL-12, tumor necrosis factor alpha (TNF-α), and C-reactive protein (CRP), while examples of anti-inflammatory messengers include IL-4 and IL-10 (Baune et al., 2008; Marioni et al., 2010).

With increasing age, there appears to be a shift toward an immunologically primed state. This has been identified in cerebral tissue, such as the hippocampus, where the inflammatory response is typically of a greater magnitude and duration in older adults (for a review see Barrientos et al., 2015). This is an important observation in the context of neurocognitive health as data from multiple studies indicate that elevated concentrations of pro-inflammatory messengers, such as those listed earlier, are predictive of poorer cerebral structural integrity. These detrimental effects are evident as lower regional cerebral tissue volumes, increased volume of white matter lesions, as well as poorer cerebral microstructure (Baune et al., 2009; Wersching et al., 2010; Nagai et al., 2011; Bettcher et al., 2012, 2014, 2015; Miralbell et al., 2012; Satizabal et al., 2012; Arfanakis et al., 2013; Taki et al., 2013b; Sudheimer et al., 2014; Jiang et al., 2015).

Likewise, there appears to be a link between higher concentrations of pro-inflammatory messengers and poorer cognitive performance or increased rate of cognitive decline with age (Yaffe et al., 2003; Dimopoulos et al., 2006; Wright et al., 2006; Marioni et al., 2010; Economos et al., 2013; Mooijaart et al., 2013; Adriaensen et al., 2014; Tegeler et al., 2016). Greater concentration of pro-inflammatory messengers are evident in adults diagnosed with MCI or dementia compared to their cognitively normal counterparts (Dimopoulos et al., 2006; Magaki et al., 2007; Bermejo et al., 2008; Troller et al., 2010), with others having identified an increased risk of dementia in conjunction with elevated inflammation (Schmidt et al., 2002; Engelhart et al., 2004).

Interestingly, some studies appear to demonstrate a significant reduction in the strength of the relationship between inflammation and cognition after controlling for cerebral structural integrity (Arfanakis et al., 2013; Marsland et al., 2015). These results suggest that inflammation may detrimentally influence cognitive function, in part by promoting the deterioration of cerebral tissues. As such, the identification of interventions efficacious for modulating inflammatory messengers may be paramount for supporting cerebral structure, and subsequently cognitive function, in older adults.

GPL Supplementation and Inflammation

Data from earlier pre-clinical studies demonstrate that GPL supplementation may modify the concentrations of pro-inflammatory messengers. In several studies, Treede et al. (2007, 2009) observed that administering TNF-α to caco-2 cells (human intestinal epithelial cells) triggered the activation of nuclear factor kappa-B (NF-KB), an important factor associated with the transcription of genes coding for pro-inflammatory messengers and the subsequent upregulation of these signaling messengers. However, simultaneous treatment with PC prevented NF-KB activation and subsequently lower concentrations of pro-inflammatory messengers (such as additional TNF-α) relative to control cultures. Similarly, cultures of human microglial cells exposed to with liposomes comprising PS and PC (sourced from pig brain or egg yolks, respectively), prior to the application of amyloid-β and interferon-γ, demonstrated lower concentrations of TNF-α (Hashioka et al., 2007b).

Building on the pre-clinical literature, Hartmann et al. (2009) use a rodent model of arthritis (involving injection of a solution containing carrageenan and kaolin into the knee joint). The

injection of this solution triggered an inflammatory response consistent with arthritis—specifically increased heat, swelling and pressure sensitivity, though only within the joints that received the injection. Importantly, these effects were abated via oral administration of a non-steroidal anti-inflammatory drug or PC (dose of 150 mg/Kg)—though only the latter decreased the number of adherent leukocytes in the affected joints. In another study, increased concentrations of TNF-α and IL-6 were elicited in rodents following injection of LPS. However, rodents fed an experimental diet fortified with PC (1%) demonstrated significantly lower concentrations of TNF-α following LPS injection when compared to control rodents (Tokes et al., 2011). Similarly, consumption of a diet fortified with soybean derived PC (1%) facilitated anti-inflammatory effects in the gastrointestinal tract of dogs following small intestine ischemia (Ghyczy et al., 2008). In other work, the concentrations of both TNF-α and IL-6 were reduced in rodents which received PC supplementation for 3 days prior to, and 5 days after, an enema with trinitrobenzenesulfonic acid (Kovacs et al., 2012). Further, Jung et al. (2013) modeled multiple organ injury via LPS injection in otherwise healthy rodents. LPS injection triggered an increase in pro-inflammatory messengers such as TNF-α and IL-6, as well as the anti-inflammatory messenger IL-10. However, in rodents administered PC, a significant reduction in both TNF-α and IL-6 was apparent, while concentrations of IL-10 were unaffected.

Although the weight of research examining the concentration of inflammatory messengers in response to GPL focuses on PC, and to a lesser extent PS, several studies have also investigated PE. Despite observing anti-inflammatory effects in response to PC supplementation, Treede et al. (2007, 2009) were unable to replicate these effects when supplementing with PE. In a later study, Eros et al. (2009) modeled pleurisy (inflammation of lung tissue and tissue lining the chest cavity) in rodents via injection of 100 μL saline containing 2% carrageenan into the thoracic cavity at the 6th intercostal space. Carrageenan injection triggered an increase in total leukocyte count and as well as elevated leukocyte accumulation within lung tissue. Importantly, inflammation was reduced in rodents fed a special diet containing PC, PE and N-acylphosphatidylethanolamine or NAPE (diet composition-−1% PC, 0.4% PE, and 0.1% NAPE) for the 7 days prior to injection of carrageenan and 48 h thereafter. However, it is difficult to disentangle the anti-inflammatory effects associated with PC and those potentially from either PE or NAPE, as only combined supplementation was investigated.

GPL Supplementation and Inflammation—Potential Pathways of Effect

The available literature suggests that PC supplementation may be capable of beneficially modifying inflammation. These effects are potentially due to PC being a significant source of choline (Cho et al., 2006; Chiuve et al., 2007) and therefore stimulating the cholinergic anti-inflammatory pathway. In this pathway, efferent fibers of the vagus nerve interact with acetylcholine receptors, notably α7 nicotinic acetylcholine (α7nACh) receptors on macrophages and other non-neural cytokine producing cells. The release of acetylcholine from vagus nerve activates these receptors which in turn facilitates inhibitory effects upon NF-KB, resulting in downregulated transcription of genes associated with pro-inflammatory messengers (Gallowitsch-Puerta and Pavlov, 2007).

In several studies, choline has been observed to function as an α7nACh receptor agonist. Parrish et al. (2008) cultured rodent macrophage cells with LPS in the presence or absence of choline for 4 h prior to measuring TNF-α concentrations. Choline treatment appeared to lower the concentrations of TNF-α following the presentation of LPS in a dose-dependent manner. This decline in TNF-α was found to have occurred because of reduced NF-KB activity. Parrish et al. then proceeded to demonstrate these effects in rodents, through intraperitoneal injection of choline (5 or 50 mg/kg) or saline at two time points (6 h as well as 30 min) prior to an injection of endotoxin (6 mg/kg). The higher choline dose reduced TNF-α concentrations in rodents previously injected with endotoxin, due to reduced NF-KB activity following activation of α7nACh receptors. Importantly, these results were then replicated in human whole blood and cultured macrophages. Similar observations were also made by Gurun et al. (2009) following administrations of CDP-choline (precursor to PC, but also containing choline) in rodents via intraplantar injection. These authors also observed that the anti-inflammatory effects of choline occurred in response to choline stimulating α7nACh receptors and subsequently suppressing NF-KB activity and therefore production of pro-inflammatory messengers. Though more work is needed to understand this potential mechanism, it is possible that PC, as a significant source of choline may influence the concentrations of pro-inflammatory messengers through the cholinergic anti-inflammatory pathway. This likely accounts for the empirical observations discussed earlier, whereby PC administration was associated with reduced concentrations of pro-inflammatory messengers such as TNF-α (Treede et al., 2007, 2009).

An additional pathway through which GPL supplementation may influence inflammation is through modifying the fatty acid composition of cell membranes, including inflammatory cells. Previous work has indicated that supplementation with ω3-PUFA primarily within PL facilitates increased bioavailability of ω3-PUFA within serum and membrane bound PL (Ramprasath et al., 2013). Increased ω3-PUFA within membrane GPL typically occurs at the expense of ω6-PUFA such as ARA (Calder, 2015). As such, there may be a proclivity toward the release of ω3-PUFA such as EPA or DHA, rather than the ω6-PUFA ARA, through the action of phospholipase A_2, during the inflammatory response. Once released, these fatty acids may be metabolized by cyclooxygenase (COX) and/or lipoxygenase (LOX) enzymes resulting in the production of eicosanoids. Eicosanoids resulting from ARA metabolism are pro-inflammatory as they stimulate the production of inflammation inducing messengers such as TNF-α, IL-6, IL-8, and IL-1β (Wall et al., 2010; Serhan and Petasis, 2011). However, eicosanoids produced through metabolism of ω3-PUFA, particularly EPA, exert far less potent pro-inflammatory effects than those associated with ARA (Wall et al., 2010; Calder, 2015). Moreover, the metabolism of EPA or DHA by COX and LOX enzymes facilitates the synthesis of powerful anti-inflammatory and inflammation resolving

agents such as resolvins, maresins and protectins (Bannenberg and Serhan, 2010; Serhan and Petasis, 2011; Calder, 2015). Furthermore, increased bioavailability of ω3-PUFA may help facilitate reduced nuclear translocation of NF-KB and therefore the transcription of genes associated with pro-inflammatory messengers (Wall et al., 2010; Calder, 2015).

Overall, it is anticipated that supplementation with GPL, especially those delivering choline and/or ω3-PUFA such as DHA, will beneficially modify the concentrations of inflammatory messengers. As chronic exposure to elevated concentrations of pro-inflammatory messengers appears to predict poorer cerebral structural integrity in older adults, it is predicted that lowering exposure to pro-inflammatory messengers will benefit cerebral structure. Subsequently, we would expect these effects to further benefit cognitive function in older adults.

Cardiovascular and Cerebrovascular Function

Cardio/Cerebrovascular Function and Cerebral Structure in Older Adults

Cardio- and cerebrovascular function are also pertinent factors predicting cerebral structure in older adults. One common measure of cardiovascular function is blood pressure (BP), specifically systolic and diastolic pressures (SBP or DBP, respectively). In middle aged and older adults, elevated BP (systolic and/or diastolic) or hypertension (clinically diagnosed high SBP and DBP) have been observed to predict poorer cerebral macrostructural integrity reflected as reduced total and regional cerebral volume (Den Heijer et al., 2003a, 2005; Raz et al., 2003; Beauchet et al., 2013), cortical thinning (Leritz et al., 2011; Alosco et al., 2014; Gonzalez et al., 2015), but also greater severity of cerebral white matter lesions or hyperintensities (Goldstein et al., 1998; Guo et al., 2009; Allan et al., 2015). Elevated BP also appears to predict poorer cerebral microstructural integrity (Kennedy and Raz, 2009; Leritz et al., 2010; Salat et al., 2012). An additional measure of cardiovascular function is arterial stiffness (AS). Increased AS has been observed to predict lower cerebral volume (Tsao et al., 2013; Lilamand et al., 2016) as well as increased severity of cerebral white matter lesions or hyperintensities (Henskens et al., 2008; Mitchell et al., 2011; Singer et al., 2014; van Sloten et al., 2015; Tsao et al., 2016). Both elevated BP and AS may contribute to poorer cerebral structural integrity, in part, due to chronically elevated pulsatile pressures damaging cerebral microvasculature. This may in turn facilitate reduced cerebral perfusion leading to ischemia and subsequent cerebral tissue decay (Gonzalez et al., 2015; van Sloten et al., 2015; Lilamand et al., 2016).

With an apparent association between poorer cardiovascular function and cerebral structural integrity, it follows that poorer cardiovascular function is predictive of reduced cognitive function. Elevated BP has been observed to predict poorer cognitive performance as well as an increased risk of developing MCI or dementia (Launer et al., 2000; Whitmer et al., 2005; Köhler et al., 2014; Chen et al., 2015; Alipour and Goldust, 2016) with similar effects having been observed in adults with elevated AS (Singer et al., 2014; Hajjar et al., 2016; Lim et al., 2016; Pase et al., 2016; Meyer et al., 2017). Given that there is a preponderance for increased BP (Neaton and Wentworth, 1992; Lloyd-Jones et al., 2005) and AS (Benetos et al., 2002; Mitchell et al., 2004) with age, targeting these outcomes with nutritional interventions may be benefit cerebral structural integrity, and therefore cognitive function in older adults.

Similarly, "blood-brain barrier" (BBB) integrity/permeability (an important measure of cerebrovascular function), may also influence cerebral structural integrity in older adults. The BBB is comprised of tightly compacted endothelial cells of capillaries perfusing cerebral tissue. Gases such as O^2 and CO^2 may freely diffuse across these capillary endothelial cells along their concentration gradients. However, the presence of "tight junctions," composed of transmembrane proteins such as occluding and claudins, but also junction adhesion molecules, limits the paracellular flow of water, ions and other large molecules. Larger molecules may cross the BBB (either entering or leaving cerebral tissue), though movement of such molecules is normally dependent upon complex receptor and transporter systems (Popescu et al., 2009; Zeevi et al., 2010).

Maintenance of BBB integrity and therefore selective permeability is essential for ensuring optimal health of cerebral tissues. However, there appears to be a tendency toward reduced BBB integrity and increased permeability with older age. Pelegri et al. (2007) and Del Valle et al. (2009) have both observed increased BBB permeability with age in a rodent model of accelerated senescence (SAMP8). Likewise, reduced BBB permeability was identified in older relative to younger human adults in a systematic review and meta-analysis published around the same time (Farrall and Wardlaw, 2009).

Changes in BBB permeability with age appears to be predictive of the integrity of cerebral tissue, but also cognitive function, in older adults. In their systematic review, Farrall and Wardlaw (2009) identified a number of studies showing that elevated BBB permeability was correlated with increased severity of cerebral white matter lesions. Likewise, elevated BBB permeability is predictive of poorer memory (Wang et al., 2006) and performance on the mini-mental state examination (van de Haar et al., 2016). There also appears to be a preponderance for elevated BBB permeability in adults with either dementia (Alzheimer's and vascular subtypes) or MCI, relative to cognitively normal adults (Farrall and Wardlaw, 2009), particularly within the hippocampus, but also cerebral cortex and deep gray matter (Wang et al., 2006; Montagne et al., 2015; van de Haar et al., 2016). It is possible that increased BBB permeability detrimentally influences cognitive function through deleterious effects upon cerebral structure. Further work utilizing advanced structural neuroimaging methods, computerized tasks sensitive to subtle cognitive change, and importantly larger (and cognitively diverse) participant samples, will provide further insight into how changes to BBB permeability influence cerebral structural integrity and cognitive function with age.

GPL Supplementation and Cardio/Cerebrovascular Function

As indicated earlier, GPL derived from marine sources (e.g., krill), but also mammalian brain or soybean may function as a

source of ω3-PUFA. There is evidence suggesting that increased ω3-PUFA intake, or bioavailability, is beneficial to BP (Mori et al., 1999; Paschos et al., 2007; Takeuchi et al., 2007; Theobald et al., 2007; Witte et al., 2013) though such effects may be limited to adults with untreated (or poorly treated) hypertension (Campbell et al., 2012; Miller et al., 2014; Minihane et al., 2016). Similar benefits may also be observed for AS. In a systematic review and meta-analysis, Pase et al. (2011) identified that supplementation with ω3-PUFA was efficacious for improving pulse wave velocity and arterial compliance, indicating reduced AS. In a later trial, Pase et al. (2015) observed that daily supplementation of 6 g fish oil (480 mg DHA + 480 mg EPA) for 16 weeks significantly lowered central AS as measured by aortic augmentation pressure. Additional support for an association between ω3-PUFA intake/bioavailability and levels of arterial stiffening is provided by several studies with middle aged and older adults whereby greater tissue concentrations of ω3-PUFA predicted lower AS (Anderson et al., 2009; Sekikawa et al., 2013; Reinders et al., 2015; Lee et al., 2016). Given that prior work has indicated that GPL supplementation may effectively elevate the concentrations of ω3-PUFA within biological tissues (Ramprasath et al., 2013), it is anticipated that supplementation with GPL containing ω3-PUFA would also benefit measures of cardiovascular function such as BP or AS.

To date, very few studies have investigated whether GPL supplementation influences cardiovascular function in older humans. In one study, Vakhapova et al. (2011) administered a daily dose of PS (300 mg PS and 79 mg DHA and EPA; ratio of 3:1) to 157 older adults (mean age was ∼72 years) daily for a period of 15 weeks. Following an initial 15-week double blind treatment phase, no differences were evident between treatment or control groups for either SBP or DBP. However, the trial was continued for an additional 15-weeks in an open label extension, though with a reduced dose of PS (100 mg PS and 26 mg DHA and EPA). The extension included 121 adults from the original trial phase, separated into two groups. Adults who previously received the PS supplement were designated as "continuers" whereas those originally receiving the placebo were termed "naïve." Following an additional 15 weeks' intervention, Vakhapova et al. (2011) identified that in the adults designated as "continuers" DBP was significantly reduced from baseline, whereas no change was identified in the "naïve" group. However, it cannot be determined whether these effects are attributable to PS or the additional ω3-PUFA (or both), as simultaneous administration was the only experimental treatment. In another open label trial, Richter et al. (2013) administered a daily dose of 300 mg PS derived from soybeans (thereby containing ALA, which may be converted to longer chain ω3-PUFA such as DHA) to 30 adults aged between 50 and 90 years, for a period of 12 weeks. Following treatment, Richter et al. (2013) identified significant reductions to both SBP and DBP.

Although beneficial effects to cardiovascular function have been observed following GPL supplementation, contrary data are available. Jorissen et al. (2002) reported data relating to the safety of soybean derived PS when administered to older adults with AAMI in doses of either 300 or 600 mg, for a period of 12 weeks. The authors report that there was an absence of any significant group differences in any BP measure upon completion of the supplementation period. However, the absence of beneficial effects may be due to important methodological factors. In an earlier publication using data from the same study, Jorissen et al. (2001) suggested that a lack of cognitive benefits following GPL supplementation may be due to their PS having degraded by 50% by 15 months' post production. Although Jorissen et al. (2001) report that PS administration ended ∼11 months after the treatments were manufactured, degradation may still have been advanced enough to minimize the likelihood of cardiovascular effects. Similar to Jorissen et al. (2002), Richter et al. (2013) administered PS in the form of gelatin capsules, though Richter et al. (2013) report that their capsules were specially engineered in order to GPL stability. Another pertinent difference between these studies is their use of parallel treatment groups. Jorissen et al. (2001, 2002) utilized a double blind, parallel groups design. Vakhapova et al. (2011) appears to have only performed within groups comparisons to determine whether adults designated "continuers" or "naïve" experienced cardiovascular benefits with no additional analyses having been performed so as to identify potential between groups effects. Likewise, Richter et al. (2013) performed an open label trial with no placebo group, so changes to cardiovascular function were only determined through within groups analyses. Lastly, there were marker differences in the samples sizes between these studies. Jorissen et al. (2002) and Vakhapova et al. (2011) included approximately 130 participants each, whereas Richter et al. (2013) only included 30 (26 by end of study). These differences in trial design, and size, may begin to account for the differences in results between these studies. As such, while GPL supplementation may benefit cardiovascular functioning, additional well designed clinical trials are required to confirm these effects in older adults.

While there has been some investigation of cardiovascular benefits following GPL supplementation, there appears to have been no clinical trials do date examining how GPL supplementation influences cerebrovascular function, particularly BBB permeability. Despite this, it is plausible that benefits to BBB permeability in older adults will be apparent following chronic ingestion of GPL containing choline and/or ω3-FA. Potential mechanisms through which GPL supplementation may benefit BBB permeability are highlighted in the following section.

GPL Supplementation and Cardio/Cerebrovascular Function-Potential Pathways of Effect

Although multiple factors likely contribute to poorer cardio and cerebrovascular function, it has been suggested that both OxS and inflammation may be particularly important. Increased ROS activity, as well as OxS, are suggested to be key factors in the etiology of hypertension (Vaziri and Rodríguez-Iturbe, 2006; Briones and Touyz, 2010; Wu and Harrison, 2014). In addition, elevated inflammation is suggested to predict increased BP or the presence hypertension (Vaziri and Rodríguez-Iturbe, 2006; Dinh et al., 2014), though it may do so via reciprocal relationships with both OxS and endothelial

dysfunction (Dinh et al., 2014). Furthermore, OxS has been positively linked with elevated AS (Patel et al., 2011; Kawamoto et al., 2016) potentially via remodeling of the vascular wall (Fleenor, 2013). Likewise, chronically elevated inflammation may initiate detrimental changes to the structural makeup of arterial walls, ultimately contributing to elevated AS (Jain et al., 2014).

Likewise, unbalanced ROS activity leading to OxS, but also elevated concentrations of certain pro-inflammatory messengers are suggested to facilitate increased BBB permeability (for reviews see Pun et al., 2009; Oakley and Tharakan, 2014; Rochfort and Cummins, 2015). Among other mechanisms, detrimental changes to tight junction proteins are often highlighted as a cause of increased BBB permeability following OxS or elevated concentrations of pro-inflammatory messengers such as TNF-α or IL-6 (Pun et al., 2009; Coisne and Engelhardt, 2011; Elahy et al., 2015; Varatharaj and Galea, 2017). Elevated HcY also predicts elevated BBB permeability in rodents (Kamath et al., 2006; Lominadze et al., 2006; Beard et al., 2011; Rhodehouse et al., 2013). Reduced BBB permeability has also been observed alongside elevated HcY in adults with MCI (Lehmann et al., 2003). Each of these risk mechanisms has been shown to be modifiable through supplementation with GPL containing choline and/or ω3-PUFA (see previous sections). Subsequently, it is anticipated that GPL supplementation, would further benefit BBB permeability in older adults. This remains to be investigated in well-designed clinical trials.

Although much more work is required, there is both direct and indirect evidence to suggest that GPL supplementation may benefit measures associated with cardio- and cerebro-vascular function in older adults. *As such, it is hypothesized that chronic supplementation with GPL containing choline and/or ω3-PUFA will improve the aforementioned measures of cardio- and cerebro-vascular function in older adults, thereby supporting cerebral structural integrity and cognitive functioning in older adults.*

Summary

Chronic supplementation with GPL has been observed to benefit cognitive function in animals and older adults (see introduction, as well as **Tables 1–3**). There is also data supporting the notion that GPL supplementation is beneficial to cerebral structure, though to date these effects have only been examined for in rodents. Despite a paucity of work in older humans, it is plausible that beneficial effects to cerebral structure are achievable, especially if the GPL administered contains choline and/or ω3-PUFA (DHA, EPA, or ALA). Long-term supplementation of GPL containing the aforementioned nutrients is expected to elevate the bioavailability of these nutrients, thereby resulting in modification of, or protection against, a number of factors associated with the destruction of cerebral tissues (see sections Homocysteine, Oxidative Stress, Inflammation, and Cardiovascular and Cerebrovascular Function). By minimizing or improving the factors outlined in this review, it is possible that GPL supplementation will lower the rate of cerebral structural decline over time. There

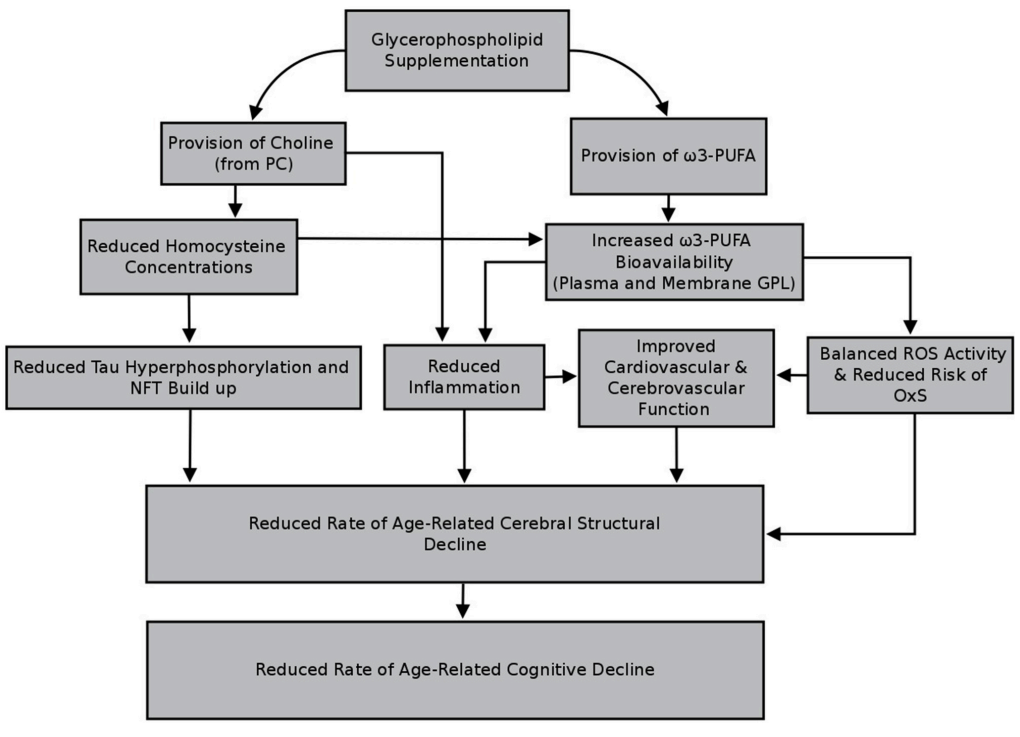

FIGURE 3 | Select pathways through which glycerophospholipid supplementation may benefit cerebral structure and cognitive function in older adults.

is also the possibility that supplementation may facilitate at least mild repair to cerebral tissue given that GPL are major components of cellular membranes. Whether it be by reduced rate of decline, repair, or a combination of both, it is anticipated that by supporting cerebral structure, GPL supplementation may also influence the trajectory of cognitive decline with increasing age. **Figure 3** outlines select pathways through which GPL supplementation may benefit cerebral structure, and subsequently cognitive function, in older adults. These effects should be examined in future well-designed clinical trials incorporating the use of advanced structural neuroimaging methods.

CONCLUSION

There is substantial research indicating that cerebral structural integrity, at both the macro- and microstructural levels, is reduced with age. Modifying nutritional intake is quickly becoming recognized as a means of supporting cerebral structure with age, with a number of trials indicating that chronic supplementation with B vitamins, ω3-PUFA, or resveratrol, mediates reduced cerebral deterioration over time, perhaps even facilitating repair. This review discusses a number of different pathways through which benefits to cerebral structure may occur in response to GPL supplementation, thereby providing a theoretical basis for future human clinical trials.

Given that cerebral macro- and microstructural integrity is a pertinent predictor of cognitive function in older adults, it is plausible that through supporting cerebral structure, a reduced rate of cognitive deterioration may become apparent. Improving the trajectory of age-related cerebral deterioration and therefore cognitive decline through readily accessible interventions such as nutritional supplementation, may help lower the risk, and delay the onset, of age related conditions such as AAMI and MCI. Moreover, it may be possible to delay the onset of pathological conditions such as dementia, thereby contributing to a reduced incidence of this disease (Brookmeyer et al., 2007). Given the ease at which nutrition can be modified, and the relative absence of harmful side effects, nutritional supplementation, particularly with GPL, may well be a useful intervention for supporting neurocognitive health with increasing age.

AUTHOR CONTRIBUTIONS

JMR: Drafted the manuscript, with all additional authors (DJW, HM, AS, and AP) making significant contributions to the overall planning and development of the manuscript.

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frontiers in Aging Neuroscience

GENERAL COMMENTARY
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Commentary: Effect of Probiotic Supplementation on Cognitive Function and Metabolic Status in Alzheimer's Disease: A Randomized, Double-Blind and Controlled Trial

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A commentary on

Effect of Probiotic Supplementation on Cognitive Function and Metabolic Status in Alzheimer's Disease: A Randomized, Double-Blind and Controlled Trial
by Akbari, E., Asemi, Z., Daneshvar Kakhaki, R., Bahmani, F., Kouchaki, E., Tamtaji, O. R., et al. (2016). Front. Aging Neurosci. 8:256. doi: 10.3389/fnagi.2016.00256

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Akbari and colleagues (Front. Aging Neurosci. 8:256. doi: 10.3389/fnagi.2016.00256) in their randomized, double blind clinical trial demonstrate that probiotic administration for 12 weeks had favorable cognitive and metabolic effects in their patients with Alzheimer's disease (AD). Most impressive was the significant improvement in the mini mental state examination [MMSE ($p < 0.001$)]. Some metabolic parameters such as plasma malondialdehyde, markers of insulin metabolism and serum triglycerides were also significantly different in the AD patients compared with the control group, and probably caused by probiotic treatment. Additionally, serum high sensitive C-reactive protein (hs-CRP) significantly changed after probiotic supplementation ($p < 0.001$). However, changes in other biomarkers of oxidative stress and inflammation were negligible. The authors concluded that evaluation of other biomarkers of inflammation and oxidative stress would be informative.

Concerning this suggestion several different fecal and serum inflammation markers in correlation to intestinal bacterial strains were investigated in our recent study on the role of gut microbiota in patients with cognitive decline (Leblhuber et al., 2017). From a subgroup of 23 patients (9 females, 14 males, aged 78 ± 8.5 years) out of 55 consecutive outpatients with symptoms of cognitive decline, intestinal bacterial taxa and immune system as well as inflammation biomarkers in serum and stool specimens were investigated.

Confirming our earlier findings (Widner et al., 2000; Wissmann et al., 2013; Leblhuber et al., 2015), signs of immune activation could be detected: serum neopterin was found elevated as well as the Kyn/Trp ratio, an index of tryptophan breakdown by enzyme indoleamine 2,3-dioxygenase-1 (IDO). Most interestingly, a close correlation was found between fecal S100A12 and serum neopterin ($p < 0.001$, see **Figure 1**), indicating coincident low grade systemic and intestinal

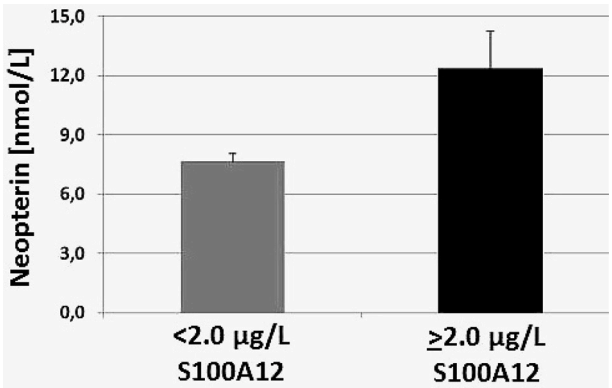

FIGURE 1 | Serum neopterin concentration (mean ± SEM) in patients suffering from cognitive decline grouped according to fecal S100A12 concentrations ($p < 0.001$), modified from Leblhuber et al. (2017).

inflammation (Caracciolo et al., 2014). There was no influence of gender. These findings again underline the role of gut inflammation as a possible pathogenic cofactor in cognitive deterioration and dementia.

In an earlier study (Shepherd et al., 2006), the potential role of pro-inflammatory S100A9 and S100A12 proteins in the pathogenesis of AD was described. Circulating CRP, known to affect cognition negatively, was elevated in our series (1.6 ± 2.3 mg/L) without clinical signs of acute infection as in the study of Akbari et al. but further indicating low grade inflammation ("inflammaging") (Caracciolo et al., 2014) in this group of patients. We found pro-inflammatory *Clostridium Cluster I* significantly correlated with anti-inflammatory *Faecalibacterium prausnitzii* ($p < 0.01$).

In a recent paper (Cattaneo et al., 2017) the stool abundance of selected bacterial stool taxa including *F. prausnitzii* and the blood levels of pro- and anti-inflammatory cytokines in cognitively impaired patients and in a group of controls was measured. Amyloid positive patients showed higher levels of pro-inflammatory cytokines compared with both controls and with amyloid negative patients. A possible causal relation between gut microbiota related inflammation and amyloidosis was suspected in this study.

In our series *F. prausnitzii* correlated with MMSE ($p < 0.05$), with *Akkermansia muciniphila* ($p < 0.01$) and with serum neopterin ($p < 0.05$). Further, a strong correlation was found between anti-inflammatory α1-antitrypsin and pro-inflammatory S100A12 ($p < 0.001$) in the fecal specimens of our cognitively impaired patients. The anti-inflammatory action of α1-antiptrypsin on microglial mediated neuroinflammation could be shown *in vitro* (Gold et al., 2014). In our series, α1-antitrypsin was also correlated with zonulin ($p < 0.01$), a protein modulating tight junction permeability between cells of the digestive tract. All these findings together may indicate changes in the microbiota-gut-brain-axis correlated to neuroinflammation during cognitive decline. Because neuroinflammation is an early event in the pathogenesis of dementia (Caracciolo et al., 2014) these markers may be important in the very beginning of this devastating process.

The increased immune activation and inflammation in AD could indeed relate to the age-related changes of gut microbiota as is indicated by the close relationship between fecal S100A12 and serum neopterin concentrations (Leblhuber et al., 2017). Unfortunately, in the above mentioned study (Akbari et al., 2016) these and additional inflammation markers were not measured except hs-CRP; as the authors stated the measurement of fecal bacteria loads before and after probiotic supplementation was "very difficult" in their study.

Overall, the role of probiotics in preventing dementia seems promising and should be further elucidated in future studies. These investigations should include the bacterial taxa as well as the serum and intestinal inflammation markers mentioned above together with the metabolic parameters mentioned by Akbari et al. (2016). A possible personalized therapy for cognitive decline and dementia as well as for establishing effective nutritional interventions with pre- and probiotics for healthy brain aging should be considered (Zamroziewicz and Barbey, 2016).

AUTHOR CONTRIBUTIONS

All authors have made substantial contribution and critical revision to this manuscript.

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ORIGINAL RESEARCH
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Effects of Lutein/Zeaxanthin Supplementation on the Cognitive Function of Community Dwelling Older Adults: A Randomized, Double-Masked, Placebo-Controlled Trial

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Background: High levels of xanthophyll carotenoids lutein (L) and zeaxanthin (Z) in the central nervous system have been previously correlated with improved cognitive function in community-dwelling older adults. In this study, we tested the effects of supplementing L and Z on older men and women with a range of baseline cognitive abilities.

Objective: The purpose of this study was to determine whether or not supplementation with L+Z could improve cognitive function in community-dwelling, older adults.

Design: Double-masked, randomized, placebo-controlled trial. A total of 62 older adults were randomized into groups receiving either 12 mg L+Z or a visually identical placebo. Data from 51 participants (M = 73.7 years) were available for analysis. Retinal L+Z levels (macular pigment optical density, MPOD) were measured psychophysically using heterochromatic flicker photometry as a biomarker of cortical L+Z levels. Cognitive function was measured using the CNS Vital Signs computerized test platform.

Results: Participants receiving the active L+Z supplement had statistically significant increases in MPOD ($p < 0.03$) and improvements in complex attention ($p < 0.02$) and cognitive flexibility domains ($p < 0.04$), relative to participants taking the placebo. A trend was also seen for the executive function domain ($p = 0.073$). In male participants only, supplementation yielded improved composite memory ($p = 0.04$).

Conclusions: Supplementation with L+Z improved cognitive function in community-dwelling, older men and women.

Keywords: Xanthophylls, cognition, older adults, attention, cognitive flexibility

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INTRODUCTION

It has long been understood that cognitive function, along with its manifestation as behavior and subjective experience, is a product of the activity of the brain. Hebb (1949), for instance, argued that it was the interplay between networks of neurons that gave rise to mental activity. Further, deleterious change within neural cell assemblies drives many of the decrements often observed

with aging (e.g., Berlingeri et al., 2013). For example, age-related change in neural cells[1] means that older individuals must activate larger regions of these networks in order to accomplish the same task as a younger person. This additional recruitment results in slowing and the common finding that dynamic aspects of cognition are more impacted by age than more static functions (Salthouse, 1996).

If, however, these neural assemblies are the physical basis of cognition and its age-related change, then if follows that the physical factors involved in forming and maintaining those physical structures would, inevitably, influence the end-product itself. Such factors are often systemic. For example, interferon-Y, a component of the immune system, has recently been shown to regulate neural connectivity and social behavior in mice (Filiano et al., 2016). Many neurotransmitters are synthesized within the gut, and the physical structure of the brain itself is dependent on dietary and immune factors originating in this distal tissue (Cryan and Dinan, 2012). In an environment of high fat (some 60% by volume) and oxygen (25% respiratory intake), the brain must concentrate high levels of antioxidants (both dietary and endogenous, like superoxide dismutase) to prevent peroxidation (Chakrabarti et al., 2011). If antioxidants are missing in the diet, then higher levels of oxidative stress exist within the brain (Rao and Balachandran, 2002). Over time, peroxidation of brain lipids likely results in losses such as the decrease in the quantity and integrity of white matter often seen with aging (Bennett and Madden, 2014), likely due to alterations in the lipid-rich axonal myelin.

Lutein, a dietary antioxidant, could help maintain brain structure by lowering chronic oxidative stress (Erdman et al., 2015). The brain is also susceptible to damage due to chronic inflammation and L and Z are known to be potent anti-inflammatories (Kijlstra et al., 2012). Such mechanisms, however, are largely prophylactic. It is reasonable to question then whether preventive measures are effective later in life. Is there value, for instance, in increasing dietary intake of food components thought to prevent loss after someone has likely already suffered many decades of loss?

We do know that older and diseased brains (and retinas) are under higher oxidative and inflammatory stress. Late stage intervention could possible lower such stressors helping to retard the cascade that ultimately accelerates the degenerative process (Hammond et al., 1998; Joseph et al., 2005). We also know that older brains are still capable of some neurogenesis (especially within the hippocampus; Kempermann et al., 2002). If supplemental L, through diet or purified supplements, could both decrease age-related inflammatory and oxidative stress while simultaneously stimulating regenerative processes, supplementing L certainly could be a useful strategy. There is some, limited, data that are consistent with this possibility.

For instance, preliminary data suggests that, in younger individuals, supplementing L and Z increases systemic levels of brain-derived neural growth factor (Stringham et al., 2016) when compared to placebo. A number of clinical trials have shown, mostly in the young, that L and Z supplementation increases visual processing speed and reaction times (Bovier et al., 2014; Bovier and Hammond, 2015). Although there is no direct data on mechanism, it has been speculated (e.g., the neural efficiency hypothesis for L and Z) that this influence on processing speed is due to direct effects on brain connectivity (Renzi and Hammond, 2010), perhaps by enhancing gap junctions between neurons.

Whatever the mechanism, we do have empirical data on participants across the lifespan showing that L and Z supplementation has direct effects on improving cognition compared to placebos. Johnson et al., for instance, showed that L and Z, combined with DHA, improved verbal fluency, rate of learning and memory (Johnson et al., 2008). Later, this basic finding was repeated using avocados, rich in lutein and omega-fatty acids (Johnson et al., 2015). In the current study, we extend these basic findings, also using a double-blinded placebo controlled design, to test both older men and women using only L and Z (separating it from the much larger literature on omega fatty acids and cognition).

MATERIALS AND METHODS

Subjects

A total of 80 community dwelling older adults from the Athens-Clarke County, Georgia population were screened for enrollment between August 2012 and August 2014, with follow-up lasting through October 2015. This sample was part of a larger trial on xanthophyll supplementation and cognitive function. Inclusion criteria included good overall health; no xanthophyll supplementation within the 6-month period prior to study enrollment, with the exception of multivitamins that contained less than 1 mg L+Z/day; best corrected visual acuity of 20:40 or better (Snellen notation); no previous history of stroke, dementia, Parkinson's disease, or any other neurological condition known to impair cognitive function, with the exception of affective disorders such as anxiety or depression; absence of gastric conditions known to impair absorption of nutritional supplements, such as gastric bypass or gastric ulcer. Inclusion criteria were verified as follows: all participants were given a medical examination by a qualified physician at the University of Georgia Health Center; self-reported health information was obtained; and all participants participated in a structured clinical interview, administered by qualified neuropsychological staff (see below).

Abbreviations: L, Lutein; Z, zeaxanthin; MPOD, macular pigment optical density; MZ, meso-zeaxanthin; CNS, central nervous system; MP, macular pigment; HPLC, high-performance liquid chromatography; FFQ, food frequency questionnaire; RCI, reliable change index; VeM, verbal memory; ViM, visual memory; R, reasoning; EF, executive function; PmS, psychomotor speed; CA, complex attention; CF, cognitive flexibility; NVRT, non-verbal reasoning test; SAT, shifting attention test; FTT, finger tapping test; SDC, symbol-digit coding; ST, Stroop test; CPT, continuous performance task; CDR, Clinical Dementia Rating Scale; NCI, Neurocognitive index.

[1]This appears to be less the result of neuronal death per se and more due to actual changes in morphology; for example, Hof and Morrison (2004) showed a 46% decrease in dendritic spine number and density when comparing younger and older adults.

Randomization Process and Intervention

Of the 80 participants that were screened for enrollment, a total of 62 participants met inclusion criteria and were randomized into one of two groups: the active supplement group, or the placebo group. Simple randomization was conducted by the clinical coordinator, who had no data collection responsibilities. A set of numerical codes was generated that corresponded with either the active supplement or the placebo. The codes were placed in an opaque envelope, and a unique code was drawn for each participant. Of the 62 participants who were randomized, 20 participants were randomized into the placebo group, and 42 participants were randomized into the active supplement group. In the placebo group, two were lost to follow-up (completed baseline but failed to attend subsequent testing sessions) and three were withdrawn due to non-compliance. In the intervention group, four were lost to follow-up and one was withdrawn.

The active supplement contained 10 mg L and 2 mg Z. The placebo was visually identical to the active supplement. Supplements and placebos (provided by DSM Nutritional Products Ltd., Kaiseraugst, Switzerland) were contained in identical opaque, sealed bottles with labels that were visually identical, with the exception of the randomization code on the label, and contained instructions for one tablet to be taken from the bottle, daily, with a meal. Compliance to the intervention was monitored by bi-monthly telephone calls and pill counts from bottles returned by the participants during study visits.

Baseline Characteristics of the Analyzable Sample

Of the 62 participants who were randomized, a total of two participants from the placebo group and four participants from the active supplement group were lost to follow-up over the year of intervention. Three participants from the placebo group and 1 participant from the active supplement group were withdrawn by study personnel for either failure to maintain inclusion criteria, or because of non-compliance with the study regimen, determined by self-reported failure to take study supplements on a minimum of four of the compliance telephone calls. Baseline characteristics of the analyzable sample ($N = 51$) are presented in **Table 1.** There were no significant differences between the placebo and active supplement groups in gender distribution, age ($p > 0.48$), or education level ($p > 0.89$).

With respect to cognitive function, significant differences were detected at baseline between male ($M = 88.19$, $SD = 8.79$) and female ($M = 96.60$, $SD = 7.61$) participants in the composite memory domain ($p < 0.05$). No other significant differences were present between male and female participants at baseline.

Ethics

The tenets of the Declaration of Helsinki were adhered to at all times during the course of this study. All participants issued written and verbal informed consent prior to study enrollment, and consent documents were administered by trained study personnel. The University of Georgia Institutional Review Board approved all study-related documents and procedures prior to study initiation, and all study personnel received training in ethical principles and procedures in human subject's research.

Methods

Confirming Enrollment Criteria

In order to be enrolled in this study, participants went through a three-step process to confirm eligibility. Participants were initially recruited using newspaper advertising and print advertisements posted throughout the community. When participants contacted study personnel to express interest, a telephone screener was used to collect self-report data on past supplement use and a brief history of neurological and ocular disorders. If the participant passed the first eligibility screen, visits were scheduled with the University of Georgia Health Center for the medical examination, as well as the University of Georgia Neuropsychology and Memory Assessment Laboratory. If the study medical team confirmed eligibility, the participant progressed to the neuropsychological screen / clinical interview.

Participants were asked to bring a legally authorized representative to the interview, who could serve as a collateral source of information. In addition to baseline cognitive functional testing on the primary test battery (CNS Vital Signs; Morrisville, NC), participants and collaterals were given the Clinical Dementia Rating Scale (CDR) as part of the larger interview (Morris, 1993). Potential participants with CDR sum of boxes scores of 1.0 or higher were excluded from participation. Participants with sums of boxes equal to 0.5 (mild impairment, O'Bryant et al., 2008) were included in the study sample, in order to include participants with a wider range of baseline cognitive abilities.

Retinal L ± Z levels

Retinal L+Z levels, as macular pigment optical density (MPOD) were measured psychophysically using customized

TABLE 1 | Baseline characteristics of the analyzable study sample.

Study group	Age (years)	Gender	Years of education	Dietary intake of fruits and vegetables (servings/day)	Baseline macular pigment optical density (MPOD)
All analyzable participants	73.74 ± 8.20	30 female; 21 male	16.34 ± 3.01	5.26 ± 1.48	0.49 ± 0.18
Active Supplement Group	72.51 ± 6.24	19 female; 17 male	16.37 ± 3.21	5.25 ± 1.40	0.51 ± 0.19
Placebo Group	70.93 ± 5.70	11 female; 4 male	16.25 ± 2.53	5.26 ± 1.56	0.42 ± 0.16

All participants reported ethnic and racial information as "Non-Hispanic/White." Values are presented as Mean, ± Standard Deviation.

heterochromatic photometry (cHFP) (Wooten et al., 1999; Stringham et al., 2008). This procedure has been described previously (Vishwanathan et al., 2014) and was modified slightly for this study. First, rather than the five trials per condition that are typically used to measure macular pigment, nine trials were completed both centrally, at 30-min of eccentricity along the horizontal meridian of the temporal retina, and parafoveally, at 7° of eccentricity. These trials were completed using a test stimulus that consisted of a waveband peaking at 460 nm (strongly absorbed by MP) that alternated in counterphase with a reference waveband peaking at 570 nm. Data collection was also limited to three skilled experimenters.

cHFP is the gold standard for measuring MPOD (Hammond et al., 2005) and has been used previously in participants with poor ocular health (e.g., with cataract and age-related macular degeneration) (Ciulla et al., 2001; Stringham et al., 2008), children (McCorkle et al., 2015), and participants with mild cognitive impairment (Renzi et al., 2014). In order to further confirm reliability of cHFP in this sample, an additional five central trials and five parafoveal trials were conducted using a different test stimulus, which consisted of a waveband peaking at 490 nm that alternated in counterphase with the same 570 nm reference waveband. Past research using *ex vivo* absorption spectra (Snodderly et al., 1984) and *in vivo* psychophysical methods (Snodderly et al., 2004; Wooten and Hammond, 2005; Stringham et al., 2008) for MP suggests that at 490 nm, absorbance is reduced by approximately half. Consequently, MPOD using the 490 nm test stimulus should be

TABLE 2 | Individual tests administered during the computerized cognitive functional test battery, and computed domain scores analyzed for older adult participants.

Domain	Description	Tests used to compute the domain	Computation
Verbal Memory (VeM)	Ability to remember words presented in a list vs. distractor words, immediately after list presentation and after a 30-minute delay.	Verbal Memory Test	Correct hits for presented words + correct passes on distractors for tests immediately after presentation and after a 30-min delay.
Visual Memory (ViM)	Ability to remember arbitrary visual shapes and symbols vs. distractor shapes and symbols immediately after presentation and after a 30-minute delay.	Visual Memory Test	Correct hits for presented shapes and symbols + correct passes on distractors for tests immediately after presentation and after a 30-min delay.
Reasoning ®	Ability to perceive and understand the meaning of abstract concepts and recognize the relationships between abstract concepts.	Non-Verbal Reasoning Test (NVRT)	Correct responses on the NVRT – commission errors on the NVRT.
Executive Function (EF)	Ability to recognize and act upon sets with randomly shifting rues in the presence of other simultaneously occurring tasks and pieces of information.	Shifting Attention Test (SAT)	Correct responses on the SAT – errors on the SAT.
Psychomotor Speed (PmS)	Ability to rapidly preform motor tasks in absence of sensory stimuli.	Finger Tapping Test (FTT) Symbol-Digit Coding Test (SDC)	Average number of taps on the FTT with the right hand + average number of taps with the left hand + number of correct responses on the SDC
Complex Attention (CA)	Ability to maintain sustained attention or vigilance in the face of changing response rules	Stroop Test (ST) SAT Continuous Performance Task (CPT)	Commission errors on the ST + Errors on the SAT + Commission and omission errors on the CPT
Cognitive Flexibility (CF)	Ability to inhibit irrelevant information and disinhibit previously "incorrect" response patterns.	SAT ST	Correct responses on the SAT – errors on the SAT – Commission errors on the ST
Neurocognitive Index (NCI)	Global cognitive functioning, takes into account all other functional domains	N/A	Average of domain scores from: ViM and VeM, PmS, Reaction Time across domains, CA, CF

TABLE 3 | Serum levels of L, Z, and L+Z at baseline and over the course of the study intervention, stratified by intervention group.

		Baseline	4-months	8-months	12-months
Lutein (ng/μL)	Active	0.15 ± 0.08	0.66 ± 0.34**†	0.55 ± 0.27**†	0.59 ± 0.23**†
	Placebo	0.15 ± 0.06	0.25 ± 0.17	0.17 ± 0.09	0.14 ± 0.07
Zeaxanthin (ng/μL)	Active	0.03 ± 0.02	0.15 ± 0.09**†	0.12 ± 0.05**†	0.13 ± 0.06**†
	Placebo	0.03 ± 0.01	0.04 ± 0.03	0.03 ± 0.01	0.03 ± 0.01
Lutein + Zeaxanthin (ng/μL)	Active	0.18 ± 0.11	0.81 ± 0.39**†	0.66 ± 0.32**†	0.72 ± 0.28**†
	Placebo	0.18 ± 0.07	0.29 ± 0.20	0.21 ± 0.10	0.17 ± 0.08
Macular Pigment (optical density)	Active	0.52 ± 0.19	0.51 ± 0.18	0.58 ± 0.22*†	0.59 ± 0.22†
	Placebo	0.42 ± 0.16	0.39 ± 0.21	0.38 ± 0.17	0.47 ± 0.20

Data are presented as M ± SD.

**Denotes significant difference between active and placebo group ($p < 0.05$).*

***Denotes a significant difference between active and placebo group ($p < 0.01$).*

†*Denotes a significant change from baseline ($p < 0.05$).*

approximately half of the value at 460 nm if participants were successfully able to understand and complete the psychophysical task.

Serum L ± Z levels

In addition to measuring retinal L+Z levels, which relate strongly to cortical L+Z levels in human subjects (Vishwanathan et al., 2016) and are used as a biomarker of cortical L+Z in this and other studies (e.g., Feeney et al., 2013; Renzi et al., 2014; Vishwanathan et al., 2014), L and Z were also measured in serum via high-performance liquid chromatography. The methods used to acquire and analyze the serum in this study have been prevented previously (Lindbergh et al., 2017).

Cognitive Function

Cognitive function was measured using a computerized test battery (CNS Vital Signs; Morrisville, NC) at four different time points: baseline, and after 4-, 8-, and 12-months of taking the study intervention. Participants were tested in a low distraction environment, in full ambient room lighting, in the presence of a trained research assistant who could answer questions about the test procedures should they arise. Each participant completed a practice session prior to each individual test.

Raw scores from individual functional tests taken during the test session were used to compute performance on the following larger cognitive domains: Verbal Memory (VeM), Visual Memory (ViM), Reasoning Ability (R), Executive Function (EF), Psychomotor Speed (PmS), Cognitive Flexibility (CF), and the Neurocognitive Index (NCI). For example, in order to gauge performance in CF, errors on the shifting attention test and commission errors on the Stroop task were subtracted from correct responses on the shifting attention test. For a complete list of cognitive tests administered during the battery, as well as functional domains computed and analyzed, see **Table 2**.

Statistical Analyses

Statistical analyses were performed using SPSS version 23 (IBM), with $\alpha = 0.05$. Tests of cognitive function were one-tailed, as *a priori* hypotheses were directional in nature (i.e., increasing MPOD by supplementation will improve cognitive function). Prior to enrolling subjects, a power analysis was conducted to determine what sample size would yield $1-\beta = 0.80$ for a difference of 0.10 log units of MPOD. The current sample size yielded statistical power of 85.7%. A statistically significant increase in MPOD in the supplementation group was analyzed as the primary outcome variable in this study, and improvements in cognitive function in the supplemented group relative to the placebo group were analyzed as secondary variables.

RESULTS

Retinal L ± Z levels

At baseline, MPOD for the entire older adult cohort (0.49) was comparable to other published data on a different sample with approximately the same average age, recruited from the same geographic region (0.47) (Renzi et al., 2014), but was

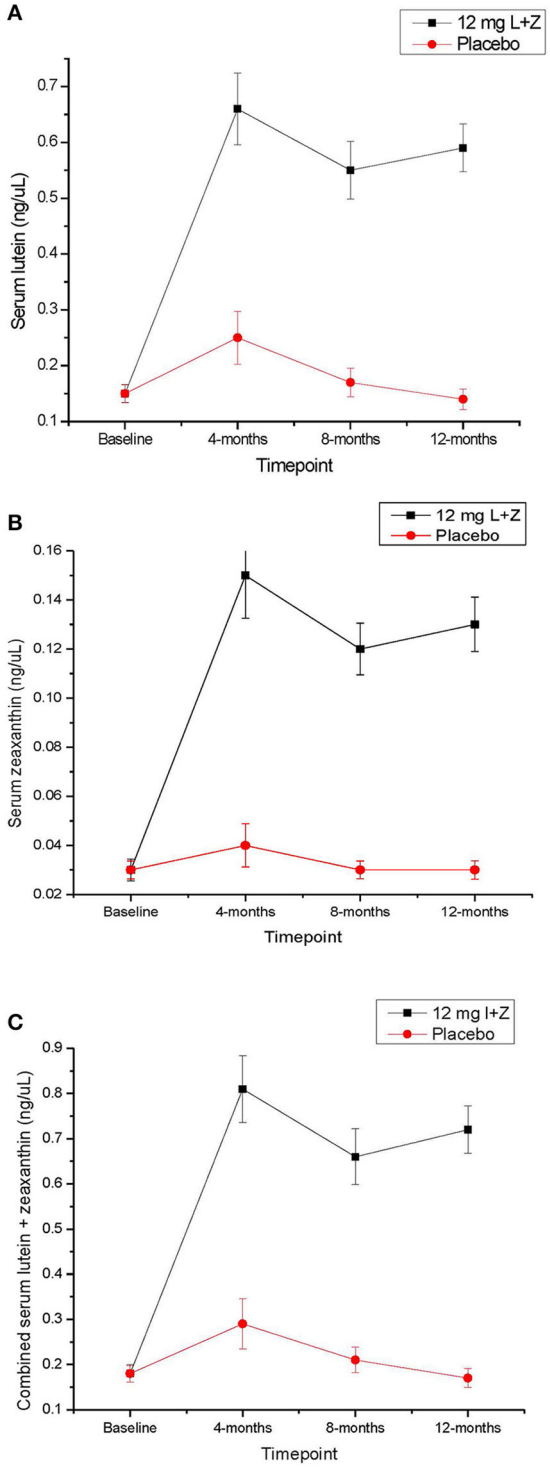

FIGURE 1 | (A) Serum lutein across the 1-year intervention time period in participants taking the active study supplement, vs. placebo. Error bars represent the standard error of the mean. **(B)** Serum zeaxanthin across the 1-year intervention time period in participants taking the active study supplement, vs. placebo. Error bars represent the standard error of the mean. **(C)** Serum lutein + zeaxanthin levels across the 1-year intervention time period in participants taking the active study supplement, vs. placebo. Error bars represent the standard error of the mean.

TABLE 4 | Cognitive domain scores at baseline for the entire sample, and for the sample stratified by supplement status.

	NCI	VeM	ViM	R	EF	PmS	CA	CF	CDR = 0.5
Whole Sample	101.10 ± 10.16	50.76 ± 5.46	42.98 ± 9.66	2.82 ± 3.88	33.41 ± 17.33	141.86 ± 19.84	12.27 ± 10.15	31.83 ± 18.23	7.69% of sample
Active Supplement Group	100.12 ± 10.45	49.91 ± 5.66	41.03 ± 6.68	2.97 ± 3.95	32.33 ± 18.33	140.24 ± 20.11	13.12 ± 11.12	30.32 ± 19.09	8.8% of sample
Placebo Group	103.33 ± 9.44	52.67 ± 5.29	43.87 ± 5.18	2.47 ± 3.83	35.87 ± 15.90	145.53 ± 19.37	10.21 ± 7.21	35.50 ± 16.01	6.67% of sample

NCI, Neurocognitive Index; VeM, Verbal Memory; ViM, Visual Memory; R, Reasoning Ability; EF, Executive Function; PmS, Psychomotor Speed; CA, Complex Attention; CF, Cognitive Flexibility; CDR, Clinical Dementia Rating Scale.
Data are presented as M ± SD.

higher than MPOD reported in similarly aged cohorts from other parts of the U.S. (0.34, 0.36; Moeller et al., 2009; Vishwanathan et al., 2014) and world (0.20; Feeney et al., 2013). MPOD at baseline was numerically but not statistically higher in the group that received the active supplement (0.51 ± 0.19) than the group that received the placebo (0.42 ± 0.16). MPOD increased significantly between the baseline and the 12-month time points ($M = 0.58$, $SD = 0.23$; $p < 0.03$) in the group that received the active supplement. The placebo group did not change significantly over the course of the year (see **Table 3**).

When MPOD at 460 nm was compared against MPOD at 490 nm to confirm validity at the 12-month timepoint, total MPOD for the study sample, regardless of group membership, was 0.53 at 460 nm. When measured at 490 nm, MPOD was 0.29, which is approximately half the 460 nm value, suggesting that participants were able to reliably perform the cHFP task.

Serum L ± Z levels

At baseline, serum L, Z, and L+Z levels were not significantly different between participants in the active supplement and placebo groups. Beginning at the 4-month time point and continuing throughout the rest of the intervention, serum L, Z, and L+Z were significantly higher in the group that received the active supplement than the placebo group ($p < 0.01$ for L, Z, and L+Z at all-time points; see **Table 3**, **Figure 1**). Participants on the placebo supplement did not show any significant changes in serum L, Z, or L+Z levels during the year-long intervention.

Cognitive Function

At baseline, participants in the active supplement group were not significantly different from participants who were randomized into the placebo group on any of the cognitive domain scores analyzed or on global cognitive health, as measured by the CDR (see **Table 4**). At baseline, trends for relationships between MPOD and the memory, executive function and cognitive flexibility domains were present in the sample as a whole, but not statistically significant ($p > 0.05$). Given the relatively low sample size present in this study, the lack of statistical significance is not surprising.

When correlations between MPOD and cognitive function were analyzed at the 12-month time point, MPOD was significantly related to performance in the reasoning domain ($r = 0.45$, $p = 0.04$), and a trend was seen for errors of attention in the complex attention domain ($r = -0.18$, $p = 0.08$). A trend for relation between MPOD and verbal memory ($r = 0.31$, $p = 0.07$) was also present for those participants whose MPOD improved from the baseline to the 12-month time point, regardless of whether or not they received the supplement. Within the supplementation group, participants with the greatest changes between the baseline and 12-month time points in cognitive function in the reasoning ($r = 0.34$, $p = 0.02$) and complex attention ($r = -0.31$, $p = 0.04$, expressed as errors in complex attention) domains also tended to have the highest MPOD at the 12-month time point. Trends for the relationship between magnitude of cognitive change and 12-month MPOD were also seen for the visual memory ($r = 0.24$, $p = 0.09$) and cognitive flexibility ($r = 0.20$, $p = 0.10$) domains.

Given the fact that participants were tested four times throughout the course of the study, and given the fact that participants were given practice sessions prior to each active test session, practice effects were anticipated. In order to determine whether or not change was reliable and meaningful, the Reliable Change Index (RCI) was computed for each cognitive index and group, with a standard criterion of 1.96. Participants who took the active supplement had significantly improved performance in complex attention ($p < 0.02$; RCI = 3.71 for the active group and 0.34 for the placebo group) and cognitive flexibility ($p < 0.04$; RCI = 6.31 for the active group and 0.84 for the placebo group) domains, relative to participants taking the placebo. A trend was also present ($p = 0.07$; RCI = 5.64 for the active group and 1.27 for the placebo group) for the executive function domain (see **Figure 2**), by the end of the 12-month period. When male and female participants were analyzed separately, male participants who received the active supplement improved significantly in the composite memory domain ($p = 0.04$).

DISCUSSION

This study was designed as a year-long intervention with the dietary carotenoids L and Z. These plant pigments, long known for their effects on systemic and ocular health, have been identified in brain (Craft and Dorey, 2004; Vishwanathan et al.,

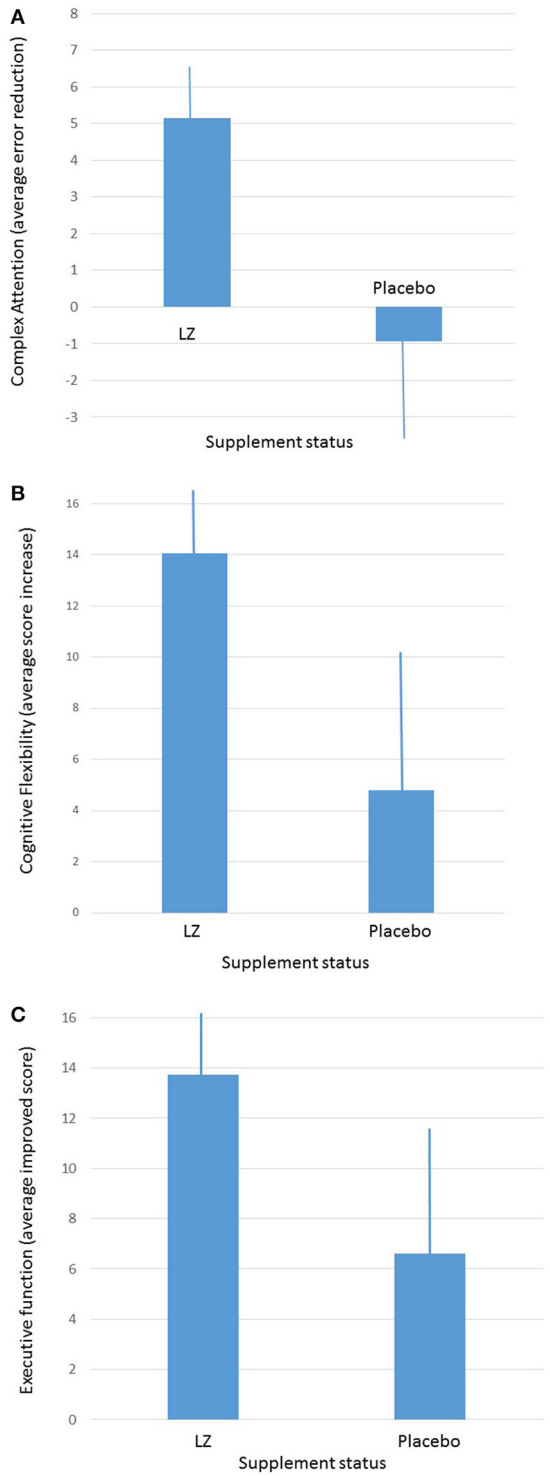

FIGURE 2 | (A) Average improvements in complex attention between participants taking the active supplement and participants taking the placebo after 1-year of intervention. **(B)** Average improvements in cognitive flexibility between participants taking the active supplement and participants taking the placebo after 1-year of intervention (average and ± SEM). **(C)** Average improvements in executive function between participants taking the active supplement and participants taking the placebo after 1-year of intervention (average and ± SEM).

2016). Accumulating evidence has shown that LZ may influence various aspects of brain function ranging from visual-motor to executive functions (e.g., Johnson et al., 2008; Feeney et al., 2013; Bovier et al., 2014; Renzi et al., 2014; Vishwanathan et al., 2014). L and Z, being lipid-soluble, easily pass the blood-brain and blood-retinal barriers and tend to deposit within central nervous system tissues with high specificity (e.g., in the retina, they concentrate toward the central macular region). Past study has shown that brain concentrations of L associate with higher cognitive test scores in the elderly (Johnson et al., 2013) prompting the possibility that increasing intake could lead to benefit. Johnson et al., first in 2008 using purified supplements (Land DHA), and then again in 2011 using whole food (avocados), confirmed that such interventions could lead to improved cognitive function in older subjects (Johnson et al., 2008, 2015). In the current study, we also found that, when compared to placebo, supplementing 10 mg of L and 2 mg of Z for one year led to statistically significant increases in complex attention and cognitive flexibility (with numerical increases, but not exceeding statistical criteria for significance, $p < 0.07$, in executive function) in a sample of older adults.

In many respects, it is quite surprising that simple dietary change can lead to any improvements when considering such a homogeneous (Caucasian, upper middle-class), well-nourished and educated, group such as was sampled in our study. As with any dietary experiment, there were no true placebos, as the term is typically used in pharmaceutical studies: subjects have been exposed to the "intervention" all of their life (meaning that L is present in many normally consumed foods). Even during the intervention year, the control group maintained their normal diet which contains L and Z. Hence, any effect of L and Z on the treatment group would therefore have to be simply additive. Using this framing, the research question becomes: does adding LZ in supplement form to relatively well-nourished subjects with normal LZ intake improve cognition in a sample that was already well-educated? Education tends to attenuate any relation between diet and cognition (Akbaraly et al., 2009), mostly because more educated people tend to be well-fed (hence, our relatively high baseline MP levels) and dietary effects tend to be driven by deficiency (enhancement from normal is always much harder to achieve). The optimal sample for these studies are subjects who are less well fed, more diverse, less educated, etc. Similarly, an optimal intervention would likely include whole foods as opposed to supplements. Further, our study results, like many, are limited by convenience sampling/interventions which turns out to be, of course, of limited convenience when it comes to interpreting the actual real world effects of changing diet. Given these kind of limitations, the fact that L and Z did yield some benefit, especially when coupled with the results from other labs showing similar effects, suggests that L and Z do, in fact, have a positive effect on higher level functions of the brain.

AUTHOR CONTRIBUTIONS

Authors LR, BH, and LM contributed to research study design; LR, BH, LM, CL, and CM collected study data; MB served

as clinical coordinator; LR, BH, and LM contributed to data analysis; LR and BH drafted the initial manuscript; LH, BH, and LM primarily edited the manuscript; LR, BH, LM, CL, CM, and MB assume responsibility for the final content of the manuscript.

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REGISTRY INFORMATION

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Flavonoid Hesperidin Induces Synapse Formation and Improves Memory Performance through the Astrocytic TGF-β1

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Synapse formation and function are critical events for the brain function and cognition. Astrocytes are active participants in the control of synapses during development and adulthood, but the mechanisms underlying astrocyte synaptogenic potential only began to be better understood recently. Currently, new drugs and molecules, including the flavonoids, have been studied as therapeutic alternatives for modulation of cognitive processes in physiological and pathological conditions. However, the cellular targets and mechanisms of actions of flavonoids remain poorly elucidated. In the present study, we investigated the effects of hesperidin on memory and its cellular and molecular targets *in vivo* and *in vitro*, by using a short-term protocol of treatment. The novel object recognition test (NOR) was used to evaluate memory performance of mice intraperitoneally treated with hesperidin 30 min before the training and again before the test phase. The direct effects of hesperidin on synapses and astrocytes were also investigated using *in vitro* approaches. Here, we described hesperidin as a new drug able to improve memory in healthy adult mice by two main mechanisms: directly, by inducing synapse formation and function between hippocampal and cortical neurons; and indirectly, by enhancing the synaptogenic ability of cortical astrocytes mainly due to increased secretion of transforming growth factor beta-1 (TGF-β1) by these cells. Our data reinforces the known neuroprotective effect of hesperidin and, by the first time, characterizes its synaptogenic action on the central nervous system (CNS), pointing astrocytes and TGF-β1 signaling as new cellular and molecular targets of hesperidin. Our work provides not only new data regarding flavonoid's actions on the CNS but also shed light on possible new therapeutic alternative based on astrocyte biology.

Keywords: astrocyte, synapse, hesperidin, flavonoids, memory, TGF-β1

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Abbreviations: ACM, astrocyte-conditioned medium; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; CNS, central nervous system; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DIV, days *in vitro*; DMEM/F12, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; NOR, novel object recognition test; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PSD-95, postsynaptic density protein 95; TGF-β1, transforming growth factor beta-1.

INTRODUCTION

Synapse formation and plasticity are key properties throughout the life of animals and are crucial to their cognitive abilities, such as learning and memory. In contrast, synaptic dysfunctions are involved in the pathogenesis of several neurological conditions, particularly neurodegenerative and psychiatric disorders (Buffington et al., 2014). Currently, strong evidences point to glial cells, especially astrocytes, as active participants in synapse formation and function during development and adulthood (Eroglu and Barres, 2010; Diniz et al., 2014a). Conversely, astrocyte dysfunctions are also present in brain diseases and may drive synaptic alterations and cognitive impairments in these cases (Chung et al., 2015). Nevertheless, cellular and molecular mechanisms underlying the astrocytic control of synapses in health and disease are only now beginning to be better understood.

Astrocytes closely and dynamically control synapses by two known mechanisms: expression of adhesion molecules (Hama et al., 2004) and secretion of soluble factors, such as cholesterol (Mauch et al., 2001), thrombospondin 1 (Christopherson et al., 2005), hevin (Kucukdereli et al., 2011), glypicans (Allen et al., 2012) and transforming growth factor beta-1 (TGF-β1; Diniz et al., 2012). Recently, elegant studies have pointed to the critical role of some of these molecules secreted by adult murine and human astrocytes in the formation of functional synapses (Han et al., 2013; Zhang et al., 2016). Genetic inhibition of vesicular release from astrocytes, a pathway also involved in the secretion of synaptogenic soluble factors, strongly affects the synaptic integration of adult-born hippocampal neurons (Sultan et al., 2015). Although the secretion profile of astrocytes has been investigated recently (Orre et al., 2014; Zhang et al., 2016), the mechanisms involved in the control of astrocyte secretion are still poorly known.

Natural compounds have been successfully discovered and used in "popular medicine" for thousands of years, and their mechanisms of action and therapeutics have been better described from the past decades (Ji et al., 2009). Flavonoids constitute the richest class of polyphenolic compounds in nature, with more than 4000 varieties identified. They are widely found in fruits, leaves, grains, bark, roots, stems, flowers and even in plant-derivatives such as tea and wine. They are classified into four main groups according to their molecular structures: flavones, flavanones, catechins and antocyanins (Nijveldt et al., 2001).

Currently, a growing number of clinical trials have revealed beneficial effects of flavonoids on the human brain. Adoption of a high cocoa flavanol-containing diet can ameliorate cognitive function in elderly humans (Brickman et al., 2014), as well as a higher intake of anthocyanidins, mainly found in berries, reduces cognitive decline in older women (Devore et al., 2012). It has also been shown that chronic consumption of flavanone-rich orange juice by healthy older adults improved their cognitive function (Kean et al., 2015).

Most of the beneficial effects of flavonoids on cognition in animal models have been related to their antioxidant activity and their ability to control neuronal function, survival, synaptic plasticity and long-term potentiation (LTP; Bhullar and Rupasinghe, 2013). Indeed, neuronal-binding sites and signaling pathways have been described to be modulated by flavonoids. It is well know that isoflavones, such as genistein and daidzein, behave as phytoestrogens and are able to interact and activate estrogen receptors (Rickard et al., 2003; Adams et al., 2012). Besides, the first flavonoid described as an agonist of TrkB receptors was 7,8 dihydroxyflavone, which is able to modulate neuronal survival and function through the activation of this pathway (Jang et al., 2010; Zhang et al., 2014). Despite this information, flavonoid's cellular targets and molecular mechanisms of action remain poorly understood, especially concerning their actions on non-neuronal cells. Glial cells are highly responsive to flavonoids (Matias et al., 2016), as these compounds may: (1) modulate the redox state of astrocytes through an up-regulation of antioxidant enzymes and genes (Bahia et al., 2008); (2) reduce neuroinflammation by controlling astrocyte reactivity and secretion of pro-inflammatory cytokines (Khan et al., 2016; Rehman et al., 2017); and (3) regulate the secretion of trophic factors by astrocytes (Xu et al., 2013a; Wang et al., 2014).

We previously showed that hesperidin, the major flavanone glycoside present in citrus fruits (Garg et al., 2001), promotes neuronal differentiation and survival (Nones et al., 2011) and also enhances the neuroprotective capacity of astrocytes, by inducing them to secrete soluble factors involved in neuronal survival *in vitro* (Nones et al., 2012b). Nevertheless, the identity of astrocyte-secreted factors induced by hesperidin remains unknown, as well as its impact on astrocyte function.

Here, we hypothesized that hesperidin modulates cognitive ability of healthy adult mice by affecting the synaptogenic potential of astrocytes. By using different experimental approaches, we showed that the short-term treatment with the flavonoid ameliorates the memory performance of mice, which was followed by an increase in the density of hippocampal synapses *in vivo*. *In vitro* data indicated that this was mainly due to: (1) direct promotion of synapse formation and activity; and (2) induction of TGF-β1 secretion and its signaling pathway activation in astrocytes. Therefore, our work reveals new data regarding the actions of flavonoids in the central nervous system (CNS) and their cellular and molecular mechanisms underlying synapse formation.

MATERIALS AND METHODS

Animals

Embryonic day 14–15 and newborn (P0) Swiss mice were used for neuronal and astrocyte cultures, respectively. For *in vivo* experiments, we used 3-month-old male Swiss mice (CECAL, Fiocruz breeding colony). Adult animals were housed in groups of 5 mice in plastic cages (17 × 28 × 13 cm) with free access to certified food (Nuvital®) and tap water. Mice were kept at controlled room temperature (24 ± 2°C) and humidity, under a 12 h light-dark cycle (lights off at 6 pm) and were adapted to local conditions for at least 1 week before the

experiments. All procedures were previously approved by the local Animal Care Ethical Committee (CEUA-UFRJ, approval protocols DFBCICB053 and 004/16) and performed according to Brazilian Guidelines on Care and Use of Animals for Scientific and Teaching Purposes (DBCA), National Council for Animal Experimentation Control—CONCEA, 2013, and to Directive of the European Parliament and of the Council of the European Union of 22 September 2010 (2010/63/EU).

Drugs

The flavonoid hesperidin (C28H34O15, CAS number 520-26-3) was purchased from Sigma-Aldrich (St. Louis, MO, USA). For cell culture assays, hesperidin was diluted in dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO, USA) and used at a final concentration of 5 μM or 10 μM, as specified bellow. For *in vivo* experiments, hesperidin was prepared as previously described (Donato et al., 2014). Hesperidin was diluted in DMSO at a final concentration of 5%, a solution of 0, 25% polysorbate 80 at a final concentration of 20% and in saline solution to complete the total volume.

Experimental Design

Drug Administration and Novel Object Recognition Test

The novel object recognition test (NOR) is one of the most widely used behavioral tests to evaluate recognition memory in mice. We used a modified protocol from Antunes and Biala (2012), as described below: after 3 days of habituation sessions (10 min/day, low light condition), mice were treated intraperitoneally (i.p.) with hesperidin (10 mg/kg, n = 9 animals) or vehicle (control group, 10 mL/kg, n = 9 animals), 30 min before the training session. During this session, mice were placed in a circular arena (40 cm diameter, 30 cm high) in the presence of two equal objects for 10 min. After 48 h, they received one more i.p. injection of hesperidin or vehicle 30 min before the 5-min-long test session. Then, animals were placed back in the arena in which one of the objects was replaced by a novel one, unfamiliar object. The arena and objects were cleaned thoroughly between trials with 10% ethanol to eliminate olfactory cues. The time spent by the animals exploring the objects was recorded. Exploratory behavior was defined as sniffing or touching the objects with the front paws or nose. Total traveled distance (cm) and the mean locomotor velocity (cm/s) animals were evaluated in both sessions using MouseGlob software.

Immunohistochemistry and Quantification of Synaptic Markers

The animals were anesthetized i.p. with ketamine (100 mg/kg) and xylazine (10 mg/kg) and transcardially perfused with saline solution. Brains were removed and one hemisphere of each brain was fixed with 4% paraformaldeyde (PFA) for 24 h. Serial 10 μm saggital cryosections were mounted on glass slides and submitted to immunofluorescence labeling. Briefly, cryosections were dried, washed in Tris-buffered saline (TBS), permeabilized in 0.5% Triton X-100 for 30 min and then blocked in TBS containing 0.2% Triton-X, 5% goat serum (Invitrogen, Carlsbad, CA, USA) and 3% of BSA (Sigma Chemical Co., St. Louis, MO, USA) for 1 h at room temperature before incubation with the synaptic primary antibodies: mouse anti-synaptophysin (Chemicon International, Billerica, MA, USA; 1:1000), and rabbit anti-Drebrin A/E (Millipore, Darmstadt, DE; 1:1000) overnight at 4°C. Secondary antibodies were Alexa Fluor 546-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Molecular Probes, Paisley, UK; 1:1000), or Alexa Fluor 488-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Molecular Probes, Paisley, UK; 1:300). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma Chemical Co., St. Louis, MO, USA) and coverslips were mounted with Dako Mounting Media.

Hippocampal CA1 stratum radiatum regions were analyzed on a confocal microscope (Leica TCS SPE) with a 63× oil objective, using the same image parameters for all experimental groups. Synaptic density was evaluated by the Puncta Analyzer plug-in in NIH Image-J as previously described (Diniz et al., 2014b). For each confocal experiment (the comparison of synaptic markers between control and hesperidin groups), we imaged 2–4 equidistant brain tissue sections per mouse, with 2–4 images per section. Results represent the mean of four and three independent animals for control and hesperidin group, respectively.

Immunoblotting Assays

Protein concentration, in cell and hippocampal tissue extracts, was measured by the BCA™ Protein Assay Kit (Cole-Parmer Canada Inc., Montreal, QC, Canada). Forty micrograms of protein per lane were submitted to electrophoresis in a 10% SDS-PAGE gel and electrically transferred onto a Hybond-P PVDF transfer membrane (Millipore, Darmstadt, DE) for 1.5 h. Membranes were blocked in phosphate-buffered saline (PBS)-milk 5% for 1 h at room temperature. Next, membranes were incubated in block solution overnight in the presence of the following antibodies: mouse anti-synaptophysin (Chemicon International, Billerica, MA, USA; 1:1000), rabbit anti-postsynaptic density protein 95 (anti-PSD-95; Abcam, Cambridge, MA, USA; 1:1000), rabbit anti-Drebrin A/E (Millipore, Darmstadt, DE; 1:1000), rabbit anti-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (anti-AMPAR; Abcam, Cambridge, MA, USA; 1:1000), rabbit anti-glial fibrillary acidic protein (anti-GFAP; Dako, Cytomation, Glostrup, Denmark; 1:5000), rabbit anti-phospho-SMADs 2/3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200), mouse anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH; Abcam, Cambridge, MA, USA; 1:1000), mouse anti-α-Tubulin (Sigma Chemical Co., St. Louis, MO, USA; 1:5000), rabbit anti-β-actin (Abcam, Cambridge, MA, USA; 1:1000). Membranes were incubated for 1 h with IRDye 680CW goat anti-mouse antibody and IRDye 800CW goat anti-rabbit antibody (LI-COR, Lincoln, USA; 1:20,000). After washing, membranes were scanned and analyzed using Un-Scan-It gel version 6.1 (Silk Scientific, Inc., Orem, UT, USA).

Neuronal Cultures and Treatment

Neuronal cortical cultures were prepared as described previously by our group (Diniz et al., 2012). Briefly, the cerebral cortices of embryonic day 14–15 Swiss mice were removed, meninges were

carefully removed, neural tissue was dissociated in Neurobasal medium (Invitrogen, Carlsbad, CA, USA) and the cells were plated at a density of 75,000 per well of 13 mm diameter and 500,000 cells per well of 35 mm diameter, onto glass coverslips previously coated with poly-L-lysine (10 μg/mL, Sigma Chemical Co., St. Louis, MO, USA). Cultures were maintained in Neurobasal medium supplemented with B-27, penicillin, streptomycin, fungizone, L-glutamine and cytosine arabinoside (0.65 μM, Sigma Chemical Co., St. Louis, MO, USA). Cultures were kept at 37°C in a humidified 5% CO_2, 95% air atmosphere for 6 days or 12–14 days *in vitro* (DIV).

Neuronal cultures were submitted to three different type of treatments and in all of them hesperidin was used at a final concentration of 5 μM: (1) to analyze cell death, cultures were treated on the 3 DIV for 72 h; (2) to analyze synapse formation, cultures were treated for 24 h on the 12 DIV; and (3) to analyze synaptic activity, cultures were incubated with hesperidin throughout the 12 DIV.

Murine Astrocyte Cultures and Treatment

Primary cortical astrocyte cultures were derived from newborn Swiss mice as previously described (Diniz et al., 2012). Briefly, the mice were decapitated, the cerebral cortices were removed and the meninges were carefully stripped off. Tissues were maintained in Dulbecco's minimum essential medium (DMEM) and nutrient mixture F12 (DMEM/F12, Invitrogen, Carlsbad, CA, USA), enriched with glucose (3.3×10^{-2} M), glutamine (2×10^{-3} M) and sodium bicarbonate (0.3×10^{-2} M) and dissociated into single cells. Dissociated cells were plated onto glass coverslips in a 24-well plate (Corning Incorporated, Corning, NY, USA), previously coated with poly-L-lysine (Sigma Chemical Co., St. Louis, MO, USA), in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). The cultures were incubated at 37°C in a humidified 5% CO_2, 95% air atmosphere. Cell culture medium was changed 24 h after plating and, subsequently, every 2 days, until reaching confluence, which usually occurred after 7–10 days. After that, cells were subjected to one passage to generate purified astrocyte cultures (secondary cultures), which was constituted by more than 95% of GFAP-positive cells.

Astrocytes were treated with 10 μM hesperidin, as previously described (Nones et al., 2011, 2012b), for different periods (0, 30 min and 2 h) for kinetic analysis of nuclear translocation and phosphorylation of SMADs 2/3.

Astrocyte-Conditioned Medium (ACM)

Confluent secondary astrocyte cultures were washed to eliminate residual serum and incubated for an additional day in DMEM/F12 serum-free medium. Then, the culture medium was replaced by DMEM/F12 supplemented with 10 μM hesperidin to generate astrocyte-conditioned medium (ACM)-Hesperidin, or 0.1% DMSO, ACM-Control, and kept for 24 h. After that, the cultures were washed and the medium was replaced by serum-free medium and maintained for additional 24 h. Those ACM were collected, centrifuged at $1000\times g \times 10$ min to remove cellular debris and used immediately or stored in aliquots at −70°C for further use.

To investigate the effect of ACM on synapse formation, 12 DIV-neuronal cultures were treated with the ACM-Control or ACM-Hesperidin for 3 h. For the neutralization of TGF-β1 activity in the ACMs, the medium was pre-incubated with 1 μg/mL of neutralizing antibody against TGF-β1 (Abcam, Cambridge, MA, USA) for 30 min at room temperature. After that, neuronal cultures were simultaneously maintained in the presence of ACM and the neutralizing antibody for 3 h, followed by fixation and immunostaining for synaptic proteins.

Immunocytochemistry

Cultured cells were fixed with 4% PFA for 10 min and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. After, the cells were blocked with 3% bovine serum albumin and 5% normal goat serum (Sigma, St. Louis, MO, USA) in PBS (blocking solution) for 1 h and, then, incubated overnight at 4°C with the specified primary antibodies diluted in blocking solution. The primary antibodies were mouse anti-β-tubulin III (Promega, Madison, WI, USA; 1:1000), rabbit anti-cleaved caspase-3 (Cell Signaling, Beverly, MA, USA; 1:50), rabbit anti-GFAP (Dako Corporation, Glostrup, Denmark; 1:1000), mouse anti-synaptophysin (Chemicon International, Billerica, MA, USA; 1:1000), rabbit anti-PSD-95 (Cell Signaling Technology, Beverly, MA, USA; 1:100), mouse anti-SMADs 2/3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200). After primary antibody incubation, the cells were extensively washed with PBS and incubated with secondary antibodies for 2 h at room temperature. Secondary antibodies were Alexa Fluor 546 (goat anti-rabbit IgG, goat anti-mouse IgG; Molecular Probes, Paisley, UK; 1:1000) or Alexa Fluor 488 (goat anti-rabbit IgG, goat anti-guinea pig IgG, goat anti-mouse IgG; Molecular Probes, Paisley, UK; 1:300). Nuclei were counterstained with DAPI (Sigma Chemical Co., St. Louis, MO, USA). The cells were observed with the aid of a TE2000 Nikon microscope.

Presynaptic Activity Analysis

The presynaptic activity assay was performed as previously described (Diniz et al., 2012). Neurons were washed with extracellular solution (150 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4) and incubated with a despolarazing solution, a high potassium solution, (97 mM NaCl, 57 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4) containing FM1-43 (5 μM; Molecular Probes, Paisley, UK) at room temperature for 5 min. Then, neurons were washed with extracellular solution and fixed for observation in a TE2000 Nikon microscope.

Synaptical Puncta Analysis

Synapse analysis was performed as previously described (Diniz et al., 2012). Briefly, neurons were randomly identified and selected if nuclei staining (DAPI staining) were, at least, two diameters away from the neighboring neuronal nucleus. Neuronal cultures were analyzed for immunostaining

for the pre- and post-synaptic markers, synaptophysin and PSD-95, respectively. The green and red channels were merged and quantified using the Puncta Analyzer plug-in in NIH Image-J as previously described (Diniz et al., 2012). A number of 10–15 images was analyzed and experiments were done in duplicate. Each result represents the mean of at least three independent neuronal cultures.

In-Cell Western

Secondary astrocytes were grown in 96 wells-plate for 2 days in DMEM/F12 supplemented with 10% FBS. After reaching confluence, astrocytes were washed and maintained in serum-free medium for 24 h and, then, treated with 10 μM hesperidin or DMSO 0.1% for 2 h. After that, cells were fixed with 4% PFA for 20 min, washed three times with PBS-containing 0.1% triton X-100 and incubated with the Odyssey blocking buffer (LI-COR, Lincoln, NE, USA) for 1.5 h at room temperature. Primary antibodies were: anti-mouse TGF-β1 (Abcam, Cambridge, MA, USA; 1:100) and anti-rabbit cyclophilin B (Sigma Chemical Co., St. Louis, MO, USA; 1:1000) overnight at 4°C. Plates were washed with PBS containing 0.1% tween-20 for three times, followed by incubation with IRDye 680CW goat anti-rabbit and IRDye 800CW goat anti-mouse antibodies (LI-COR, Lincoln, NE, USA; 1:800) for 1 h at room temperature. Plates were scanned with the Odyssey Infrared Imaging System and analyzed using the program Un-Scan-It gel version 6.1 (Silk Scientific, Inc., Orem, UT, USA).

Statistical Analysis

Statistical analysis was done by Student's *t*-test and one-way analysis of variance (ANOVA) followed by Newman-Keuls Multiple Comparison Test, using the Graphpad software version 5.0 (GraphPad Software, La Jolla, CA, USA). A confidence interval of 95% was used, and a *P*-value <0.05 was considered statistically significant. Data are reported as means ± SEM. For each result, exact number of independent experiments and animal samples are described in the legend and in the "Materials and Methods" Section.

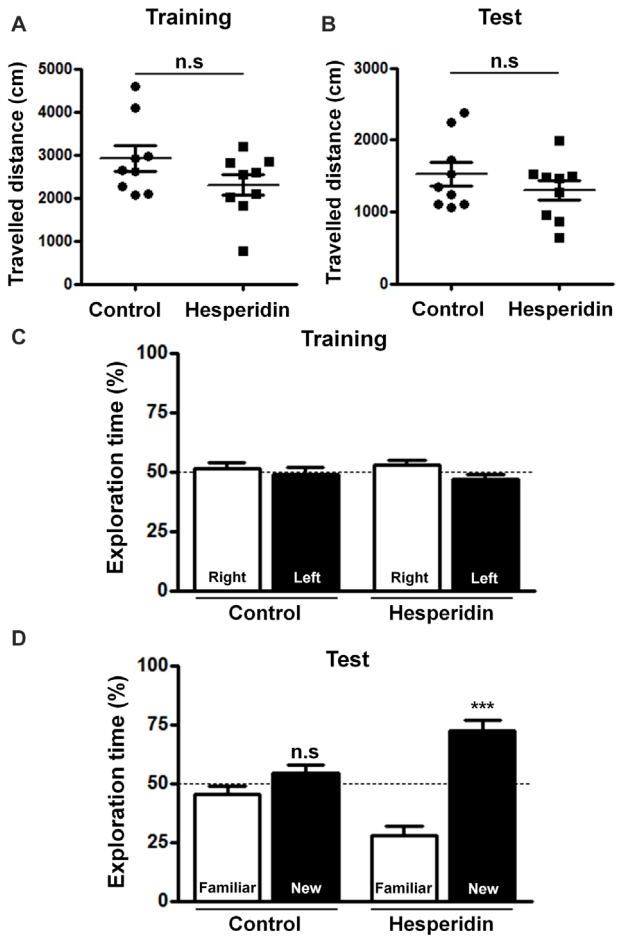

FIGURE 1 | Hesperidin improves memory performance in adult mice. Animals were treated i.p with vehicle (control group) or 10 mg/kg of hesperidin before the training session and, then again, before the object recognition test. Traveled distance during the training **(A)** and the test session **(B)** and recognition memory of objects during training **(C)** and test session **(D)** were measured. Hesperidin significantly improved recognition memory performance of mice. ***$p < 0.001$, $n = 9$ per experimental group. Student's test compares the mean exploration time for each object with a fixed value of 50%.

RESULTS

Hesperidin Promotes Hippocampal Synaptogenesis and Improves Memory in Healthy Mice

Beneficial functions of flavonoids on cognition in animal models for neural disorders have been subjected of several studies (Williams and Spencer, 2012). However, little is investigated about their possible beneficial effects in non-pathological conditions. In order to test whether hesperidin affects memory performance in healthy adult mice, we used a modified protocol from the NOR, by using a longer retention interval between the training and test phase.

We have not observed any differences in the traveled distance (**Figures 1A,B**), total exploration time (Supplementary Figures S1A,B) and mean locomotor velocity (Supplementary Figures S1C,D) between the hesperidin and control groups during training and test sessions, indicating that hesperidin has no effect on locomotor/exploratory activities. Additionally, during training, mice from both groups had no preference for the left or right objects (**Figure 1C**). However, during the test, mice treated with hesperidin spent more time exploring the new object than the familiar one, compared with vehicle treatment (**Figure 1D**). Results suggest that hesperidin increases the long-term memory performance of healthy adult mice.

In order to better understand the underlying mechanisms of hesperidin actions on memory, we analyzed the distribution and levels of synaptic proteins in the hippocampus. We observed that hesperidin remarkably increased synaptophysin and drebrin immunoreactivity (**Figures 2A,B**), as well as the number of synapses, represented by the colocalization of those two markers,

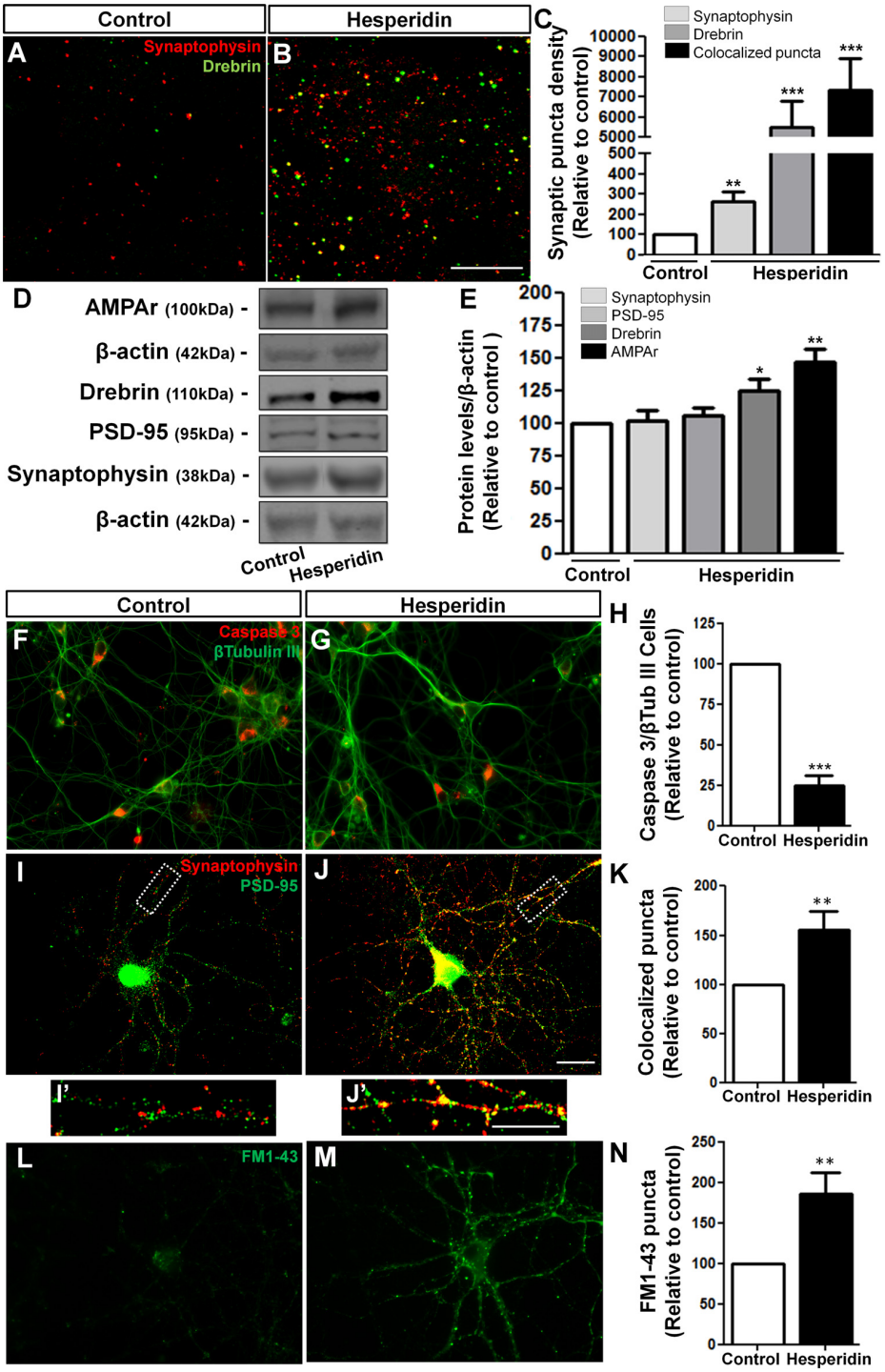

FIGURE 2 | Hesperidin increases synapse formation *in vivo* and *in vitro*. Adult mice treated i.p with vehicle (control group) or 10 mg/kg of hesperidin were submitted to immunohistochemistry analysis for the pre- and post-synaptic markers, synaptophysin and drebrin, respectively **(A–C)** in the hippocampal CA1 area, and western blotting analysis of the levels of the synaptic proteins, synaptophysin, postsynaptic density protein 95 (PSD-95), drebrin and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) **(D,E)**. Increased synapse formation **(A–C)** and levels of synaptic proteins **(D,E)** were observed in the hippocampus of mice treated with hesperidin. Cortical neurons treated with hesperidin were analyzed by immunocytochemistry for Cleaved Caspase 3 and β-Tubulin-III **(F–H)**, synaptophysin and PSD-95 **(I–K)** and were submitted to the FM1-43 assay **(L–N)**. Neurons treated with the flavonoid showed reduced cell death **(F–H)**, and increased synaptogenesis **(I–K,I′,J′)**, as well as increased pre-synaptic activity **(L–N)**. Scale bars, 20 μm, 20 μm and 10 μm, respectively. $^{*}p < 0.050$, $^{**}p < 0.010$ and $^{***}p < 0.001$, n = 3–5 animals per experimental group for the *in vivo* experiments and n = 3–6 independent neuronal cultures for the *in vitro* experiments. Student's *t* test.

in the hippocampal CA1 area (**Figure 2C**). These effects were accompanied by a 25% and 46% increase in the total protein levels of drebrin and AMPA receptor in the hippocampus, respectively (**Figures 2D,E**).

To test the direct action of hesperidin on neurons and synaptogenesis, we first analyzed the effects of hesperidin in the survival of cultured cerebral cortical neurons. To do that, neurons cultured for 3 DIV were then treated for 72 h with hesperidin. Under normal culture condition, the rate of neuronal death is low, approximately 5%. We observed that neurons treated with hesperidin showed increased survival, represented by a reduction in the number of cleaved Caspase 3/β-tubulin III positive cells compared to control (**Figures 2F–H**). To analyze synapses, 12 DIV neurons were treated for 24 h with the flavonoid. We observed that hesperidin increased the number of double immunostaining puncta for the synaptic markers, synaptophysin and PSD-95, by 55%, suggesting that hesperidin also promotes synapse formation *in vitro* (**Figures 2I–K**). The synaptogenic effect of hesperidin was not related to an increase in the levels of synaptophysin and PSD-95 proteins *in vitro* (Supplementary Figure S2).

We next investigated whether hesperidin affects the pre-synaptic activity of cultured neurons. To do that, cerebral cortical neurons were treated with hesperidin throughout the 12 DIV and synaptic activity analyzed by the FM1-43 assay. We observed an 85% increase in the number of FM1-43 puncta on neurons treated with hesperidin (**Figures 2L–N**). Together, these results corroborate the already described neuroprotective function of hesperidin, as well as indicate its novel effect on the synapse formation and function *in vivo* and *in vitro*.

Hesperidin Increases the Synaptogenic Potential of Astrocytes through the Modulation of Astrocytic TGF-β1 Signaling

Although several studies have showed that glial cells are responsive to flavonoids in many experimental disease models (Bahia et al., 2008; Lan et al., 2016), there is a lack of evidences regarding the effects of flavonoids on astrocyte morphology and physiology in the healthy brain. To elucidate this question, we firstly analyzed the effect of hesperidin treatment on astrocytes from the hippocampal CA1 stratum radiatum of adult mice. The quantification of GFAP positive cells revealed that the treatment had no effect on the number of astrocytes (**Figures 3A–C**), as well as on the level of GFAP in the hippocampus compared to vehicle (**Figure 3G**).

In addition, we also analyzed the effect of hesperidin in cultured cerebral cortical astrocytes. To do that, astrocytes cultures were treated with 10 μM of hesperidin for 2 h, previously to morphological analysis. Under control and hesperidin treatment conditions astrocytes presented similar flat-protoplasmatic morphology, characteristic of cultured astrocytes (**Figures 3D,E**). Similarly, number of GFAP positive cells (**Figure 3F**) and the levels of GFAP protein (**Figure 3G**) were not affected by the flavonoid. Together, these data suggest that hesperidin, at least in a non-pathological model, does not affect astrocytic morphology and reactivity *in vitro* and *in vivo*.

We previously showed that flavonoids, including hesperidin, enhanced the neuroprotective capacity of astrocytes through an increase in secretion of astrocyte-soluble factors (Nones et al., 2012b). However, there is no evidence regarding whether the synaptogenic ability of astrocytes is affected by flavonoids and the identity of astrocyte-derived soluble factors in this context.

TGF-β1 is a pleiotropic factor expressed by astrocytes from different brain regions (Buosi et al., 2017), widely secreted by cortical astrocytes and involved in synapse formation and function (Diniz et al., 2012, 2014b; Araujo et al., 2016). To investigate whether hesperidin affects the synaptogenic ability of astrocytes, we treated 12 DIV neuronal cultures with the ACM-Control or ACM-Hesperidin for 3 h, and then analyzed the number of synapses. ACM-Control increased the number of synapses by 80% (**Figures 4A,B,F**). Surprisingly, ACM-Hesperidin was more effective in promoting synapse formation than ACM-Control, with a two-fold increase in relation to ACM-Control (**Figures 4B,D,F**).

To verify the involvement of TGF-β1 in the synaptogenic ability of astrocytes, we pre-incubated the ACMs with neutralizing antibody against TGF-β1 (αTGF-β1) before the treatment of neuronal cultures with the ACMs. Although we have observed a decreased synaptogenic effect of ACM-Control incubated with αTGF-β1, this effect was not statistically significant (**Figures 4C,F**). However, effect of ACM-Hesperidin was strongly reduced by addition of αTGF-β1 (**Figures 4D–F**). These results suggest that hesperidin improves the synaptogenic potential of astrocytes and points to TGF-β1 as an important synaptogenic factor secreted by astrocytes in response to hesperidin.

In order to address the underlying mechanisms of hesperidin action on the synaptogenic potential of astrocytes, we treated confluent astrocyte cultures with hesperidin for 2 h and analyzed the levels of TGF-β1in these cells. We observed a slightly, but significant, increase in the level of TGF-β1 in astrocytes in response to hesperidin (**Figure 5A**).

To investigate whether hesperidin activates the TGF-β/SMADs signaling pathway, we performed kinetics assays in astrocytes treated with hesperidin for 0, 30 min and 2 h. We found a 70% increase in the phospho-SMADs 2/3 levels in astrocytes treated for 2 h with the flavonoid (**Figure 5B**). These findings were supported by the increased nuclear translocation of SMADs 2/3 in hesperidin treated-astrocytes, a hallmark of TGF-β/SMADs pathway activation (**Figures 5C–E**). Further, we also observed a 30% increase of phospho-SMADs 2/3 levels in the hippocampus of mice injected with hesperidin (**Figure 5F**).

Together, these results indicate that the TGF-β1 signaling is modulated by hesperidin *in vitro* and *in vivo* and TGF-β1 is one of the indentified molecules that mediates the synaptogenic action of hesperidin-treated astrocytes.

DISCUSSION

In the current study, we described the beneficial effects of the short-term treatment with hesperidin on memory of healthy adult mice and its underlying cellular mechanisms. We showed

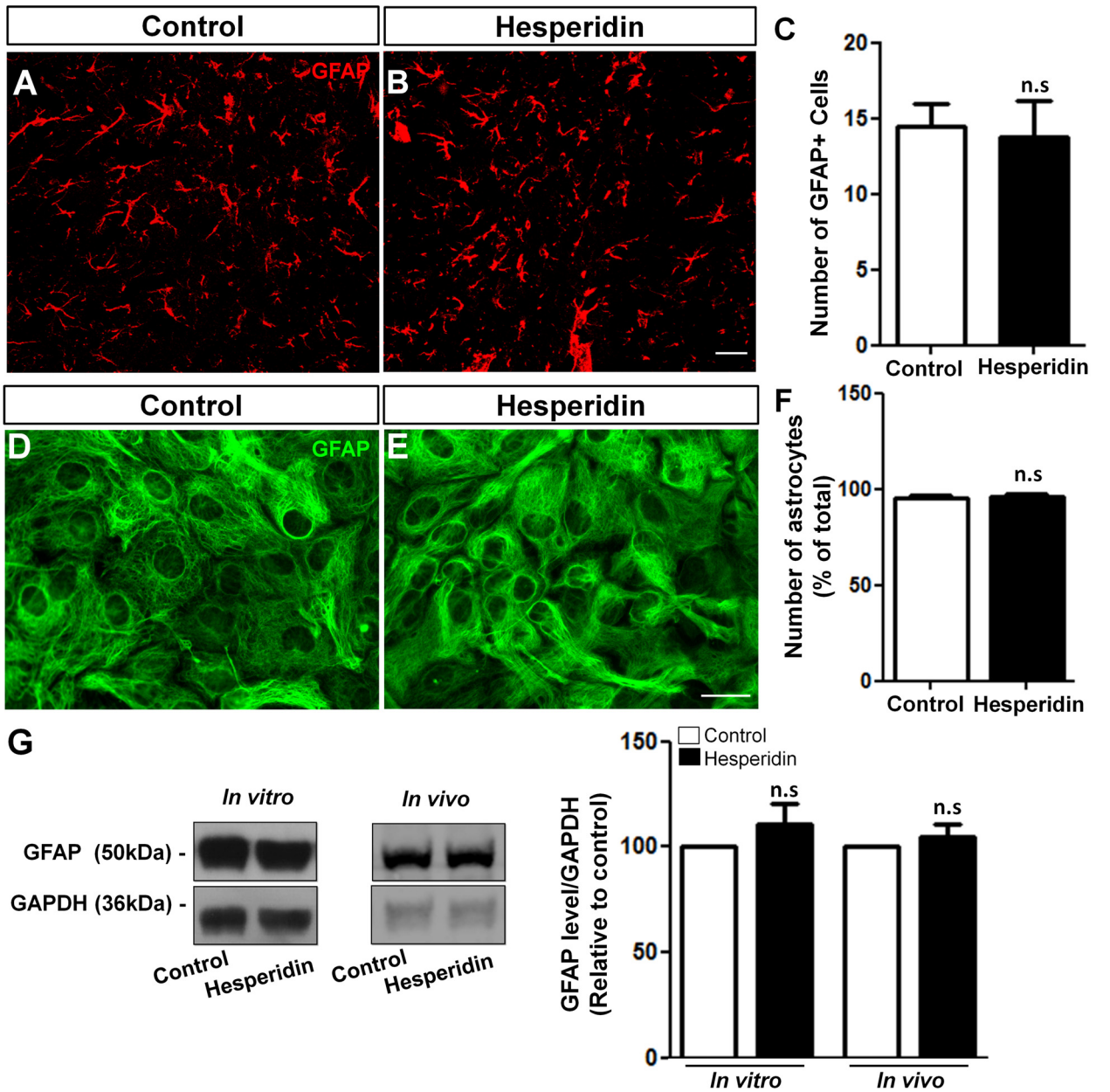

FIGURE 3 | Hesperidin does not affect astrocytes number and glial fibrillary acidic protein (GFAP) levels. Adult mice treated i.p with vehicle or 10 mg/kg of hesperidin were submitted to immunohistochemistry analysis for GFAP **(A–C)** in the hippocampal CA1 area, and western blotting analysis of the level of GFAP **(G)**. Cultured cortical astrocytes were treated with hesperidin and analyzed by immunonocytochemistry **(D–F)** and western blotting for GFAP **(G)**. The number of astrocytes and the levels of GFAP did not change between control and hesperidin groups *in vivo* and *in vitro*. Scale bar, 20 μm, respectively. n = 4–6 animals per experimental group and n = 3 independent astrocyte cultures. Student's t test.

that hesperidin promotes synaptogenesis and increases the levels of synaptic proteins in the hippocampus. We further elucidated its cellular targets and mechanisms using *in vitro* approaches and we found that hesperidin exerts two distinct effects on synapses: a direct effect, by increasing synapse formation and pre-synaptic activity; and an indirect effect, by enhancing the synaptogenic potential of astrocytes through TGF-β1 secretion and activation of TGF-β1-SMADs 2/3 signaling.

Evidences suggest that long-term ingestion of flavonoid-rich food potentially prevents age-related cognitive decline in humans and in several murine models (Kean et al., 2015; Matias et al., 2016). Most of the data are related to the potential of flavonoids to prevent cognitive deficits or rescue learning and memory impairments in pathological contexts, including animal models for Alzheimer's (Gu et al., 2016), Parkinson's (Antunes et al., 2014) and Huntington's diseases (Sandhir and Mehrotra, 2013)

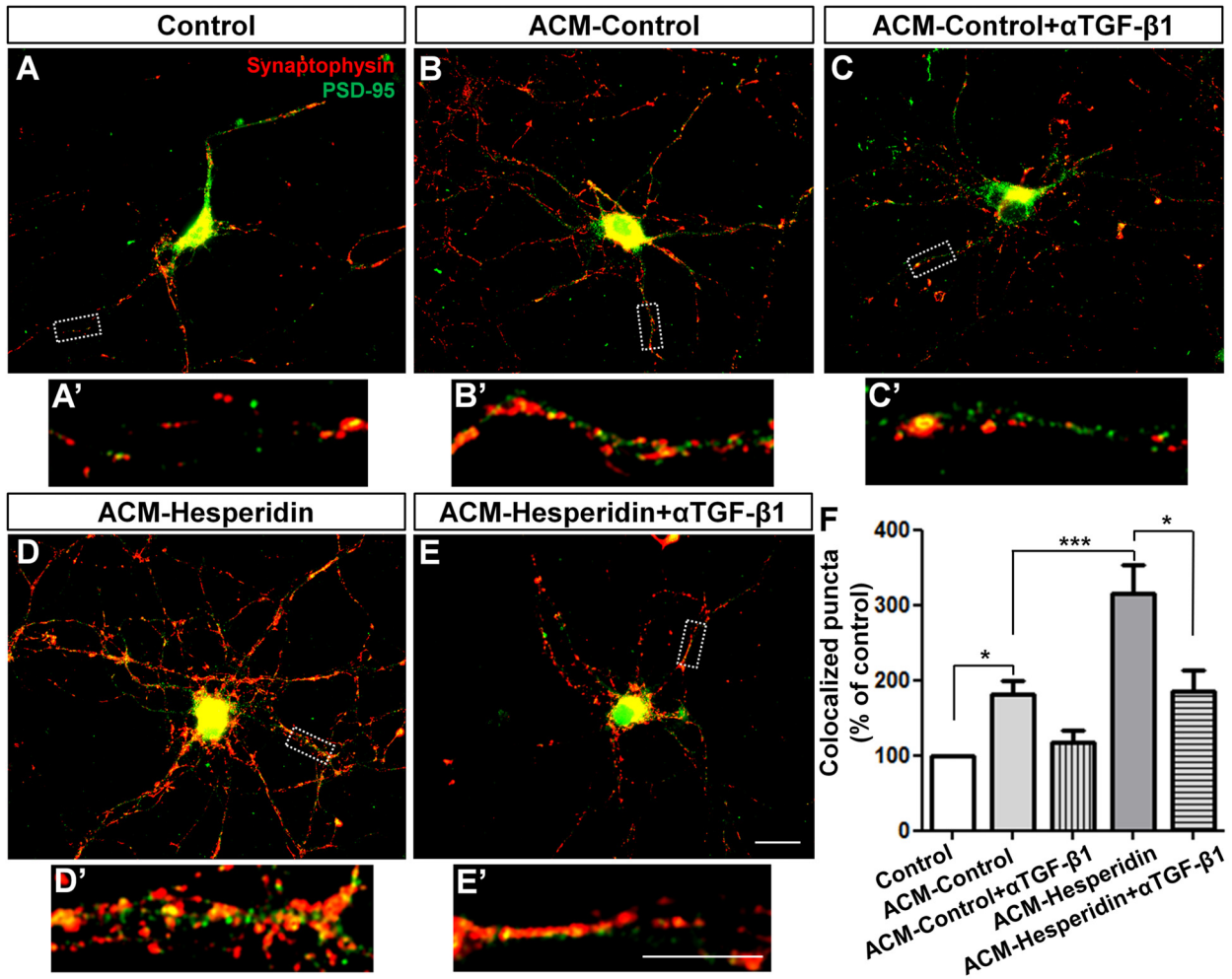

FIGURE 4 | The astrocytic transforming growth factor beta-1 (TGF-β1) mediates the synaptogenic action of astrocytes treated with hesperidin. Twelve days in vitro (12 DIV) cortical neurons were maintained for 3 h in the presence of Dulbecco's minimum essential medium (DMEM)/F12 (Control; **A**,**A′**), astrocyte conditioned medium (ACM-Control; **B**,**B′**) or astrocyte conditioned medium from astrocytes treated with hesperidin (ACM-Hesperidin; **D**,**D′**), or simultaneously with a neutralizing antibody against TGF-β1 (ACM-Control + αTGF-β1; **C**,**C′**, and ACM-Hesperidin + αTGF-β1; **E**,**E′**) synapse formation was evaluated by immunocytochemistry for the synaptic markers, synaptophysin and PSD-95. ACM-Control increased the number of synapses by two times relative to control and ACM-Hesperidin enhanced synapse formation by two times in relation to ACM-Control; whereas depletion of TGF-β1 partially blocked this effect. Scale bars 20 μm **(E)** and 10 μm **(E′).** $^{*}p < 0.050$ and $^{***}p < 0.001$; comparisons among multiple groups were analyzed using a one-way analysis of variance (ANOVA) followed by Newman-Keuls *post hoc* tests. n = 3–6 independent astrocyte cultures.

and in Multiple Sclerosis (Makar et al., 2016). Nevertheless, it has been showed that these compounds may also improve the cognitive ability of healthy young and old animals (van Praag et al., 2007; Williams et al., 2008; Rendeiro et al., 2013). Despite of this, cellular and molecular mechanisms of flavonoid's actions in these cases remain poorly understood, especially in relation to their effects in the healthy brain.

The most well described mechanism by which flavonoids can ameliorate or rescue cognitive ability is through the modulation of synaptic plasticity and function. *In vivo* evidences have showed that the chronic administration of 7,8-dihydroxyflavone improved spatial memory and mitigated dendritic spine and AMPARs loss in the hippocampus of a mouse model for Alzheimer's disease (Gao et al., 2016). Similar results have been reported in different animal models for neural diseases and in cognitively impaired aged rats treated chronically with flavonoids (Zeng et al., 2012; Tian et al., 2015; Gu et al., 2016). However, there are fewer evidences concerning the effects of flavonoids on synapses in non-pathological models.

Here, we showed that the memory performance improvement of healthy mice treated acutely with hesperidin were accompanied by a higher number of structural synapses in the hippocampal CA1 area and protein levels of drebrin and AMPARs in their hippocampus. In agreement with our data, studies have demonstrated that the dietary supplementation of flavonoids, particularly anthocyanins and flavanols, enhances spatial memory performance of healthy mice,

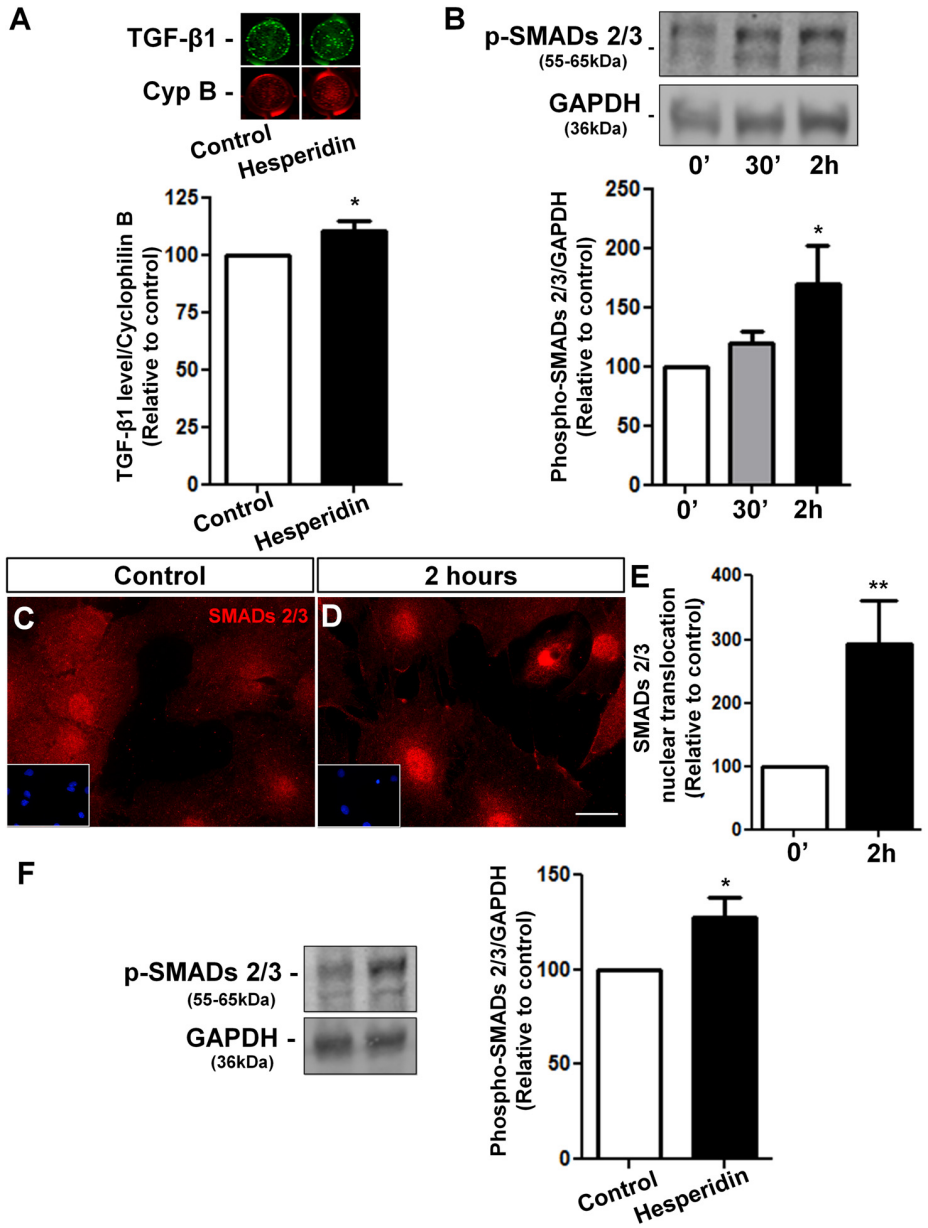

FIGURE 5 | Hesperidin increases TGF-β1 levels and activates its signaling pathway in cultured astrocytes and in the hippocampus. Cortical astrocytes were treated with hesperidin for 2 h and submitted to In-Cell western analysis for TGF-β1 **(A)**. Activation of TGF-β1 signaling pathway was analyzed by Western blotting assays for phospho-SMADs 2/3 **(B)** and by evaluation of nuclear translocation of SMADs 2/3 **(C–E)**. Levels of phospho-SMADs 2/3 were also analyzed in the hippocampus of adult mice treated with vehicle or hesperidin **(F)**. Hesperidin slightly increases the levels of astrocytic TGF-β1 and strongly induced phosphorylation and activation of the SMADs 2/3 signaling pathway *in vitro* and *in vivo*. $^{*}p < 0.050$ and $^{**}p < 0.010$. Student's *t* test was used in **(A,E,F)**. Comparisons among multiple groups were analyzed using a one-way ANOVA followed by Newman-Keuls *post hoc* test in **(B)**. $n = 4–5$ independent astrocyte cultures for the *in vitro* experiments and $n = 4–6$ animals per experimental group for the *in vivo* experiments.

events related to increased levels of hippocampal BNDF (Williams et al., 2008; Rendeiro et al., 2013). Interestingly, increased levels of NMDA-NR2B receptor have also been showed in the hippocampus of healthy young rats treated with a mix of anthocyanins and flavanols (Rendeiro et al., 2014). Therefore, our results reinforce the involvement of glutamatergic receptors and downstream signaling pathways, as well as the proper reorganization of pre- and post-synaptic proteins that could strength hippocampal connectivity and facilitate LTP in the effects of flavonoids on synapse formation.

Although *in vivo* evidences have shown many beneficial effects of hesperidin on the CNS, its cellular targets and pathways still need to be elucidated. Here, we showed that hesperidin

promoted survival and synapse formation and activity between cultured cortical neurons. Our previous data indicated that hesperidin increases neurogenesis by inducing differentiation of neural progenitors and supports neuronal survival *in vitro* (Nones et al., 2011). Whereas the neuroprotective property of hesperidin has been extensively investigated (Menze et al., 2012; Hong and An, 2015), its actions on synapses are less known. A screening of 65 flavonoids showed that hesperidin, among eight flavanones evaluated, was the only one able to increase the expression of synaptotagmin, but not PSD-95, in cortical neurons *in vitro* (Xu et al., 2013b). Here, we analyzed the total levels of synaptic proteins in neurons treated with hesperidin, and, although we have not observed differences in the levels of synaptophysin and PSD-95, we observed a higher number of synapses and pre-synaptic activity between cells, suggesting that the flavonoid modulates synapse formation and possibly synaptic function *in vitro*.

Given the remarkable role of astrocytes on synapse formation, maintenance and function, a better characterization of flavonoids actions on these cells would contribute to our knowledge about how synapses form and function. In the current study, we showed that hesperidin does not change the number of astrocytes and levels of GFAP in the hippocampus of adult mice neither in culture. These data are apparently against those obtained from murine models for age-related diseases. Under pathological contexts, flavonoids may attenuate the inflammatory profile of astrocytes, as observed by reduced expression of GFAP gene and down-regulation of pro-inflammatory pathways (Currais et al., 2014; Rehman et al., 2017). Previous data from our group, however, also showed that another flavonoid, casticin, does not affect morphology and proliferation rate of cortical astrocytes (de Sampaio e Spohr et al., 2010). Therefore, these data suggest that the morphological profile of astrocytes is not modified by flavonoids under non-pathological conditions. In contrast, in the injured brain, flavonoids may rescue physiological phenotype of these cells (Sharma et al., 2007) including up-regulation of their antioxidant capacity (Bahia et al., 2008; Park et al., 2011), events that were related to prevention of neuronal cell death *in vitro* (Vafeiadou et al., 2009; Nones et al., 2012b) and that contribute to the rescue of cognitive function in disease models (Currais et al., 2014; Rehman et al., 2017).

One of the main mechanisms by which astrocytes modulate neuronal connectivity and function is through the secretion of trophic factors (Diniz et al., 2014a). We and other workers have shown that flavonoids may modulate the secretion of neurotrophic factors by astrocytes (de Sampaio e Spohr et al., 2010; Nones et al., 2012b; Xu et al., 2013a), although the identity of these factors is not fully known. A screening of thirty-three flavonoids, including hesperidin, showed that most of them are able to increase the astrocytic levels of NGF, GDNF and BDNF secretion (Xu et al., 2013a). In agreement, *in vivo* studies showed that the long-term treatment of adult mice with hesperidin results in higher levels of hippocampal BDNF, an event related to the antidepressant-like effect of hesperidin (Donato et al., 2014; Antunes et al., 2016). Here, we demonstrated that hesperidin increased the synaptogenic potential of cortical astrocytes *in vitro* by inducing TGF-β1 synthesis and activation of its signaling pathway and in the hippocampus of adult mice.

TGF-β1 is a pleiotropic cytokine involved in several steps of brain development and function, including astrocyte generation and synapse formation (Diniz et al., 2014a; Stipursky et al., 2015). TGF-β1 signaling is expressed by astrocyte progenitors in the cerebral cortex during brain development (Stipursky and Gomes, 2007; Stipursky et al., 2012) and by mature astrocytes in different brain regions during synaptogenic period (Araujo et al., 2016; Buosi et al., 2017). Recently, we demonstrated that astrocytes control the balance between excitatory and inhibitory synapses in the cerebral cortex by activating distinct downstream TGF-β1 pathways (Diniz et al., 2012, 2014b; Araujo et al., 2016). Here, we demonstrated that TGF-β1 is essential for the increased synaptogenic potential of hesperidin-treated astrocytes. This is supported by the observation that TGF-β1 neutralizing antibody assays impaired the synaptogenic property of the hesperidin-treated astrocytes. Further, we also showed that hesperidin activates signaling pathway of the astrocytic TGF-β1. Together, we firstly provided evidence that flavonoids may improve the synaptogenic ability of astrocytes through an up-regulation of secreted-soluble factors known to be involved in synapse formation and function.

Since the effect of hesperidin on the astrocytic SMADs 2/3 activation was time-dependent, we suggest that the flavonoid does not directly binds or actives the TGF-β receptor, but rather, it probably triggers an indirect effect through the modulation of non-canonical pathways of TGF-β. The crosstalk between TGF-β with other signaling pathways is well described, such as with the mitogen-activated protein kinases (MAPKs) pathways. In these cases, activation of TGF-β receptors leads to downstream activation of MAPKs; inversely, these kinases can also regulate SMADs phosphorylation (Funaba et al., 2002; Derynck and Zhang, 2003). Indeed, previous data from our group showed that hesperidin is able to activate neuronal MAPK and PI3K pathways, which are involved in the hesperidin neuroprotective action (Nones et al., 2012a). In agreement, other flavanones, such as hesperitin and 5-nitro-hesperitin, have been described to prevent neuronal death through the activation of pro-survival Akt and ERK1/2 in cortical neurons (Vauzour et al., 2007). Together with our results, these evidences raise the possibility that hesperidin positively modulates the astrocytic TGF-β1/SMADs 2/3 signaling through involvement of non-canonical pathways. The full elucidation of this mechanism will deserve further investigation.

While most of the evidences concerning flavonoids' actions on the CNS refer to the long-term treatment or dietary intake of flavonoids, here, we shed light on the beneficial effects of these compounds in a short-term intervention. In this perspective, flavonoids present the potential to be useful as pharmacological drugs capable to directly modulate CNS function and repair. Collectively, we described hesperidin as a new molecule able to improve the recognition memory

performance in healthy adult mice, an event accompanied by an increase in hippocampal synapse formation. *In vitro* assays indicated that hesperidin exerts such effects on synapse through 2 distinct mechanisms: direct on neurons and indirect, by enhancing astrocytic synaptogenic potential and TGF-β1 signaling in these cells. These results strengthen the potential of flavonoids as new therapeutic approaches for the prevention and/or treatment of neurological disorders, which are accompanied by synaptic dysfunction and loss of memory and point to the potential of astrocytes as targets for these natural compounds.

AUTHOR CONTRIBUTIONS

IM, LPD, AB, GN, JS and FCAG: designed experiments. IM, LPD and AB: performed experiments. IM, LPD, AB, GN, JS and FCAG: analyzed data. IM, GN, JS and FCAG: wrote the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnagi.2017.00184/full#supplementary-material

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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The Role of Retinal Carotenoids and Age on Neuroelectric Indices of Attentional Control among Early to Middle-Aged Adults

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One apparent consequence of aging appears to be loss of some aspects of cognitive control. This loss is measurable as early as mid-adulthood. Since, like many aspects of cognition, there is wide variance among individuals, it is possible that behavior, such as one's diet, could drive some of these differences. For instance, past data on older humans and non-human primates have suggested that dietary carotenoids could slow cognitive decline. In this study, we tested how early such protection might manifest by examining a sample (n = 60) of 25–45 year olds. Carotenoid status was assessed by directly measuring macular pigment optical density (MPOD) which has shown to be highly correlated with the primary carotenoid in brain, lutein. Cognitive control was measured using event-related potentials during the performance of cognitive control tasks designed to tap into different aspects of attentional (i.e., selective attention, attentional inhibition, and response inhibition) control. Our results showed that, across participants, MPOD was related to both age and the P3 component of participants' neuroelectric profile (P3 amplitude) for attentional, but not response, inhibition. Although younger adults exhibited larger P3 amplitudes than their older adult counterparts, older subjects with higher MPOD levels displayed P3 indices similar to their younger adult counterparts in amplitude. Furthermore, hierarchical regression analyses showed that age was no longer a significant predictor of P3 amplitude when MPOD was included as a predictor in the model, suggesting that MPOD may partially contribute to the relationship between age and P3 amplitude. In addition, age and MPOD were shown to have independent associations with intraindividual variability of attentional control, such that younger individuals and individuals with higher MPOD showed less intraindividual variability. These results show a relationship between retinal carotenoids and neuroelectric indices underlying cognitive control. The protective role of carotenoids within the CNS may be evident during early and middle adulthood, decades prior to the onset of older age.

Keywords: cognitive aging, macular pigment optical density, lutein, carotenoids, event-related potentials, inhibition, cognitive control

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INTRODUCTION

Lutein (L) and zeaxanthin (Z) are naturally occurring carotenoids found in abundance in richly colored fruits and vegetables (e.g., spinach and kale). These pigments cannot be synthesized de novo, and thus must be obtained from the diet. Since they can also be directly and non-invasively measured in central nervous system tissue (the retina), and those levels correlate strongly with dietary intake, serum levels, and brain concentrations (Burke et al., 2005; Vishwanathan et al., 2015), they provide a powerful biomarker and means of testing how diet might influence the brain. Lutein is the predominant carotenoid in human brain tissue and greater lutein status is positively associated with better cognitive function in older adults (Johnson et al., 2008; Feeney et al., 2013; Johnson et al., 2013; Renzi et al., 2014; Vishwanathan et al., 2014a). Whether greater accumulation of retinal carotenoids is related to cognitive abilities and underlying neuroelectric function in earlier adulthood, however, has not been as thoroughly studied.

Much effort has been directed toward determining the cognitive constructs that are affected in normal aging and the underlying mechanisms that may explain age-related cognitive decline. Considerable research has shown age-related decline across a variety of cognitive domains including attention (Cabeza et al., 2004), working memory (Kirova et al., 2015), inhibition (Sebastian et al., 2013; Pettigrew and Martin, 2014), context processing (Rush et al., 2006), executive functioning (Silver et al., 2011), and processing speed (Albinet et al., 2012). Furthermore, brain-based research has begun to elucidate the mechanisms by which cognitive aging occurs. For example, fMRI work has shown that older adults tend to exhibit more widespread brain activation than younger adults during some cognitive tasks (Cabeza, 2002; Payer et al., 2006; Schneider-garces et al., 2009), suggesting the need for a greater recruitment of neural resources for successful task completion. In addition, older adults show less correlation of activation among different brain regions, suggesting less integrated functioning across the brain (Andrews-Hanna et al., 2007).

Traditionally the work on cognitive aging has been done cross-sectionally by comparing older adults with either middle aged or younger adults. There is evidence to suggest, however, that cognitive aging has its origins much earlier than older adulthood (Salthouse, 2005, 2009). For example, work done in samples of 18–84 year olds shows a significant relationship between age and a host of cognitive outcomes across the adult lifespan (Salthouse, 2001; Salthouse et al., 2003). Decreased performance in middle age may be especially prevalent when cognitive load is increased (Jain and Kar, 2014), and especially among low performers (Vuoksimaa et al., 2013). Imaging studies have identified neural patterns that mimic the kind of loss one typically sees in the elderly in non-clinical populations of early and middle aged adults including decreased grey matter volume (Sowell et al., 2003; Allen et al., 2005), whole brain volume (Fotenos et al., 2005), regional white matter volume (Pieperhoff et al., 2008), and cortical thickness (Magnotta et al., 1999; Salat et al., 2004).

Event related potential (ERP) work has shown consistent age-related changes in the P3 component across the lifespan. Early work established that age-related changes can be seen in P3 latency, with latencies decreasing over the course of adulthood, reaching a peak in early adulthood, and subsequently increasing over the course of middle to late adulthood (Courchesne, 1978; Goodin et al., 1978; Beck et al., 1980; Brown et al., 1983). Later work, however, has shown a similar trend in P3 amplitudes, with amplitudes increasing throughout childhood and decreasing beginning in middle age. Mullis et al. (1985), for example, showed that in a sample of 8–90 year old participants completing a modified oddball task, participants showed increasing P3 amplitudes to target stimuli up to 30–35 years of age, after which amplitudes decreased through older age. More recent work has shown greater differentiation in P3 amplitudes to target and standard stimuli across the lifespan and found decreased differentiation as a function of age beginning in middle age and increasing into older adulthood. This effect was driven by an age-related trend of larger amplitudes elicited for standard stimuli and smaller amplitudes for targets across middle and older age (Mott et al., 2014). Furthermore, it has been shown that the scalp distribution of the P3 changes across the lifespan, with healthy younger adults showing a posterior maxima which becomes increasingly central and more widespread with age (Fabiani and Friedman, 1995; Friedman et al., 1998; Lorenzo-López et al., 2007; Vallesi, 2011). The N2, a component thought to be a marker of inhibition (Jodo and Kayama, 1992), is shown to peak in amplitude and latency during childhood and subsequently decrease in adolescence through early adulthood (Johnstone et al., 2005). A continued slowing of the N2 response has been shown in older adulthood for visual stimuli (Falkenstein et al., 2002).

If, in fact, cognitive loss can begin so early, it is reasonable to question whether such changes can be delayed or prevented through changes in one's lifestyle. Much of this work has focused on the role of physical fitness and activity, indicating that older adults who are physically active or have superior cardiovascular health show fewer age-related declines (van Boxtel et al., 1997; Pontifex et al., 2009; Wendell et al., 2014; Bula, 2016), and that exercise interventions may serve to protect against cognitive aging (Colcombe and Kramer, 2003). These effects have been shown across the lifespan (van Boxtel et al., 1997; Hillman et al., 2006). Work on dietary interventions (Johnson et al., 2008; Tucker, 2016) is more recent. The majority of work in this field has targeted specific nutrient supplementation, with evidence suggesting that vitamin D (Hooshmand et al., 2014), vitamin B (Erickson et al., 2008; Clarke et al., 2014), and omega 3 fatty acids (Conklin et al., 2007; Dangour et al., 2012; Konagai et al., 2013) act as neuro-protective agents in aging adults. Carotenoids have also been studied with most attention being focused on L and Z. These antioxidant pigments have had a long history of being studied for their role in ocular function and disease (e.g., Stringham et al., 2008; Tan et al., 2008; Hammond et al., 2012), as well as a multitude of systemic health issues such as cancer, cardiovascular disease, and neurodegenerative disease (Krinsky and Johnson, 2005).

Macular pigment optical density (MPOD), a cumulative biophysical measure of carotenoids known to accumulate in the retina (i.e., L, Z, and mesozeaxanthin), has been specifically

linked to cognitive and brain health (Johnson, 2014). As such, greater MPOD has been correlated with superior visual processing abilities (Renzi and Hammond, 2010) and decreased risk of macular degeneration (Snodderly, 1995; Tan et al., 2008). However, in addition to accumulation in the macula, L has been shown across brain cortices in both infants and older adults (Craft and Dorey, 2004; Vishwanathan et al., 2014b; Erdman et al., 2015; Lieblein-boff et al., 2015). L and Z have anti-oxidative and anti-inflammatory properties (Beatty et al., 2000; Pintea et al., 2011) but also likely influence brain function through a number of other possible mechanisms (reviewed by Johnson, 2014). For example, Vishwanathan et al. (2014a) tested older adults in a battery of cognitive tasks and found that their retinal L and Z levels significantly corresponded to global cognitive abilities including verbal learning, verbal fluency, memory recall, processing speed, and perceptual speed. L and Z levels have also been shown to protect cognitive function in older adults with mild cognitive impairment (Renzi et al., 2014) and supplements of L, Z and DHA have shown to improve verbal fluency and memory in healthy older women (Johnson et al., 2008). However, the extent to which retinal carotenoids may interact with age on specific aspects of cognitive function remains inadequately studied, particularly among young or middle-aged adults.

The aims of the present study were to: (1) establish any independent relationships between age, retinal carotenoid levels (MPOD), and ERP indices in early and middle-aged adults (25–45 years); and (2) investigate the nature of the relationship between age, MPOD, and neuro-cognitive indices during tasks eliciting selective attention, attentional inhibition, and response inhibition. We hypothesized that age would be negatively associated with MPOD and positively associated with behavioral performance in both tasks, as well as to the corresponding ERP indices. Further, we hypothesized that both age and MPOD would be related to neuro-cognitive indices, but that the adjusting of MPOD would significantly influence the contribution of age.

MATERIALS AND METHODS

Participants

Cross-sectional data were collected from 60 adult participants between the ages of 25–45 years old living in the Eastern-Central Illinois region. To qualify for the study, participants had to provide all demographic data, complete the Kaufman Brief Intelligence Test (KBIT), a measure of intelligence quotient (IQ) (Kaufman and Kaufman, 1990), provide a readable EEG recording, have normal or corrected to normal vision based on the minimal 20/20 standard, be free of diagnosed neurological disorders and diseases (e.g., ADD/ADHD and autism). Participants were excluded if they did not complete all relevant aspects of testing, if they were not in the selected age range, if they were pregnant or nursing, or if they were currently taking any anti-psychotic, anti-depressant, or anti-anxiety mediations. All participants provided verbal and written consent in accordance with the University of Illinois' Institutional Review Board and the Declaration of Helsinki.

MPOD Assessment

Macular pigment optical density was measured with a customized hetero-flicker photometry (cHFP) technique and a macular densitometer (Macular Metrics Corporation, Rehoboth, MA, United States). This technique and its underlying principles have been described previously (Wooten et al., 1999; Hammond et al., 2005). In short, participants are asked to view stimuli peaking at a measuring wavelength of 460 nm that flickers in counterphase with a 570 nm reference (flicker rate being optimized for the optimal width of the subject's null zone). Participants were asked to adjust the radiance to identify a null flicker zone by indicating when they could no longer detect the flicker. The task is done while the stimulus is centrally fixated (measuring MP where it is most dense) and at 7° in the para-fovea (where density is minimal). The MPOD is calculated by subtracting the foveal from the parafoveal log sensitivity measurements after normalizing at 570 nm.

Cognitive Tasks

A two-stimulus oddball task was used to assess selective attention. In this task, participants viewed a series of large and small circles presented serially. Large circles (5.5 cm diameter) served as the "rare" stimulus and were presented on 20% of trials in a random order, whereas the "frequent" small circle stimuli (3 cm diameter) were presented in the remaining 80% of trials. Participants were to respond to the rare target trials with a button press. Participants were presented with a practice block of 30 trials followed by 200 experimental trials. Stimuli were presented for 100 ms with a 1000 ms response window and an inter-trial interval of 2000 ms. This task is described in detail in Pontifex et al. (2009).

The Eriksen flanker task was used to assess attentional inhibition. In this task, participants viewed a sequential array of visually presented arrows. The arrows were white line drawings presented on a black background for 83ms. Participants were asked to attend to a centrally located target arrow presented amid an array of four task-irrelevant distractor arrows that flank the target on both sides. The task was to quickly and accurately respond to the directionality of the centrally located target arrow with a button press. The target was presented as either a congruent trial in which the flanking arrows face in the same direction as the target (>>>>>), or as an incongruent trial in which the flanking arrows face in the opposite direction in relation to the target (>><>>). After receiving instructions, participants completed a block of 40 practice trials. Following the practice trials, participants completed 200 experimental trials (2 blocks of 100 trials each), made up of an equiprobable distribution of congruent and incongruent trials as well as left and right target trials presented in a random order. The trials were presented with randomly selected inter-trial intervals of 1100, 1300, and 1500 ms.

A go/no-go task was used to assess response inhibition. In this task, participants were presented with the same large and small stimuli that were used in the oddball task (described

above). However, in this task, participants are presented with a "go" stimulus (the 3 cm circle) in which they are to respond with a button press, as well as randomly interspersed "no-go" stimulus (the 5 cm circle) that they must ignore by inhibiting the established pre-potent, button-pressing response. As in the oddball task, participants were presented with a practice block of 30 trials followed by 200 experimental trials, and stimuli were presented for 100 ms with 1000 ms response windows and inter-trial intervals of 2000 ms. It should be noted that the go/no-go task was always presented directly after the oddball task, creating an even stronger pre-potent response to the rare (no-go) stimuli.

ERP Recording Technique

Electro-encephalographic (EEG) activity was recorded via a Neuro-scan Quik-cap with 64 scalp electrodes arranged in the international 10–10 system. A midline sensor placed between Cz and CPz served as a reference and AFz served as the ground. Using a Neuroscan Synamps2 amplifier, continuous EEG signal was digitized at a sampling rate of 500 Hz, amplified 500 times to 70-Hz filter with a direct current and a 60-Hz notch filter. Electro-oculographic (EOG) activity was recorded with a set of four electrodes placed at the outer canthus of each eye and above and below the left orbit.

Offline, continuous data were re-referenced to the average mastoids. An independent components analysis (ICA) was used to systematically reject eye-blink artifacts from the data. Data were submitted to a 0.1 Hz high-pass filter before being submitted to the ICA. If a component identified during the ICA were correlated at or above 0.35 with the vertical EOG channel, it was considered an eye-blink and subsequently rejected. –200 to 1200 ms around stimulus onset was used as a time window for creating stimulus-locked epochs with a –200 to stimulus onset used for baseline correction. A 30-Hz zero phase shift low-pass filter was employed. Only trials that were responded to correctly were included in ERP analysis. Epochs were excluded if the moving window peak-to-peak amplitude exceeded $\pm$100 100 μV using a 100 ms window and a 50 ms window step. ERP variables of interest were the P3 peak amplitude and peak latency. In the flanker task, the P3 was defined as the localized peak and corresponding latency occurring between 300 and 600 ms after stimulus onset; in the Oddball and Go/No-go tasks, it was defined as the localized peak and corresponding latency occurring between 350 and 750 ms after stimulus onset. The N2 in the go/no-go task was defined as the localized negative peak and corresponding latency occurring between 200 and 350 ms after stimulus onset.

Statistical Analysis

All analyses were conducted using SPSS version 24 (IBM). Bivariate correlations were used to assess the relationships between the demographic variables of interest, MPOD, the cognitive variables, and the ERP variables of interest. To assess the relationships between the demographic variables of interest, MPOD, and the cognitive and ERP variables, three sets of bivariate correlations were conducted, the first using cognitive and ERP data from the oddball task, the second from the flanker task, and the third from the go-nogo task. The demographic variables of interest submitted to correlational analyses were participant age, sex, KBIT score, and family income. The cognitive variables of interest in the flanker task were mean reaction time, response accuracy, inverse efficiency (reaction time/accuracy), and coefficient of variation (CV) (SD reaction time/M reaction time). Inverse efficiency is a way to examine how quickly participants respond accounting for accuracy. Higher numeric values for inverse efficiency indicate less efficient response patterns. CV is a means of examining the degree of intraindividual variability or consistency in response times for correct trials. Previous work indicates that measures of dispersion such as intraindividual variability may be more sensitive to cognitive dysfunction than measures of central tendency (Leth-Steensen et al., 2000; Hervey et al., 2006; MacDonald et al., 2006). Higher CV values indicate less consistent response patterns and represent a hallmark of several conditions in which attention regulation is diminished including old-age and neurological disorders (Kelly et al., 2008). For both the oddball and the go/no-go tasks, mean reaction times, response accuracies, and inverse efficiency scores were used. For all performance indices, the accuracy is recorded as the percentage correct, and reaction time is based on the mean of trials that were responded to correctly in milliseconds. The ERP variables of interest for all tasks were the peak amplitude and peak latency at the PZ electrode, where P3 is maximal in typical healthy adults (Polich, 2007). For the go/no-go task, N2 is also reported at the FCZ electrode, as it is considered to index inhibitory control in this task (Jodo and Kayama, 1992). Two-tailed tests are reported, with alpha of.05 for determining statistical significance. Outliers were calculated and subsequently removed based on the dependent variables of interest. The outlier labeling rule (Hoaglin and Iglewicz, 1987), which creates high and low cutoff scores based on the overall distribution of data, was used to identify outliers.

In order to draw more specific conclusions regarding the role of age and MPOD in predicting the neuro-cognitive outcomes, a series of hierarchical linear regression (HLR) analyses were conducted. The variables included in these models were derived from the bivariate correlations. Of particular interest were variables significantly associated with both age and MPOD. In the flanker task, two neuro-cognitive outcomes were associated with age and MPOD: The peak amplitude of the incongruent flanker trials at the PZ electrode, and the CV for the incongruent flanker trials. Subsequently, HLR models were run with each of these variables as the dependent measure. In each case, age was entered as a predictor into block 1 of the model, and MPOD was entered as a predictor into block 2 of the model. Standardized betas with corresponding t values are reported for predictors and F values with R^2 changes are reported for overall model fit. For the go-nogo task, no variables were significantly correlated with both age and MPOD scores; therefore, no subsequent HLR analyses were conducted.

RESULTS

Participants spanned early to middle adulthood, ranging in age from 25 to 45 years ($M = 33.8$, $SD = 5.7$, 31 females) and were

normally distributed in terms of their KBIT scores ($M = 110.2$, $SD = 13.3$) and MPOD ($M = 0.49$, $SD = 0.25$) values (Shapiro Wilk values = 0.986,0.983, $p = 0.711$,0.554, respectively). Age and income, however, were not normally distributed, with our sample representing a disproportionately younger demographic. The distribution of family income was even, suggesting that our sample equally represented adults of various income classes ($N \leq \$50{,}000 = 34$, $N > \$50{,}000 = 22$).

Oddball Task

Means and standard deviations of performance on the oddball task are shown in **Table 1**; results of the bivariate correlations are shown in **Table 2**. Waveforms, separated across the median values for age and MPOD, can be seen in **Figure 1**. The results indicate that participants performed well on the task overall, and while P3 amplitudes were numerically higher and latencies numerically lower on the targets compared to standards, as expected, neither were correlated with any of the demographic variables of interest. However, age was inversely correlated with response accuracy to standard stimuli ($r = –0.266$, $p = 0.040$), suggesting that younger adults had higher scores when asked to withhold responses to frequent, standard stimuli. MPOD was positively associated with both reaction time ($r = 0.366$, $p = 0.004$) and inverse efficiency ($r = 0.257$, $p = 0.047$) to target stimuli, suggesting that those with higher MPOD scores likely adopted a response set strategy in which they responded more slowly and less efficiently than their peers with lower MPOD. Finally, KBIT was positively associated with both the response accuracy to standard stimuli ($r = 0.342$, $p = 0.007$) and with reaction time to target stimuli ($r = 0.468$, $p \leq 0.001$).

Flanker Task

Means and standard deviations of the flanker performance and ERP indices are presented in **Table 3**. Flanker performance was skewed in favor of high performance, a pattern expected in typically functioning adults. An inclusion criterion of 50% accuracy was used for flanker performance, and no participants were excluded for low performance.

The results of the bivariate correlation analysis are included in **Table 4**. The correlation analysis indicated a number of cognitive performance variables associated with age including reaction times for congruent and incongruent trial types (r congruent = 0.271, $p = 0.036$; r incongruent = 0.290, $p = 0.025$), inverse efficiency for congruent and incongruent trial types (r congruent = 0.260, $p = 0.045$; r incongruent = 0.260, $p = 0.045$) and co-efficient of variation for incongruent trials ($r = 0.450$, $p < 0.001$). Thus, age was associated with less efficient cognitive processing as indicated by a number of performance indices. MPOD values, on the other hand, were inversely correlated only with CV for incongruent trials ($r = –0.306$, $p = 0.018$) suggesting that individuals with higher MPOD scores were more likely to process flanker information more efficiently. The processing benefit was seen specifically in terms of reliability of response patterns and only on trials that required selective processing to inhibit irrelevant distractor stimuli (i.e., during the incongruent trials). Sex and KBIT were associated with response accuracy for congruent trials ($r = 0.260$, $p = 0.045$; $r = 0.277$, $p = 0.032$) suggesting that males and individuals with higher IQ scores responded correctly to more congruent items. Sex was associated with CV for incongruent trials ($r = –0.285$, $p = 0.027$) and KBIT was associated with CV for both congruent ($r = –0.277$, $p = 0.032$) and incongruent trials ($r = –0.394$, $p = 0.002$).

The ERP waveforms at the PZ electrode can be seen in **Figure 2**. The bivariate correlations on the ERP data revealed that age was reliably correlated with peak amplitude for incongruent trials ($r = –0.255$, $p = 0.050$) and peak latency for congruent trials (0.300, $p = 0.020$) suggesting that the older participants showed decreased amplitudes for incongruent trials, and more delayed neural responses to congruent trials. MPOD was associated with peak amplitude for incongruent trials ($r = 0.259$, $p = 0.045$), indicating that individuals with higher MPOD values showed larger amplitudes. No other demographic variables were significantly associated with the P3 data.

To further elucidate the relationships between age, MPOD, and the cognitive and P3 variables, HLRs were conducted on the two variables that were significantly associated with age and MPOD: peak amplitude and CV for incongruent trials. The results of these models are summarized in **Table 5**. When peak amplitude was used as the dependent variable and age was entered as step 1, the resulting model was statistically significant ($R^2 = 0.065$, $F = 4.020$, $p = 0.05$), and age served as a significant predictor of peak amplitude ($\beta = –0.255$, $t = –2.005$, $p = 0.05$, CI = –0.334,0.000). However, when MPOD was added into step 2, both age ($\beta = –0.212$, $t = –1.664$, $p = 0.102$,CI = –0.307,0.028) and MPOD ($\beta = 0.218$, $t = 1.710$, $p = 0.093$, CI = –0.563, 7.161) failed to significantly predict peak amplitude, though the overall model was significant ($R^2\ \Delta = 0.046$, $F = 3.539$, $p = 0.036$), suggesting that the relationship between age and PZ peak amplitude is at least partially accounted for by MPOD.

When CV was used as the dependent variable, step 1 resulted in a statistically significant model ($R^2 = 0.189$, $F = 14.749$, $p = .000$) and age was a significant predictor of CV ($\beta = 0.450$, $t = 3.840$, $p = 0.000$, CI = 0.001,0.005). Step 2 of the model resulted in a significant model ($R^2\ \Delta = 0.058$, $F = 9.597$, $p = 0.000$). In addition, age remained a statistically significant predictor ($\beta = 0.406$, $t = 3.476$, $p = 0.001$, CI = 0.001,0.004) whereas the added effect of MPOD was only moderately significant ($\beta = –0.226$, $t = –1.935$, $p = 0.058$, CI = –0.070,0.001).

Go-Nogo

Only a subset of participants had go-nogo data appropriate for analysis ($N = 55$, 27 females). Three participants did not

TABLE 1 | Behavioral performance and event related potential (ERP) peak indices for the PZ electrode in the oddball task.

	Targets	Standards
Response accuracy (% correct)	84.6 (15.4)	90.4 (15.7)
Reaction time (ms)	477.9 (67.9)	–
Inverse efficiency	6.0 (2.0)	–
Peak amplitude (μV)	13.3 (8.6)	7.4 (9.2)
Peak latency (ms)	543.4 (124.9)	521.5 (131.8)

Means are presented with standard deviations in parentheses.

TABLE 2 | Bivariate correlations between participant demographic characteristics, MPOD, and the neuro-cognitive data from the oddball task.

	Age	MPOD	Sex	KBIT	Income
Accuracy					
Targets	0.184	−0.167	−0.093	−0.095	0.085
Standards	−0.266*	0.162	−0.094	0.342**	0.147
Reaction Time					
Targets	−0.195	0.366*	0.014	0.468**	0.005
Standards	–	–	–	–	–
Inverse Efficiency					
Targets	−0.094	0.257*	−0.014	0.222	0.051
Standards	–	–	–	–	–
Peak Amplitude					
Targets	−0.096	0.090	−0.214	−0.199	−0.060
Standards	−0.100	0.170	−0.111	−0.247	−0.158
Peak Latency					
Targets	−0.104	−0.066	0.069	−0.236	−0.238
Standards	−0.029	−0.039	−0.121	−0.136	0.049

** denotes statistical significance at the 0.05 level (two-tailed); ** denotes statistical significance at the 0.01 level (two-tailed).*

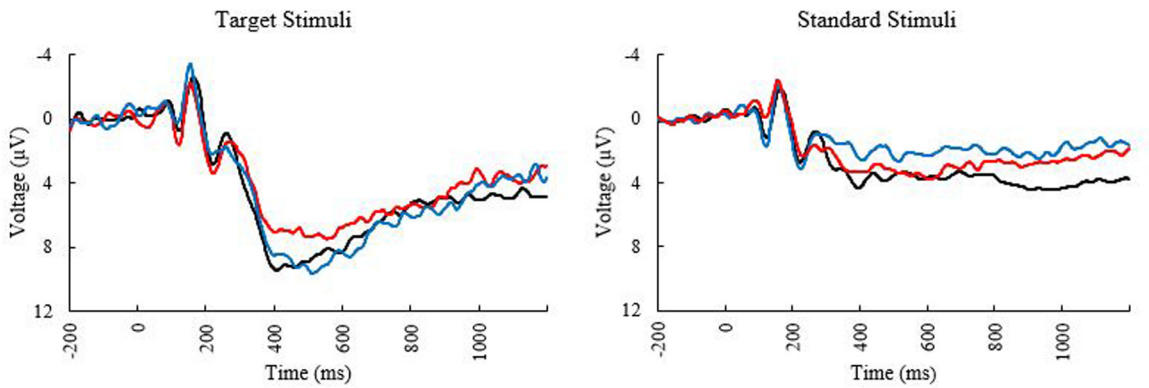

FIGURE 1 | Event related potential (ERP) Waveforms at electrode PZ elicited during the oddball task. Black line, younger adults; blue line, older adults with low MPOD; red line, older adults with high MPOD.

complete the task or had unusable ERP data and two participants were excluded for having peak amplitudes that were outliers (Hoaglin and Iglewicz, 1987). Means and standard deviations can be seen in **Table 6**. KBIT and MPOD scores were normally distributed (Shapiro–Wilk ≥ 0.984, $p \geq 0.687$). Age and income were not normally distributed, and the corresponding plots suggested good distribution of ages and incomes. The waveforms elicited at the PZ and FCZ electrodes can be seen in **Figure 3**. The results of the bivariate correlations (**Table 7**) revealed no significant correlations between the demographic variables of interest and the amplitudes or latencies of either the P3 or N2 components of the ERP waveform. (all p's ≥ 0.095).

TABLE 3 | Behavioral performance indices and ERP peak indices forth PZ electrode in the modified flanker task.

	Congruent trials	Incongruent trials
Response accuracy (% correct)	97.8 (3.4)	92.7 (5.1)
Reaction time (ms)	406.3 (45.2)	476.8 (43.1)
Inverse efficiency	4.2 (0.5)	4.9 (0.5)
Coefficient of variation (CV)	0.17 (0.04)	0.18 (0.04)
Peak amplitude (μV)	10.2 (3.4)	10.7 (3.7)
Peak latency (ms)	394.1 (52.0)	460.5 (55.3)

Means are presented with standard deviations in parentheses.

DISCUSSION

The aim of the present study was to investigate the relationships between participant age, retinal carotenoids, and inhibition-related components of the ERP waveform throughout early and middle adulthood. Our findings are consistent with our hypotheses that age and retinal carotenoid accumulation are differentially related to performance and neurolectric indices of attentional control. However, the benefits of greater retinal carotenoids appeared to be selective for intraindividual variability and attentional resource allocation during the flanker task, rather than the in the oddball or the go/nogo tasks. Our data

TABLE 4 | Bivariate correlations between participant demographic characteristics and the neuro-cognitive data from the flanker task.

	Age	MPOD	Sex	KBIT	Income
Accuracy					
Congruent	−0.040	−0.006	0.260*	0.277*	0.122
Incongruent	0.008	0.152	0.248	0.220	0.068
Reaction Time					
Congruent	0.271*	0.072	−0.066	−0.048	0.107
Incongruent	0.290*	0.027	−0.138	−0.054	0.112
Inverse Efficiency					
Congruent	0.260*	0.071	−0.142	−0.131	0.060
Incongruent	0.260*	0.034	−0.217	−0.148	0.050
CV					
Congruent	0.140	−0.090	0.002	−0.277*	−0.019
Incongruent	0.450**	−0.306*	−0.285*	−0.394**	0.142
Peak Amplitude					
Congruent	−0.148	0.151	−0.118	0.105	−0.088
Incongruent	−0.255*	0.259*	−0.001	0.115	−0.074
Peak Latency					
Congruent	0.300*	−0.065	−0.113	−0.200	0.100
Incongruent	0.154	−0.033	0.028	−0.046	−0.110

** denotes statistical significance at the 0.05 level (two-tailed); ** denotes statistical significance at the 0.01 level (two-tailed).*

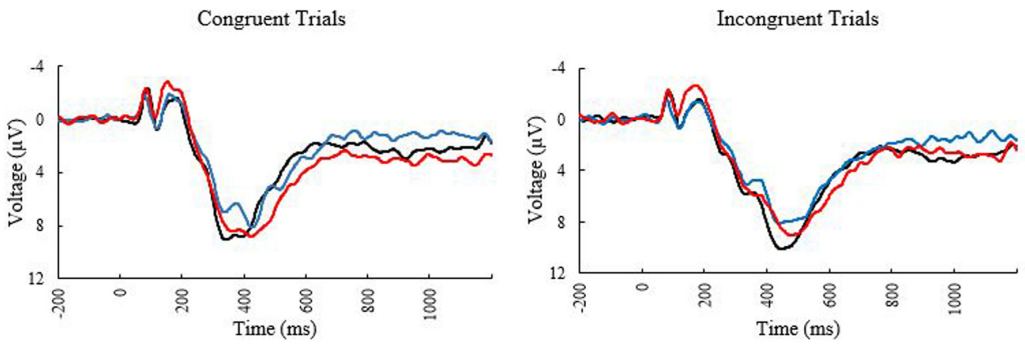

FIGURE 2 | Event related potential waveforms at electrode PZ elicited during the Flanker task. Black line, younger adults; blue line, older adults with low MPOD; red line, older adults with high MPOD.

TABLE 5 | Summary of regression analyses for the effects of age and MPOD on neuro-cognitive flanker variables.

	Incongruent peak amplitude			Incongruent coeff of variation		
Step and Variable	β	ΔR^2	**Model *p***	β	ΔR^2	**Model *p***
Step 1		0.065	0.050		0.203	0.000
Age	−0.255	–	0.050	0.450	–	0.000
Step 2	–	0.046	0.036	–	0.049	0.000
Age	−0.212	–	0.102	0.406	–	0.000
MPOD	0.218	–	0.093	−0.226	–	0.058

revealed that there were general performance benefits of age and MPOD, and that these effects were present by middle adulthood. Younger participants were more likely to perform the flanker task more quickly, more efficiently, and more consistently than older participants. MPOD scores were also associated with better performance, but the benefit of MPOD appeared more selective than that of age. Participants with higher MPOD scores were more likely to perform the flanker task with a consistent speed, although only in incongruent flanker trials, suggesting that the effect may be specific to conditions in which attentional inhibition demands are high. Secondly, participants' P3 indices for the flanker task were correlated with age and MPOD. Younger adults were more likely to show larger P3 amplitudes, as were participants with higher MPOD scores, suggesting that those who

TABLE 6 | Behavioral performance and ERP peak indices in the go-nogo task.

	Go/NoGo Task	
	Go stimuli	**Nogo stimuli**
Response accuracy (% correct)	92.2 (8.9)	65.9 (18.9)
Reaction time (ms)	415.0 (61.3)	–
Inverse efficiency	4.6 (1.0)	
Peak amplitude (μV)		
N2	−3.1 (3.6)	−4.5 (5.1)
P3	6.5 (3.5)	11.0 (5.7)
Peak latency (ms)		
N2	249.9 (31.9)	257.7 (37.7)
P3	509.1(123.4)	527.1(101.7)

The N2 indices are reported for electrode FCZ and P3 indices are reported for the PZ electrode. Means are presented with standard deviations in parentheses.

were younger and had higher levels of retinal carotenoids were able to dedicate greater cognitive resources in the task. These results were selective to incongruent trials, where attentional inhibition is employed and were not shown in the go-nogo task indexing response inhibition.

These findings are consistent with previous literature in cognitive aging and health. A substantial body of work demonstrates that older adults show a smaller P3 peak amplitude compared to younger adults. This has been shown in a variety of paradigms and across sensory modalities (Smith et al., 1980; Pfefferbaum and Ford, 1988; Kugler et al., 1993; Morgan et al., 1999; Murphy et al., 2000). Furthermore, several studies have examined the P3 across adulthood and suggest that P3 aging effects may begin before the onset of late adulthood (Picton et al., 1984; Mullis et al., 1985; Iragui et al., 1993; Polich, 1996; Mott et al., 2014), as our data would suggest. It is possible that the smaller P3 amplitudes shown in older adults are the result of a lack of compensatory mechanisms employed that emerge as early as middle adulthood. Prior work comparing young and old high and low cognitive performers showed the increased amplitude in older high performers, but lower amplitudes in low performers which suggests that ERP amplitude may be associated with neural compensatory mechanisms that appeared lacking in our sample (Riis et al., 2008). This view is consistent with some explanations of fMRI work showing increased activity across brain regions in elderly samples (Cabeza, 2002). There has also been substantial literature to indicate that latency effects are present in cognitive aging (Pfefferbaum et al., 1980; Picton et al., 1984; Pfefferbaum and Ford, 1988; Curran et al., 2001; Walhovd and Fjell, 2003; Vallesi, 2011), effects consistent with the significant relationship between age and latency to congruent flanker stimuli in our data.

The relationship between MPOD and enhanced neuro-cognitive function is also consistent with prior literature. Several studies have established a relationship between retinal carotenoids and superior cognitive processing in older (Johnson et al., 2008, 2013; Feeney et al., 2013; Renzi et al., 2014; Vishwanathan et al., 2014a) and middle aged (Kelly et al., 2008) adults. Our data are consistent with these findings and suggest that the age-related protective properties of L and Z may begin in early to middle adulthood. This finding is not surprising. Higher MPOD is directly related to a healthy diet (Krinsky and Johnson, 2005) and healthy eating patterns have been shown to be related to better cognition across adulthood (e.g., Holloway et al., 2011). While ours is the first study to examine the relationship between retinal carotenoids and an in-vivo, brain-based measure of neuro-cognition like ERPs, the finding that higher MPOD was related to larger P3 amplitudes is an effect consistent with prior work in health-related fields. However, our results extend the existing literature by providing novel evidence for the age-related protective effects of MPOD on attentional control in young and middle-aged adults.

The results of the regression models suggest that the roles of MPOD and age in explaining variance in neuro-cognitive performance differs depending on the domain that is examined. In the behavioral domain, where CV was used as the dependent measure, age showed a robust effect independent of the effect of MPOD. This suggests that while MPOD was a moderately associated with CV performance, its effects are independent of the impact of age. Similar aging effects have been seen in other domains of cognition, such as IQ (Ardila, 2007). In the neuroelectric domain, however, the addition of MPOD into the model weakened the influence of age on P3 amplitude, suggesting that MPOD may partially influence the relationship between age and P3 amplitude and may serve as a protective factor against typical aging effects. This result is consistent with reports suggesting that cognitive aging is mediated by sensory acuity. For example, Lindenberger and Baltes (1994) found that 49% of the variance in age-related change in IQ scores was accounted for by auditory and visual acuity. Lovden and Wahlin (2005) likewise showed that visual acuity accounted for roughly half of the variance in general cognitive ability across adulthood. In this view, it is reasonable that MPOD, which has been consistently linked to visual health, (Tan et al., 2008; Junghans et al., 2001; Pintea et al., 2011; Ozawa et al., 2012; Vishwanathan and Johnson, 2013) may be an indicator of visual acuity. Indeed, higher levels of lutein and zeaxanthin have been directly linked to superior visual performance (Stringham and Hammond, 2008; Johnson, 2014; Schalch, 2014).

Our data also suggest that domains in which aging and MPOD have some combined effects on cognition are relatively selective. In our cognitive performance indices, age and MPOD were associated independently with very few measures of the oddball and none of the measures of the go-nogo task. This is perhaps unsurprising, since one might expect age effects in trials in which inhibition demands are high, such as those in the go-nogo task. However, because participants are asked to inhibit a behavioral response (i.e., pressing a button to a rare target), behavioral data are not collected on the nogo stimuli that require the highest levels of inhibitory control. Thus, the go-nogo task may not have sufficiently sensitive performance indices. However, one may still expect to see aging effects in regard to the N2 and P3, since the neural signature is present without a motor response. This effect was not shown in our data. Thus, our data suggest that the protective role of MPOD against aging-related effects may be selective for attentional

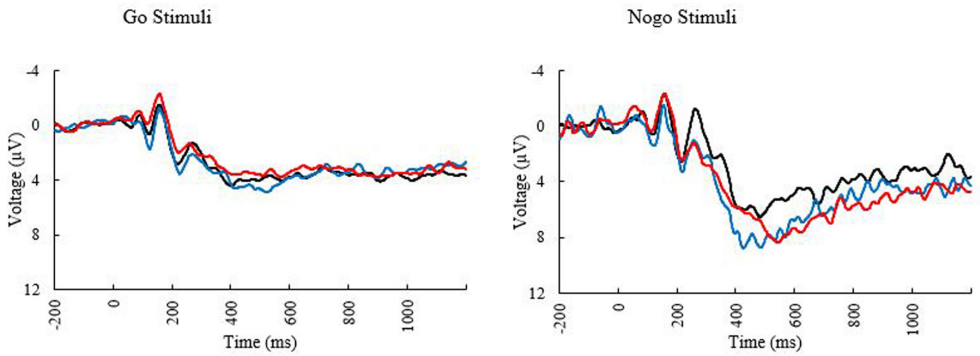

FIGURE 3 | Event related potential waveforms at electrode PZ elicited during the go/no-go task. Black line, younger adults; blue line, older adults with low MPOD; red line, older adults with high MPOD.

TABLE 7 | Bivariate correlations between participant demographic characteristics, MPOD, and the neuro-cognitive data from the go-nogo task.

	Age	MPOD	Sex	KBIT	Income
Accuracy					
Go Stimuli	−0.046	−0.107	0.142	0.228	0.226
Nogo Stimuli	0.142	0.023	−0.137	0.024	0.113
Reaction Time					
Go Stimuli	0.210	0.172	−0.043	−0.036	0.114
Nogo Stimuli	–	–	–	–	–
Inverse Efficiency					
Go Stimuli	0.152	0.198	−0.104	−0.145	−0.061
Nogo Stimuli	–	–	–	–	–
Peak Amplitude					
P3					
Go Stimuli	−0.169	0.243	0.017	−0.240	−0.002
NoGo Stimuli	−0.070	0.102	0.088	−0.163	−0.035
N2					
Go Stimuli	0.067	0.059	−0.120	−0.227	−0.127
NoGo Stimuli	0.095	0.177	−0.137	−0.189	−0.002
Peak Latency					
P3					
Go Stimuli	−0.057	0.016	0.104	−0.185	−0.192
Nogo Stimuli	−0.130	0.167	0.192	−0.001	−0.220
N2					
Go Stimuli	−0.003	0.054	−0.002	−0.104	−0.040
Nogo Stimuli	−0.186	−0.089	0.074	−0.079	−0.040

** denotes statistical significance at the 0.05 level (two-tailed); ** denotes statistical significance at the 0.01 level (two-tailed).*

inhibition, rather than for response inhibition or general selective attention as indexed by the oddball task. Prior behavioral and ERP work has shown that various forms of cognitive inhibition may be affected differentially and independently in aging, (Andrés et al., 2008; Anguera and Gazzaley, 2012), although none of these studies have used the flanker task as an index of inhibition. Furthermore, no studies to date have examined different forms of cognitive inhibition in relationship to retinal carotenoids. Thus, it is unclear whether the specificity seen in our data is a result of task demands, a result of MPOD selectively affecting attentional inhibition over selective attention or response inhibition, or a result of attentional inhibition being impacted by cognitive decline at an earlier point in the course of adulthood than the other attentional constructs measured.

There are several limitations to the present study and future work could benefit from expanding on the work in a number of ways. The present is study is cross-sectional in design and therefore is not suitable for directly answering questions regarding causality. While we attempted to elucidate on the role of age and MPOD using statistical measures (i.e., HLR), to determine true causal mechanisms it is necessary to implement interventions involving L and Z rich diets or supplementation. Furthermore, our study was limited in the age range that was

utilized. While aging effects were seen even in our sample of early to middle aged adults, a more conclusive picture would emerge from examining participants across the lifespan in both cross-sectional and longitudinal designs. Longitudinal studies would also serve to answer important questions about the developmental trajectory of nutritional effects on neuro-cognitive function. Finally, there is a need to examine more exhaustive cognitive domains and paradigms in order to ascertain the reliability of the selectivity of the associations that were shown our data.

CONCLUSION

Through our study, we sought to explore the relationships between aging, MPOD, and the cognitive and underlying neuro-electric indices in three attentional tasks. Our results showed robust age affects in the attentional inhibition task, but no relationships between aging and our response inhibition task. Furthermore, MPOD appeared to influence the relationship between aging and P3 amplitude in the attentional inhibition task suggesting that diet may serve a protective role in typical aging effects on inhibition. The results reported herein have important implications for the association between neuro-cognitive health and retinal carotenoids, and dietary quality by extension. While some age-related cognitive decline is to be expected in healthy aging, our data suggest that these effects may be less pronounced among adults with greater retinal carotenoid status, a marker of dietary patterns characterized by greater intake of green and leafy vegetables. Furthermore, these practices may provide neuro-cognitive benefit before the onset of older age, in early to middle adulthood. Future experimental clinical trials are needed to determine whether changes in retinal carotenoid status moderate the influence of age-related neurocognitive decline across the lifespan.

ETHICS STATEMENT

The study was approved by the Institutional Review Board at the University of Illinois. Participants provided verbal and written consent.

AUTHOR CONTRIBUTIONS

AW analyzed the data and prepared the first draft of the manuscript. CE, NB, MC, AC, and GR implemented data collection and contributed to the manuscript development. BH and LR-H provided important contributions to the content of and assisted in revising the manuscript. NK conceptualized and supervised the design and implementation of the study and manuscript. All authors contributed to, and accept responsibility for, the research described in this manuscript.

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Corrigendum: Chronic Pyruvate Supplementation Increases Exploratory Activity and Brain Energy Reserves in Young and Middle-Aged Mice

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In the Original Research article there was an error in the Section "Treatment" under the section "Methods" about the estimated daily intake of pyruvate:

"With the average food intake of 4 g this corresponds to 800 mg of pyruvate/day, which is at the upper range of effective pyruvate doses in earlier *in vivo* studies (Suh et al., 2005; Fukushima et al., 2009; Isopi et al., 2014)."

As correctly stated in the Abstract, the estimated dose was 800 mg of pyruvate/kg/day.

The corrected version of this section is shown below:

Treatment

Chronic Pyruvate Administration

The test group (PYR) received experimental chow supplemented with 0.6 % (w) of Na-pyruvate (Safe Diets, Augy, France). The control group (STD) received the same basic rodent chow (A04, Safe Diets). With the average food intake of 4 g this corresponds to 800 mg of pyruvate/kg/day, which is at the upper range of effective pyruvate doses in earlier *in vivo* studies (Suh et al., 2005; Fukushima et al., 2009; Isopi et al., 2014). *Acute pyruvate administration.* The mice received Na-pyruvate (Sigma, St. Louis, MO, USA) 500 mg/kg i.p. or the same molar concentration of NaCl (260 mg/kg i.p.). This single dose affords neuroprotection against cortical concussion injury and increases brain glucose and pyruvate levels as measured by *in vivo* microdialysis (Fukushima et al., 2009).

All cage labels about the treatment groups were coded so that the researchers running behavioral tests or assays on post-mortem samples were blinded as to the treatment.

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

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The Walnuts and Healthy Aging Study (WAHA): Protocol for a Nutritional Intervention Trial with Walnuts on Brain Aging

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Introduction: An unwanted consequence of population aging is the growing number of elderly at risk of neurodegenerative disorders, including dementia and macular degeneration. As nutritional and behavioral changes can delay disease progression, we designed the Walnuts and Healthy Aging (WAHA) study, a two-center, randomized, 2-year clinical trial conducted in free-living, cognitively healthy elderly men and women. Our interest in exploring the role of walnuts in maintaining cognitive and retinal health is based on extensive evidence supporting their cardio-protective and vascular health effects, which are linked to bioactive components, such as n-3 fatty acids and polyphenols.

Methods: The primary aim of WAHA is to examine the effects of ingesting walnuts daily for 2 years on cognitive function and retinal health, assessed with a battery of neuropsychological tests and optical coherence tomography, respectively. All participants followed their habitual diet, adding walnuts at 15% of energy (≈30–60 g/day) (walnut group) or abstaining from walnuts (control group). Secondary outcomes include changes in adiposity, blood pressure, and serum and urinary biomarkers in all participants and brain magnetic resonance imaging in a subset.

Results: From May 2012 to May 2014, 708 participants (mean age 69 years, 68% women) were randomized. The study ended in May 2016 with a 90% retention rate.

Discussion: The results of WAHA might provide high-level evidence of the benefit of regular walnut consumption in delaying the onset of age-related cognitive impairment and retinal pathology. The findings should translate into public health policy and sound recommendations to the general population (ClinicalTrials.gov identifier NCT01634841).

Keywords: cognitive decline, Alzheimer's disease, age-related macular degeneration, dietary intervention, walnuts, randomized trial, aging

INTRODUCTION

The worldwide expansion of aging populations has become a major public health challenge (Song and Chen, 2015). Increased lifespan has resulted in a steady rise of debilitating diseases related to aging, including neurodegenerative disorders, such as Alzheimer's disease (AD), the most common type of dementia (Alzheimer's Association, 2015), and age-related macular degeneration (AMD), the leading cause of visual loss and blindness worldwide (Lim et al., 2012). Unfortunately, to date there is no effective treatment for AD (Iqbal et al., 2014) or even mild cognitive impairment (MCI), its precursor stage (Cooper et al., 2013). Likewise, no effective preventive strategies exist for AMD, although nutritional and behavioral modifications can delay disease progression once initiated (Lim et al., 2012). In the continuum from normal cognition to MCI and dementia there is age-related cognitive decline, the onset and progression of which depends on a number of factors, including genetic variation, the level of education, environmental factors, particularly dietary habits, and the presence of cardiovascular risk factors or disease (Baumgart et al., 2015). Similar risk factors, albeit with a different genetic background, play a role in the pathophysiology of AMD (Lim et al., 2012). Optimal cognitive and visual functions are essential for quality of life and developing effective primary prevention strategies to reduce the economic and societal burden of such common age-related diseases would be of utmost public health importance.

Oxidative stress and inflammation are thought to play a pivotal role in precipitating neurodegenerative diseases, including AD (Schrag et al., 2013; Heneka et al., 2015) and AMD (Khandhadia and Lotery, 2010). Consequently, as derived from epidemiologic studies, a promising hypothesis in recent years has been that antioxidant-rich foods, such as fruit, vegetables, and particularly seeds and berries (Nooyens et al., 2011; Valls-Pedret et al., 2012; Ros and Hu, 2013; Barbour et al., 2014; O'Brien et al., 2014), and plant-based dietary patterns, especially the Mediterranean diet and the DASH (Dietary Approaches to Stop Hypertension) dietary pattern (Otaegui-Arrazola et al., 2014; Tangney et al., 2014) may protect from cognitive decline and AD. Nutrients such as n-3 polyunsaturated fatty acids (n-3 PUFA) and antioxidants, namely polyphenols, carotenoids and vitamins C and E, may have a role in preventing both cognitive impairment (Macready et al., 2009; Dangour et al., 2012; Valls-Pedret et al., 2012; Devore et al., 2013; Barnes et al., 2014; Otaegui-Arrazola et al., 2014) and macular degeneration (Age-Related Eye Disease Study Research Group, 2001; Parisi et al., 2008; Tan et al., 2009; Majumdar and Srirangam, 2010; Merle et al., 2013). Nuts in general and walnuts in particular have a rich matrix of these bioactive components and through additive effects have the potential to beneficially impact neuronal function in the brain and the retina (Poulose et al., 2014). Prospective studies have provided support for the association between nut consumption and improved cognitive performance (Valls-Pedret et al., 2012; O'Brien et al., 2014) and lower incidence of AMD (Tan et al., 2009; Amirul Islam et al., 2014). Among the different tree nuts, walnuts contain n-3 PUFA, specifically α-linolenic acid (ALA; C18:3n-3). ALA has long been believed to improve brain health indirectly via modest conversion to docosahexaenoic acid (DHA; C22:6n-3) (Domenichiello et al., 2015), which has been found to modulate brain plasticity and counteract neuroinflammation in experimental studies (Dyall, 2015). However, studies in rodents uncovered brain benefits of ALA by itself, including increased brain plasticity (Blondeau et al., 2009), reduced cell death and calcium dysregulation (Carey et al., 2013), and reduced amyloid-beta deposition (Gao et al., 2016). Other bioactive compounds in walnuts, such as arginine, tocopherols, folate, melatonin, and polyphenols also support neurological health and cognitive wellness by modulating blood pressure, HDL function, glucoregulation, endothelial vasodilator function, arterial compliance, oxidative status, and vascular inflammation (Carey et al., 2013; Del Rio et al., 2013; O'Brien et al., 2014). In support, experimental feeding studies in aged rodents have shown that walnuts improve age-related motor and cognitive deficits as assessed by rod walk, plank walk, and Morris water maze tests (Willis et al., 2009).

Easy to implement lifestyle modifications that might help prevent chronic non-communicable disorders need to be uncovered, with the concept that even small changes in the right direction can lead to substantial benefit for global health. Thus, delaying the onset of AD by only 5 years would significantly reduce its prevalence by the next decade (OECD, 2014). Thus far randomized controlled trials (RCTs) testing various interventions in patients with established AD have failed to show any benefit, probably because at this stage neuropathology is far advanced and irreversible. This underlines the need to conduct RCTs in individuals at risk but cognitively healthy, when there are little or no underlying brain changes.

Although not primarily focused on neurodegenerative disorders, one such study, the PREvención con DIeta MEDiterránea (PREDIMED) study, is a landmark RCT conducted in older individuals at high cardiovascular risk that has shown beneficial effects of Mediterranean diets supplemented with extra-virgin olive oil or mixed nuts on several age-related disorders after intervention for ∼5 years (Martínez-González et al., 2015). In the PREDIMED trial, the Mediterranean diets reduced the incidence of cardiovascular diseases by 30%, compared with a low-fat control diet, while the risk of stroke was reduced by 34% by the olive oil diet and by 49% by the nuts diet (Estruch et al., 2013). In a PREDIMED sub-study, we showed better cognitive performance associated with consumption of polyphenol-rich Mediterranean foods at baseline (Valls-Pedret et al., 2012) and cognitive improvement in participants allocated the two Mediterranean diets compared with those in the control group after 4.1 years of follow-up (Valls-Pedret et al., 2015). Thus, data from PREDIMED suggest that nuts, including walnuts, are a good option for cardiovascular and brain health. Therefore, it is reasonable to surmise that usual walnut consumption may be an effective approach to preserve cognitive abilities and visual function. However, while there is sufficient rationale for the role of walnuts in neuroprotection, direct clinical evidence is lacking. The Walnuts and Healthy Aging (WAHA) study is the first RCT assessing cognitive function and macular health

in an elderly cohort following daily ingestion of walnuts for 2 years.

METHODS

Study Design

The WAHA study is a dual center, single blind, randomized 2-year clinical trial conducted in free living, cognitively healthy elderly individuals[1]. The study is carried out in two centers: Loma Linda University, CA, USA (LLU) and Hospital Clínic, Barcelona, Spain (BCN). With a parallel design, participants were randomized to either the walnut group (consuming walnuts daily) or control group (abstaining from walnuts). Otherwise participants followed their habitual diet throughout the study. Primary aims are to assess changes in cognitive function and retinal integrity. Secondary aims relate to effects on cardio-metabolic risk factors, body weight and composition, and circulating markers of oxidation/inflammation. Additional secondary aims (BCN center) are changes in brain magnetic resonance imaging (MRI), ultrasound-assessed carotid atherosclerosis, blood pressure by 24-h ambulatory monitoring, bone mineral density, leukocyte telomere length, and microRNAs (miRNAs) related to lipoprotein metabolism.

Participants and Eligibility Criteria

Recruitment and selection of participants took place between May 2012 and May 2014; the trial ended May 31, 2016. Participants were healthy elderly men and women with normal cognitive and visual function at the time of recruitment. Inclusion criteria were age between 63 and 79 years, apparently healthy, and equally willing to be in either of the two groups. Exclusion criteria included inability to undergo neuropsychological testing; morbid obesity (BMI $\geq$ 40 kg/m^2); uncontrolled diabetes (HbA1c $>$ 8%); uncontrolled hypertension (on-treatment blood pressure $\geq$ 150/100 mmHg); prior stroke, significant head trauma or brain surgery; relevant psychiatric illness; major depression; cognitive deterioration or dementia with a score $<$ 24 on the Mini-Mental State Examination (MMSE) (Folstein et al., 1975); other neurodegenerative disorders like Parkinson's disease; advanced AMD or eye-related conditions precluding ophthalmological evaluation; prior chemotherapy; chronic illness with projected shortened lifespan; allergy to walnuts; customary use of fish oil and/or tree nuts ($>$ 2 servings/week) and/or other relevant sources of ALA, such as flaxseed oil or soy lecithin.

Eligible participants were recruited via mailing study brochures (LLU) or through the non-profit organization Institute of Aging (BCN), advertisements in the study centers, and word of mouth. Interested individuals attended an informational group meeting, completed a short medical questionnaire and signed the informed consent. Next candidates had a face-to-face interview with the study clinician, who assessed potential compliance, reviewed the medical history, inclusion and exclusion criteria, and recent blood work and use of medications or supplements, and administered the MMSE. Eligible participants were scheduled to have baseline tests (neuropsychological and ophthalmologic evaluations and collection of fasting blood and urine) and were then randomized to either the control or walnut group using a computerized random number table with stratification by center, sex, and age range. Couples entering the study were treated as one number and were randomized into the same group.

Intervention

Participants received 15% of daily energy intake as walnuts for 2 years or abstained from walnuts. To estimate the required amount of walnuts, participants completed a 3-day diet history and a physical activity questionnaire at baseline. The physical activity factor and the energy requirements were obtained using the World Health Organization formula for energy needs for adults $>$ 60 years (World Health Organization, 1985). The estimated amount of walnuts ranged from 1 to 2 oz/day ($\approx$30–60 g/day). Sachets for daily consumption containing 30, 45, or 60 g of raw, pieced walnuts were provided as 8-week allotments to the participants in the walnut group at the time of their 2-monthly clinic visits with the dietitians. Instructions were given to eat walnuts daily, preferably as the raw product, either as a snack or by incorporating them into shakes, yogurts, cereals, or salads. To improve participants' compliance, 1-kg extra walnut allowances were provided every 2 months to take into account family needs. Participants in the control group were advised to abstain from eating walnuts for the duration of the study. Use of ALA-rich canola and soybean oils was restricted.

Once randomized, participants were scheduled for 2-month visits with the study dietitians aimed at assessing compliance, increasing retention, and collecting data on diet adherence, medication changes, anthropometry, and clinical blood pressure. For the walnut group participants, the dietitians noted any side effects and collected used walnut sachets as a measure of compliance. The rapport built by the dietitians with the participant during the periodic scheduled visits was critical to retain them for the entire length of study. Since participants in the control group had no active intervention, to improve retention, quarterly educational group activities unrelated to the study were offered to them.

Outcomes

Primary outcomes are changes from baseline in the composite score of all neuropsychological tests for cognition and in the average thickness of the retinal nerve fiber layer of each eye, as assessed by optical coherence tomography (OCT). As secondary cognitive outcomes, we will analyze composites for different cognitive domains, including memory, language, perception, and frontal functions (for further detail, please see section Cognitive Testing); assess the incidence of AD according to NINCDS-ADRDA criteria (McKhann et al., 1984); and evaluate the incidence of MCI, defined by Petersen's diagnostic criteria (Petersen et al., 2001). Secondary OCT outcomes are macula cube thickness, cube volume, and central thickness.

Other secondary outcomes for the whole cohort are changes in anthropometric measures, lipid profile, serum inflammatory

[1]https://clinicaltrials.gov/show/NCT01634841

and oxidative stress markers, red blood cell (RBC) fatty acids, and urinary polyphenols. At the BCN site, the following secondary outcomes will be assessed: structural and functional brain MRI variables, carotid intima-media thickness (IMT) and plaque burden, body composition, bone mineral density, 24-h ambulatory blood pressure, leukocyte telomere length, and serum miRNAs.

Measurements

Table 1 shows the variables measured in the WAHA trial and how often they were measured.

Measurements at Both Sites

Dietary assessment

In BCN, a food frequency questionnaire (FFQ) including 44 food groups was administered to all participants at baseline and end of year 2. In LLU, after the 1st study year, participants filled in a FFQ validated in a pilot sample of elderly persons (Segovia-Siapco et al., 2007). During the study, dietary intake was monitored by conducting five unannounced 24-h diet recalls over 2 years (LLU) or 3-day food records every 6 months (BCN). Participants allocated to the walnut diet who had difficulty chewing due to dental problems were given a coffee grinder at no cost, with instructions on how to consume the ground walnuts by incorporating them to semifluid foods such as yogurt.

Clinical evaluation

A clinician saw the participants at baseline, when a general questionnaire was filled in, and at the end of the study, when a follow-up questionnaire was administered to assess final changes in clinical status and medication and adverse events. In the same visit clinical blood pressure was measured following the recommendations of the European Societies of Hypertension and Cardiology (ESH/ESC) (Mancia et al., 2013): after a 5-min rest with the patient seated in a quiet environment, three measures of BP were taken at 2-min intervals with a validated semiautomatic oscillometer (Omron 705-CP, Omron Healthcare Group, Kyoto, Japan). The mean of the last two measurements of systolic and diastolic blood pressure was recorded as office blood pressure. An electrocardiogram (ECG) was performed at baseline and end of study.

Physical activity, anthropometry, and body composition

Physical activity was evaluated at baseline, year 1, and the end of the study with a validated short version of the Minnesota questionnaire (Elosua et al., 1994). The dietitians measured height at baseline and 2 years using a wall mounted stadiometer, body weight at baseline and every 2 months by calibrated scales, and waist and hip circumferences every 6 months by using an anthropometric tape midway between the lowest rib and at the iliac crest and at the widest point in the hips, respectively. Body composition was assessed at baseline and 2 years by bioelectric impedance using a body composition analyzer (Tanita, Model TBF-300A, Arlington Heights, IL, USA) at LLU and by dual energy X-ray absorptiometry (DEXA) (Whole body scanner GE-Lunar IDXA, GE Healthcare, Madison, WI, USA) at BCN.

Cognitive testing

A comprehensive neuropsychological test battery evaluating several cognitive domains was administered at baseline and at the end of the trial. Neuropsychologists who were masked to participant's group assignment conducted the cognitive tests. The instruments used were Block design from the Wechsler Adult Intelligence Scale (WAIS III) (Wechsler, 1997), Rey-Osterrieth Complex Figure (ROCF) (Rey, 1941), Rey Auditory Verbal Learning Test (RAVLT) (Rey, 1958), Boston Naming Test (Kaplan et al., 2001), Semantic category evocation of animals (Ramier and Hécaen, 1970), Number location and incomplete letters from the Visual Object and Space Perception Battery (VOSP) (Warrington and James, 1991), Trail Making Test, parts A and B (Partington and Leiter, 1949), Phonemic fluency (FAS) (Benton and Hamsher, 1976), Stroop Color Word Test (Stroop, 1935), Symbol Digit Modalities Test (SDMT) (Smith, 1973), Digit span forward and backward from the WAIS III (Wechsler, 1997), and the Conners Continuous Performance Test (CPT II) (Conners and Staff, 2000).

Subjects' raw test punctuations were standardized to z scores to generate a global cognition composite by computing the mean standardized changes of all neuropsychological tests. This composite was pre-specified as the primary outcome of the study. Moreover, composites of cognitive domains analyzed separately were calculated. First, the memory composite included the mean standardized individual change scores of the RAVLT (immediate and delayed recall) and the 3-min recall of ROCF. Language composite included scores from animal semantic fluency and the Boston Naming Test. Perception composite included scores from number location and incomplete letters from VOSP battery and block design from WAIS-III battery. Finally, a composite score related to frontal functions was created including scores from TMT parts A and B, FAS, Stroop, SDMT, digit span from WAIS-III, and CPT-II. As secondary outcomes, composites assessing cognitive domains separately were defined.

In addition, information about cognitive reserve and mood was collected using a cognitive reserve questionnaire (Solé-Padullés et al., 2009) and the Hamilton Depression Rating Scale (Hamilton, 1967), respectively. Premorbid intelligence was assessed with the American National Adult Reading Test (Grober and Sliwinski, 1991) at LLU site and the Word Accentuation Test (Del Ser et al., 1997) at BCN site.

Ophthalmologic evaluation

Retinal examinations were performed at baseline and end of the study by ophthalmologists (BCN) and trained technicians under the supervision of an ophthalmologist (LLU). The central and average thickness and volume of the macular neurosensorial retina and the thickness of the retinal nerve fiber layer were ascertained by OCT (Cirrus HD OCT, Zeiss, Germany).

Biochemical measurements

Fasting blood samples and morning spot urine samples were collected at baseline and end of years 1 and 2, and aliquots of EDTA plasma, serum, buffy coat for DNA recovery, whole blood, and urine samples are kept frozen at −80°C. Participants

TABLE 1 | Measurements in the Walnuts and Healthy Aging (WAHA) study.

Measurements	Content	Baseline	Year 1	Year 2
Eligibility questionnaire	Sex, age, inclusion, and exclusion criteria	X		
General/quality of life questionnaire*	Marital and socioeconomic status, medical conditions, medication	X		X
Follow-up questionnaire, including withdrawal	Symptoms and conditions, tolerance, medication changes		X	X
Food frequency questionnaire (FFQ)†	Multiple food groups and foods	X		X
Diet recalls‡	All daily foods and beverages	X	X	X
Physical activity questionnaire	Short-version of the Minnesota questionnaire	X	X	X
Clinical blood pressure		X	X	X
Electrocardiogram¶		X		X
Anthropometric measurements	Height, body weight, waist, and hip circumferences, % body fat§	X	X	X
Neuropsychological evaluation	A complete test battery to assess cognitive function	X		X
Ophthalmological examination	Optic nerve and macular optical coherence tomography	X		X
Standard blood chemistry	Lipid profile, glucose, insulin, renal function, liver function, blood count, others	X	X	X
Serum inflammatory and oxidative stress markers	CRP, E-selectin, others	X		X
Urine chemistry	Albumin, total polyphenols, ellagitannin, urolithins, others	X	X	X
Red blood cell fatty acids	Measure of compliance, performed in 30% of the cohort	X	X	X
Genomic studies	*APOE*, others	X		
Brain MRI¶	Performed in a subset ($n = 120$)	X		X
B-mode ultrasonography of carotid arteries¶	Carotid intima-media thickness and plaque burden	X		X
DEXA§	Body composition and bone mineral density	X		X
Twenty-four-hour ambulatory blood pressure monitoring¶	Performed in 80% of the cohort	X		X
Leukocyte telomere length¶	Performed in all participants at baseline and only in those with brain MRI at 1 and 2 years	X	X	X
Serum miRNAs¶	miRNAs involved in lipoprotein metabolism	X	X	

BCN, Barcelona; LLU, Loma Linda University; CRP, C-reactive protein; MRI, magnetic resonance imaging; DEXA, dual-energy X-ray absorptiometry; miRNAs, microRNAs.
**The general questionnaire was collected at baseline at both sites. At LLU this questionnaire was called the Quality of Life questionnaire and was collected at end of year 2 also and included more questions about the subjects' mental health and well-being.*
†Forty-four-food groups and items FFQ at baseline and year 2 at BCN; 150-food group and items FFQ once during the 2nd year at LLU.
‡Three-day (including one weekend day) self-recorded recalls in BCN at baseline, years 1 and 2; five unannounced 24-h recalls by telephone at LLU over 2 years.
§ Percent body fat measured by bioelectric impedance at LLU and by DEXA in BCN.
¶ Determined only at the BCN center.

reported on the assigned days after fasting for a minimum of 12 h. An experienced phlebotomist drew blood and samples were centrifuged and aliquoted for the various assays and stored immediately at −80°C. All assays except routine chemistry for safety assessment and determination of the *APOE* genotype were performed at the end of the study to control for between-assay variability. Extra aliquots were stored for additional outcomes of interest to the investigators that might arise during or after the completion of the study. Samples were shipped overnight on dry ice to the appropriate laboratories for biochemical determinations once the study was completed. Plasma and/or serum samples will be used to determine changes in lipid profile, glycemic control, liver and renal function tests, and inflammation and oxidation biomarkers. Extra aliquots of all samples have been stored at −80°C. To reduce assay variability, all samples for a specific assay will be run together in the same laboratory.

An objective biological marker of compliance with walnut consumption, the RBC fatty acid proportions of ALA (Sala-Vila et al., 2011), was assessed in a randomly selected subset (30% of participants) at baseline and years 1 and 2. Given that walnuts have one of the highest polyphenol content of all edible plants (Carey et al., 2013), the urinary content of total polyphenols (Valls-Pedret et al., 2012) will also be used as an indirect marker of compliance.

Genetic testing

The *APOE* genotype will be determined by using the method of Hixson and Vernier (Hixson and Vernier, 1990).

Measurements Only at BCN Site

Some techniques were performed only at the BCN center based on prior expertise, availability, and cost.

Brain magnetic resonance imaging

Approximately 120 participants underwent brain MRI at baseline and 2 years using a 3-tesla scanner (Magnetom Trio Tim, Siemens, Germany). The protocol included high-resolution 3D structural datasets, a diffusion tensor imaging sequence, a Pulsed

Arterial Spin Labeling (PASL)-MRI perfusion sequence, a fluid attenuated inversion recovery (FLAIR) image sequence, and a functional sequence with an n-back working memory task.

Carotid ultrasonography

Bilateral carotid artery ultrasound imaging was performed at baseline and 2 years according to a standardized protocol (Sala-Vila et al., 2014). Main outcome measurements were mean and maximum carotid IMT and plaque presence, maximum height and area. Briefly, patients underwent sonographic assessment with an Acuson X300 ultrasound system (Siemens, Germany) equipped with a VF 10-5 linear multifrequency transducer (frequency range 5–10 MHz) and ECG synchronization. The same certified sonographer performed all examinations without knowledge of group allocation. Secondary outcomes were mean and maximum IMT at each carotid segment. IMT was defined as the average of multiple distance readings between the far wall lumen–intima and media-adventicia interfaces taken bilaterally at common carotid artery 1 cm pre-bifurcation, bifurcation, and internal carotid artery 1 cm after the flow divider. Plaques were sought by using B-mode and color Doppler examinations in both longitudinal and transverse planes to take into consideration circumferential asymmetry and were defined as a focal wall thickening encroaching into the arterial lumen by at least 50% of the surrounding IMT value or with thickness of at least 1.5 mm as measured from the media adventitia interference to the intima-lumen surface. IMT and plaque measurements were taken offline by using edge-finding software in the predefined segments of the arterial wall. Plaque height was recorded at the more appropriate view, either longitudinal or transversal. Plaque area was determined by the technique of Spence et al. (2002). Plaque burden was recorded for all study subjects and defined in two ways: the sum of maximum heights of all plaques and the sum of areas of all plaques.

The same certified sonographer blinded to treatment allocation performed all scan readings. Consistency (reliability or repeatability) of ultrasound carotid wall measurements was evaluated by comparing results from repeated examinations in 14 subjects performed 3 days apart. Intraclass correlation coefficient ranged from 0.92 to 0.96 for IMT mean (average of right and left) and IMT maximum (maximum value from either right or left) in common, bulb, and internal carotid segments.

DEXA studies

Total body lean and fat mass distribution and bone mineral density were assessed by DEXA at baseline and 2 years. This is a standard procedure performed using the whole-body scanner GE Lunar iDXA (GE Healthcare, Madison, WI, USA) according to the manufacturer's specifications. The iDXA is a narrow fan-beam DXA instrument with a high weight limit (204 kg) and a relatively wide scanning space (66 cm) designed to accommodate obese subjects. The subjects were positioned in the center of the table for each scan. The appropriateness of patient's position was further assessed by the DXA software by means of an automatic detection system. The instrument has three scan modes that adjust the X-ray attenuation for the thickness of each patient. For this study, scans were performed using the default scan mode automatically selected by the DXA software. The GE Lunar Body Composition Software was used to obtain fat-free mass (FFM) and fat mass (FM) measurements, as well as segmental FM and FFM distribution (trunk, right and left upper limb, right and left lower limb, android, and gynoid) (Hull et al., 2009).

Ambulatory blood pressure monitoring

Twenty-four-hour ambulatory blood pressure was monitored in 67% of participants selected at random at baseline and 2 years, following the ESH/ESC recommendations (Mancia et al., 2013; Doménech et al., 2014). The devices were Spacelabs 90207/90217 (Spacelabs® Inc., Richmond, WA, USA), programmed for blood pressure lectures every 20 min during the day and every 30 min at night. Each participant kept a diary of daily activities and times of going to bed and waking up. The report included the duration of monitoring (h), the proportion of valid blood pressure registers, and the mean values of systolic and diastolic blood pressure during periods of activity, rest and the total recording period.

Telomere length

Leukocyte telomere length was determined in all participants at baseline and in those undergoing sequential brain MRI at the end of years 1 and 2. Fresh peripheral blood mononuclear cells were separated from 5 mL of EDTA-collected blood by Ficoll gradient centrifugation and also stored at −80°C until determination of telomere length by HTQFISH, which involves *in situ* hybridization of telomere repeats to fluorescent primer and analysis by fluorescence quantification of confocal images.

MicroRNA

Finally, at baseline and 1 year circulating miRNAs potentially involved in lipoprotein metabolism will be screened by qRT-PCR in plasma samples. The procedure requires a sample of ∼1 mL plasma per measurement, kept in Trizol® and stored at −80°C until the moment of the analysis.

Statistical Analyses

Sample size calculation was based on prior results from the PREDIMED study (Valls-Pedret et al., 2015). We used as reference the 2-year unadjusted changes in RAVLT (total learning) scores in the Mediterranean diet enriched with nuts compared to the control diet. In order to have a 90% power to detect differences in the contrast with the null hypothesis, assuming mean changes of 1.05 and 2.10 points in the control and intervention groups, respectively, with a standard deviation of 4.00, the total number of participants required was 308 per group. Considering an estimated dropout rate of 10%, we needed to include a total of 686 participants.

All data collected in the study were entered into an online database (Onto CRF, Costaisa, Spain) managed for both centers. An oversight committee blinded to subject allocation monitored safety, quality of data collected, and trial's progress.

Between-group differences in baseline neuropsychological and ophthalmologic data will be examined by ANOVA and further by ANCOVA adjusting for center, sex, age, education years, *APOE4* genotype, smoking, BMI, energy intake, physical activity,

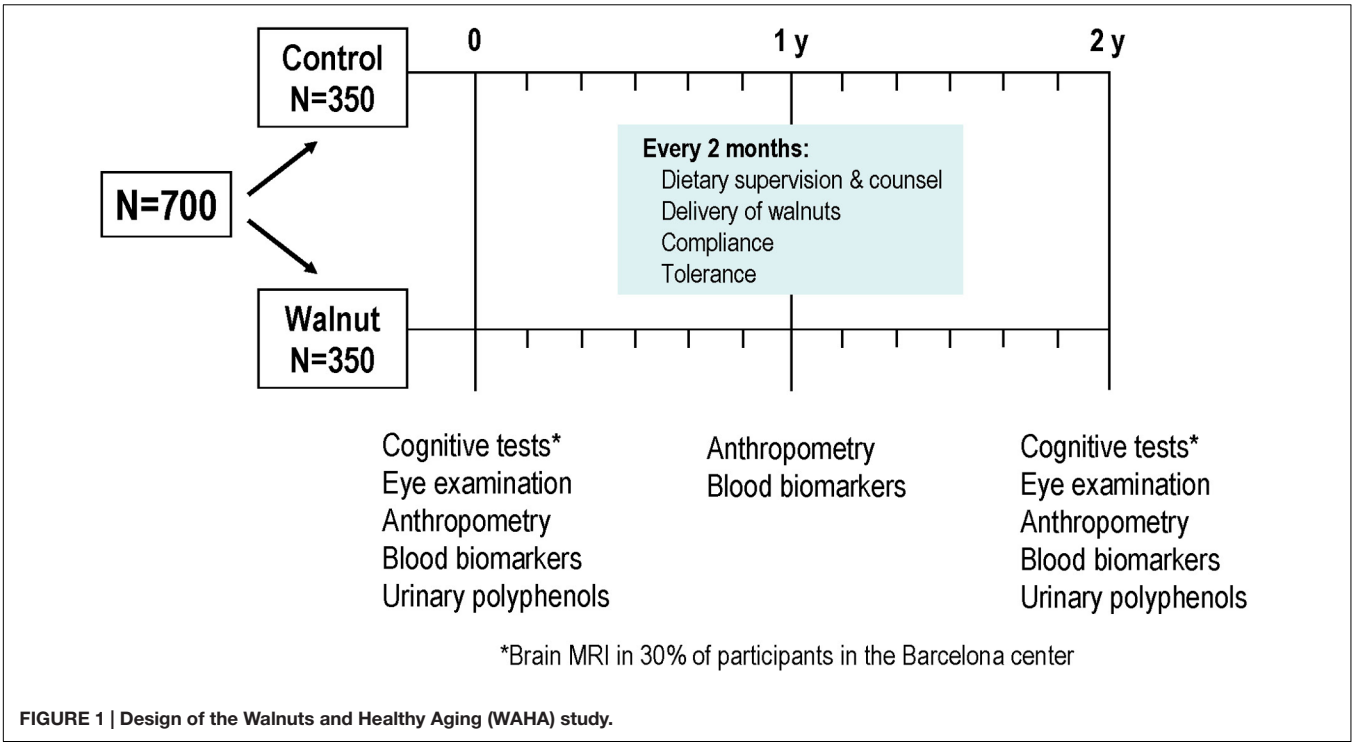

FIGURE 1 | Design of the Walnuts and Healthy Aging (WAHA) study.

diabetes, hyperlipidemia, and hypertension. Changes for each individual cognitive test, cognitive composites, ophthalmologic evaluations, and foods and nutrients ensuing intervention will be assessed by ANOVA and further by ANCOVA adjusting for the variables listed above. Statistical significance will be set at the $P < 0.05$ level. Analyses will be performed using SPSS software, version 16.0 (IBM Corp., New York, NY, USA).

STEPWISE PROCEDURES

As depicted in **Figure 1**, there are two main points of data collection, at baseline and at the end of the study after 2 years of intervention. However, there are intermediate points wherein relevant information is obtained. Thus, in mid-study (12 months) a physical activity questionnaire was administered and blood was collected for determination of safety biochemistry analyses, lipid profile, and some secondary outcome variables such as leukocyte telomere length. Nutritional information and office blood pressure was obtained every 6 months, and general health status, tolerance and side effects, medication changes, and anthropometric data were ascertained every 2 months.

The pre-intervention visit with the study's clinician lasted 1 h and consisted of a face-to-face interview with the candidate wherein complete information on the trial was provided, medical information was collected, inclusion/exclusion criteria were recorded, office blood pressure was measured, an ECG was performed, and the MMSE were administered. If the candidate was suitable for the trial and willing to participate, the informed consent was signed and blood extraction and urine collection and the first neuropsychologist and ophthalmologic visits were scheduled for next week. In the Barcelona site, one part of the informed consent concerned brain MRI, and candidates were asked to fill it in if willing to undergo the procedure. Once the pre-established number of 30% of the cohort was reached, no further candidates were offered brain MRI.

The neuropsychologist visit included the comprehensive test battery described in the Section "Cognitive Testing," which took 90 min on average to complete. Whenever a cognitive alteration was detected at this visit, the candidate was excluded from further studies and was referred to the local Neurology clinic with a report describing the test findings. In the Barcelona site, the neuropsychologist provided full details on the brain MRI examination to willing candidates and scheduled the procedure.

In the ophthalmologic visit, which lasted approximately 30 min, a general history of eye health was taken and the OCT was performed. Any candidate found to have advanced AMD or bilateral cataracts precluding retinal examination was excluded from the study and referred to the local Ophthalmology clinic.

Once candidates were considered fit to enter the study after assessment of the main outcomes, they were scheduled for the first visit with the dietitian wherein they were randomized to the walnut or control diets. Participants allocated to the walnut diet received their first 2-month allotment of walnuts plus extra packs for family needs. In the BCN site, after the first dietitian visit all participants were scheduled for the secondary outcome variables carotid ultrasound, DEXA for both body composition and bone mineral density, and ambulatory blood

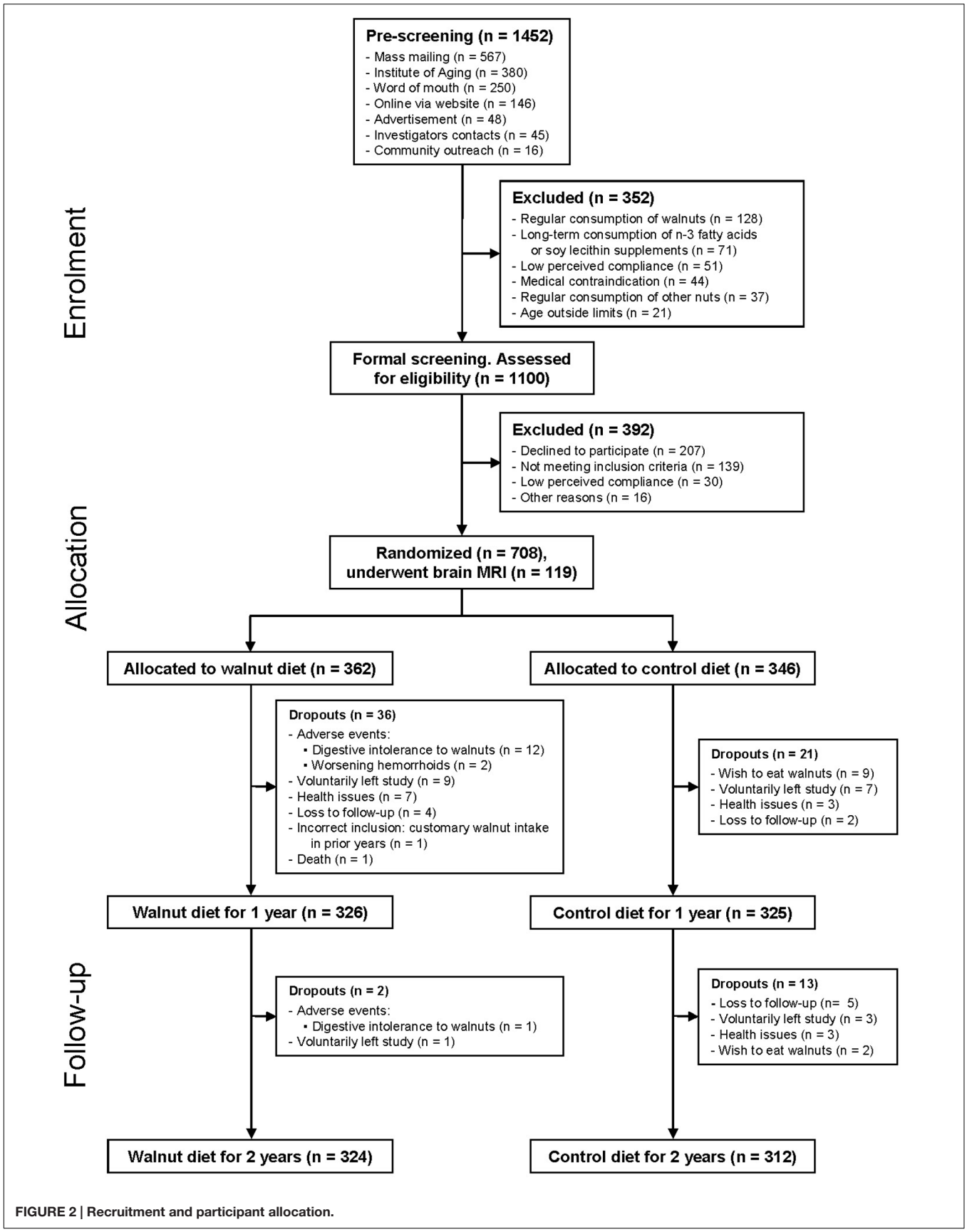

FIGURE 2 | Recruitment and participant allocation.

pressure monitoring. During the 2 years of the study, dietitians took care of all aspects of follow-up at the bimonthly visits. These included dietary compliance, tolerance and side-effects, anthropometric measurements, delivery of walnuts and recount of empty packages, and changes in clinical status and medication. Clinical and medication changes were always reported to the study's clinician for confirmation and final recording in the online study's management system.

At the end of the study, all examinations related to primary and secondary outcomes were repeated.

ANTICIPATED RESULTS

Data collection for the WAHA study was completed May 31, 2016. As shown in **Figure 2**, of the total 708 randomized subjects, 92% ($n = 651$) completed the first year of the study, while 89.8% ($n = 636$) completed the 2 years. At the end of the study, the dropout rate is 10.2%. Attrition rates were similar in the two groups, $n = 38$ in the walnut group and $n = 34$ in the control group.

The baseline characteristics of the study participants by group are provided in **Table 2**. The mean age was 69.1 years and 67.9% were women. The characteristics of participants in the two intervention groups were well balanced except for smoking status, as there were more current smokers in the walnut group than in the control group.

To objectively determine adherence to supplemental walnuts, we measured the RBC proportions of ALA, a fatty acid characteristic of walnuts, at baseline and after 1 year of intervention in a random sub-sample of participants. There were no significant differences in RBC ALA between intervention groups at baseline (**Figure 3A**). After adjusting for center, age, and sex, 1-year changes in RBC ALA were increases of 0.16% of total fatty acids (95% confidence interval, 0.144–0.183) and 0.02% (95% confidence interval, 0.001–0.039) for the walnuts and control group, respectively (P between groups < 0.001) (**Figure 3B**).

TABLE 2 | Baseline characteristics of 708 WAHA participants by treatment allocation.

Characteristics	Walnut	Control	*P*-value*
No. (%)	362 (51.1)	346 (48.9)	
Enter with partner – no. (%)	98 (27.1)	88 (25.4)	0.62
Women – no. (%)	244 (67.4)	237 (68.5)	0.76
Age – year (mean ± SD)	69.4 ± 3.8	68.9 ± 3.5	0.07
Smoking – no. (%)			0.02
Never smoker	303 (83.7)	293 (84.7)	
Former smoker	43 (11.9)	49 (14.2)	
Current smoker	16 (4.4)	4 (1.2)	
Education – no. (%)			0.83
Basic (0–4 years)	11 (3.0)	8 (2.3)	
Elementary (5–8 years)	60 (16.6)	65 (18.8)	
Secondary (9–12 years)	70 (19.3)	65 (18.8)	
Post-secondary (>12 years)	221 (61.0)	208 (60.1)	
Height – cm	164.3 ± 9.5	163.3 ± 9.0	0.14
Weight – kg	73.8 ± 15.2	73.5 ± 14.7	0.78
Body mass index – kg/m^2	27.2 ± 4.3	27.5 ± 4.4	0.42
Hypertension – no. (%)	191 (52.8)	183 (52.9)	0.97
Type-2 diabetes – no. (%)	37 (10.2)	33 (9.5)	0.76
Dyslipidemia – no. (%)	203 (56.1)	182 (52.6)	0.35

Data are no. (%) or mean ± SD, as appropriate.
**Obtained by Chi-square test or ANOVA, as appropriate.*

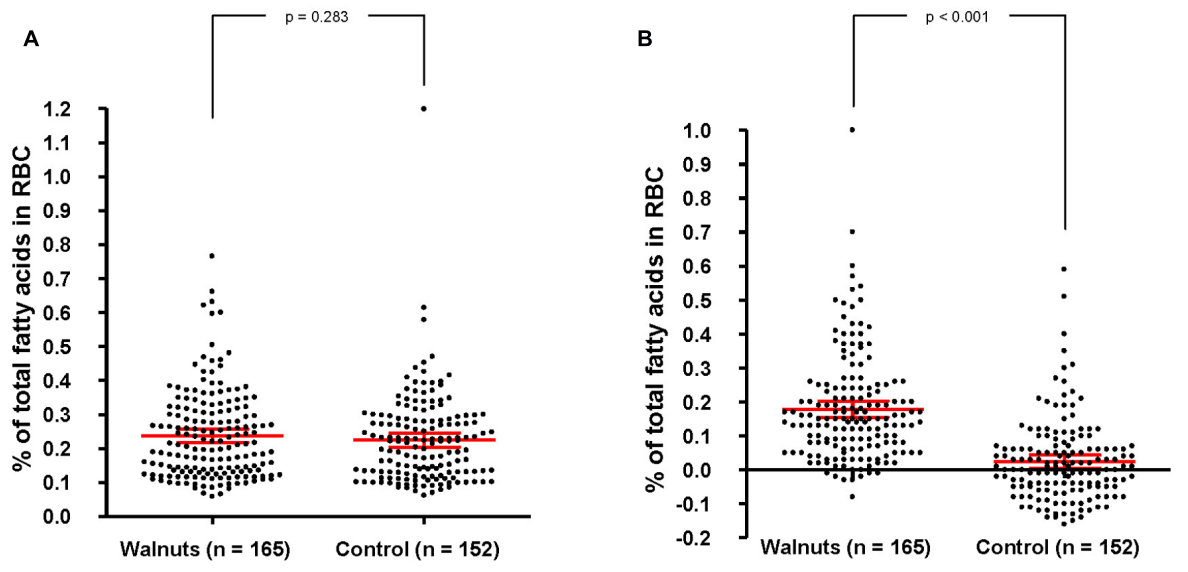

FIGURE 3 | Baseline biomarkers of adherence to walnuts and change after 1 year by intervention group. RBC, red blood cells. In red, mean and 95% CI (ANCOVA, adjusting for center, age, and sex). **(A)** Percentage of total fatty acids in RBC at baseline. Values are 0.233 (0.218–0.247) and 0.221 (0.206–0.236) for the walnut and control groups, respectively. **(B)** Percentage of change of total fatty acids in RBC after 1 year. Values are 0.163 (0.144–0.183) and 0.019 (0.001–0.039) for the walnut and control groups, respectively.

POTENTIAL PITFALLS AND COUNTERACTING MEASURES

The WAHA study was designed to assess whether a nutritional intervention with walnuts for 2 years can prevent cognitive impairment and preserve retinal health in a healthy elderly population.

An unavoidable limitation of the study is not being able to blind participants to the intervention since it consists of a whole food. Another limitation is the difficulty in keeping older, free-living volunteers in the study during 2 years and ensuring compliance with the high-fat supplemental food in the walnut diet group. The bimonthly visits by dietitians and their availability to answer questions related to diet or body weight and solve any problems with walnut ingestion contributed to ensure participants' loyalty. Thus, the final dropout rate was only 10% and there was good compliance with both eating the walnuts in the walnut group and not eating them in the control group, as attested by 1-year changes of RBC ALA, an objective biomarker of walnut consumption. Finally, by the design of the recruitment methodology, self-selected participants are in better health and have a higher educational level than the general population, which could lead to lower than expected age-related cognitive decline and a lower probability of improving cognitive function with the intervention. The mean baseline age of nearly 70 years, however, may partly overcome this healthy participant effect.

The WAHA study has several strengths. This is the first RCT that examines the effects of a single whole food, walnuts, on cognitive performance and retinal health. By being an RCT the results of the main outcomes should provide a high level of evidence, while those of secondary outcomes, such as brain MRI, carotid ultrasonography, 24-h ambulatory blood pressure, inflammatory markers, and telomere length, might shed light on the mechanisms of a putative beneficial effect of the walnut diet on age-related disorders. Having participants from two clinical centers located in different geographical regions with variations in background diet and lifestyle habits increases the external validity. We assessed the primary outcome cognitive decline with a battery of standardized neuropsychological tests designed to evaluate different cognitive domains, making it a comprehensive approach. Changes in the retina were also evaluated by a precise quantitative technique such as OCT. Finally the free-living aspect of the study makes it as close to real-life as possible since there were no major diet changes to be made except to either eat walnuts daily or abstain from eating walnuts. The results should thus be applicable to a wider population and might have a significant impact on global public health recommendations.

In summary, the WAHA study evaluates cognitive function and retinal health in an elderly cohort following daily ingestion of walnuts for 2 years. The results might provide high-level evidence of the benefit of regular walnut consumption on delaying the onset of age-related degenerative diseases. The findings should translate into public health policy and sound recommendations to the general population.

ETHICS STATEMENT

The study protocol was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the institutional review boards of each center. All participants provided written informed consent.

AUTHOR CONTRIBUTIONS

ER and JS obtained funding and supervised the study. They also developed and drafted this study protocol in consultation with SR, CV-P, MC, MS-M, AP-H, RC-M, AS-V, and MD. SJ and MC were responsible for the recruitment process. CV-P and AA were in charge of cognitive assessment. Biochemical samples were managed and analyzed by SJ, MC, AS-V, and CC. MS-M, AP-H, IR, TF-S, EH, EB, NK, and LH were involved in dietary intervention and nutritional and anthropometric data collection. RC-M, SA, and JF were in charge of ophthalmologic assessment. MD and AL-I were responsible for clinical data, medical records, and blood pressure. All authors revised the manuscript critically for intellectual content and gave final approval of the version to be published.

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Functional Brain Activity Changes after 4 Weeks Supplementation with a Multi-Vitamin/Mineral Combination: A Randomized, Double-Blind, Placebo-Controlled Trial Exploring Functional Magnetic Resonance Imaging and Steady-State Visual Evoked Potentials during Working Memory

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This study explored the neurocognitive effects of 4 weeks daily supplementation with a multi-vitamin and -mineral combination (MVM) in healthy adults (aged 18–40 years). Using a randomized, double-blind, placebo-controlled design, participants underwent assessments of brain activity using functional Magnetic Resonance Imaging (fMRI; $n = 32$, 16 females) and Steady-State Visual Evoked Potential recordings (SSVEP; $n = 39$, 20 females) during working memory and continuous performance tasks at baseline and following 4 weeks of active MVM treatment or placebo. There were several treatment-related effects suggestive of changes in functional brain activity associated with MVM administration. SSVEP data showed latency reductions across centro-parietal regions during the encoding period of a spatial working memory task following 4 weeks of active MVM treatment. Complementary results were observed with the fMRI data, in which a subset of those completing fMRI assessment after SSVEP assessment ($n = 16$) demonstrated increased BOLD response during completion of the Rapid Visual Information Processing task (RVIP) within regions of interest including bilateral parietal lobes. No treatment-related changes in fMRI data were observed in those who had not first undergone SSVEP assessment, suggesting these results may be most evident under conditions of fatigue. Performance on the working memory and continuous performance tasks did not significantly differ between treatment groups at follow-up. In addition, within the fatigued fMRI sample, increased RVIP BOLD response was correlated with the change in number of target detections as part of the RVIP task.

This study provides preliminary evidence of changes in functional brain activity during working memory associated with 4 weeks of daily treatment with a multi-vitamin and -mineral combination in healthy adults, using two distinct but complementary measures of functional brain activity.

Keywords: multi-vitamin, nutrient, functional magnetic resonance imaging (fMRI), steady-state visual evoked potentials, working memory

INTRODUCTION

Micronutrients, such as B vitamins and minerals, are critical components of healthy physiological functioning, yet humans are dependent on dietary sources in maintaining an adequate supply. B vitamins are a group of water soluble organic molecules which act as cofactors in a multitude of cellular processes. These micronutrients are implicated in catabolic reactions which support energy metabolism (Depeint et al., 2006; Huskisson et al., 2007), in addition to anabolic pathways which drive synthesis of DNA/RNA, antioxidants and neurotransmitters, whilst also reducing homocysteine to methionine through roles in one carbon transfer cycles (for a detailed review, see Kennedy, 2016). While a healthy diet may be sufficient to maintain adequate supplies of these essential micronutrients, modern Western dietary patterns may lead to a significant proportion of the population not meeting recommendations for a range of micronutrients, including B vitamins (Troesch et al., 2012).

Looking beyond the prevention of physical illness related to deficiency in specific B vitamins, there is very little data exploring optimal micronutrient levels (Neufeld and Cameron, 2012; Kennedy, 2016). Recent work investigating micronutrient supplementation with a multi-vitamin/mineral preparation (MVM) in healthy individuals provides support for a distinction between optimal and sub-optimal micronutrient status (Pietrzik, 1985; Fletcher and Fairfield, 2002) in the absence of a clinical deficiency. For example, clinical trials investigating the effects of MVM in healthy adults have generally observed a positive impact on mood, with meta-analysis indicating significant benefits to subjective stress, sub-clinical psychiatric symptoms and a range of everyday mood dimensions (Long and Benton, 2013). Indeed, mood outcomes were assessed as part of the trial which generated the functional brain activity assessments described herein, also showing significant positive effects for aspects of everyday mood (White et al., 2015).

Given the prominent role of B vitamins in cellular functions which support brain function, it follows that manipulations of micronutrients such as B vitamins via MVM supplementation may impact neurocognitive function. Trials investigating potential effects of MVM supplementation on cognitive performance amongst healthy adults have not provided a wholly consistent picture, partially driven by insufficient research (Grima et al., 2012). The potential for enhancing cognitive function amongst healthy adult males was studied by Kennedy et al. (2010). Serial subtraction task performance completed as part of a cognitive demand battery was improved following 28-days MVM supplementation. In another study, females aged 25–50 years demonstrated significant cognitive performance benefits after 63 days of MVM supplementation using a multi-tasking assessment (Haskell et al., 2010). In contrast, the largest MVM supplementation trial to date investigating cognitive changes found no evidence supporting a positive effect of such treatment in a sample of male physicians aged 65 or older over a mean follow-up intervention duration of 8.5 years (Grodstein et al., 2013). These studies not only highlight the variability in findings when considering possible cognitive function benefits associated with MVM supplementation in healthy adults, but also the differences in micronutrient doses and duration of the MVM treatment, the treatment populations, and the methods for assessing cognitive performance (for example, Grodstein et al., 2013 assessed cognitive function by telephone interview).

Recently, a parallel line of inquiry has explored potential physiological changes associated with MVM supplementation. Amongst obese females aged 18–55, daily MVM supplementation with a preparation containing a large number of minerals in addition to vitamins over 26 weeks led to an increase in resting energy expenditure, along with reductions in fat mass, body weight, and cholesterol (Li et al., 2010). Increased energy expenditure was again observed in a sample of healthy females aged 25–49 after MVM supplementation, in this case during cognitive task performance (Kennedy et al., 2016). Exploring both acute and acute-on-chronic effects of two MVM treatments, one higher dose and one lower dose with added Coenzyme Q10, Kennedy et al. (2016) reported this increase in energy expenditure during cognitive task performance after both the single acute dose and in a dose-dependent manner following 56 days of supplementation. The study also explored brain hemodynamic changes during completion of the cognitive task battery using functional near infrared spectroscopy (fNIRS), which revealed increased cerebral blood flow to prefrontal sites at the first acute assessment only with the lower MVM dose with Coenzyme Q10 and similar, albeit non-significant, trends in the high dose MVM treatment group. These hemodynamic changes were not accompanied by changes in cognitive function.

Functional Magnetic Resonance Imaging (fMRI) shares some of the properties of fNIRS but allows far better spatial resolution. Work from our laboratory has provided preliminary evidence of neurophysiological changes associated with MVM supplementation, exploring both acute doses and longer term supplementation using fMRI and Steady State Visual Evoked Potentials (SSVEP) – an electrophysiological technique with high temporal resolution (see below). In a pilot investigation of the acute effects of two MVM treatments, with or without guaraná, fMRI during completion of Rapid Visual Information Processing (RVIP) and Inspection Time tasks revealed increased blood oxygen level dependent (BOLD) response to active tasks for both

MVM treatments compared to placebo (Scholey et al., 2013). This study also reported cognitive performance and mood benefits restricted to the MVM plus guaraná treatment.

Changes in functional brain activity have been reported for both acute and chronic MVM supplementation using SSVEP methodology. Before proceeding with the outcomes of this work, a brief introduction to the SSVEP technique is provided. The SSVEP has found applications in the study of basic visual processes and perception, brain-computer interfaces and higher order cognitive functions (for reviews, see Vialatte et al., 2010; Norcia et al., 2015). One SSVEP method for probing higher cognitive functions, referred to as Steady State Topography (Silberstein et al., 1990), explores SSVEP responses to a task-irrelevant diffuse 13 Hz sinusoidal flicker superimposed onto the visual field during cognitive task engagement. SSVEP amplitude reflects summed activity of pyramidal cells firing synchronously with the 13 Hz flicker, whilst SSVEP phase reflects latency differences between the visual flicker and the SSVEP response. This phase difference is argued to reflect cortico-cortical loop transmission time, which is linked with ongoing excitatory and inhibitory processes in the underlying cortical regions such that an increase in excitatory processes will manifest as an SSVEP phase advance (or reduced latency; Silberstein et al., 1995, 2000). Using this Steady State Topography method, fluctuations in SSVEP amplitude and phase have been linked with a range of cognitive processes (Silberstein et al., 2001; Kemp et al., 2002; Ellis et al., 2006), neurophysiological changes in clinical populations (Silberstein et al., 1998, 2000), and also to probe the neurocognitive effects of a number of psychopharmacological and nutritional manipulations (Kemp et al., 2004; Silberstein et al., 2011; Camfield et al., 2012), including MVM supplementation.

The pilot fMRI findings of Scholey et al. (2013) studying acute effects of MVM administration were extended using SSVEP recordings during completion of the A-X Continuous Performance Task (A-X CPT; White et al., 2016). This study found distinct changes in SSVEP response during the A-X CPT associated with MVM supplementation containing higher B vitamin and mineral doses and a second MVM with lower micronutrient doses but additional guaraná. After the high dose MVM treatment, SSVEP amplitude reduction and phase advance were observed in frontal electrode sites during the period between the probe and target stimuli, a pattern which was correlated with better task performance. In contrast, the low dose MVM with guaraná showed little evidence of any phasic task-related SSVEP fluctuations, instead causing a generalized shift toward greater phase advance, with a diffuse topographic pattern, consistent with the general excitatory actions of the caffeine content in the treatment. Macpherson et al. (2012) explored changes in functional brain activity related to MVM supplementation over 16 weeks in females aged 64–79 using SSVEP recordings. This trial tested the effects of supplementation with an MVM preparation, which also contained 19 plant extracts and three probiotics, with changes in SSVEP studied during completion of a spatial working memory task. The study observed greater SSVEP phase lag (increased latency) during the retrieval component of the spatial working memory task, a pattern of change which was correlated with improved task performance post-treatment.

To summarize, micronutrients are essential cofactors in a host of cellular processes critical to healthy physiological and neurocognitive functioning, with an increasing effort to understand whether intake other than in the context of avoiding clinical deficiency may enhance function in otherwise healthy individuals. Where studies have considered behavioral outcomes of MVM interventions in healthy adults, results indicate potential benefits to mood, with support, though less consistent, for cognitive performance benefits. There is also evidence to suggest MVM supplementation may influence energy metabolism, and possibly more specific neurophysiological functions. In studies assessing task-related SSVEP changes following MVM administration, the technique appears sensitive to potential changes in functional brain activity related to MVM supplementation, whilst the pilot data using fMRI methods warrant further investigation. To this end, the current study aimed to utilize these complementary methods, fMRI and SSVEP, to assess functional brain activity in order to explore any potential relationship between MVM supplementation and brain function in healthy adults.

MATERIALS AND METHODS

We studied functional imaging outcomes, employing a randomized, double-blind, placebo controlled design to investigate the effects of 4 weeks daily supplementation with a MVM combination. The intervention consisted of a micronutrient preparation containing all eight B vitamins, in addition to Vitamin C and the minerals calcium, magnesium and zinc (further detailed below). The overall trial methods and outcomes relating to mood and blood biomarkers have been published elsewhere (White et al., 2015). The study was approved by the Swinburne University Human Research Ethics Committee (Ref SUHREC 2012/164) and was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12612001043820).

Participants

The study enrolled 58 healthy adult participants aged between 18 and 40 years of age (Mean age = 25.82 years, $SD = 4.87$), who were recruited from the local community via local advertisements. Participants were free of psychiatric or serious physical illnesses and had not taken medication (with the exception of the contraceptive pill or routine medications for benign conditions), herbal extracts, vitamin supplements or illicit drugs within 4 weeks prior to enrolment and for study duration. These participants completed one of three functional brain imaging assessment streams (further detailed in Supplementary Figure 3): both SSVEP and fMRI ($n = 16$, eight females, one additional participant withdrew consent prior to follow-up assessment), SSVEP assessment alone ($n = 23$, 12 female), and fMRI assessment alone ($n = 16$, eight females, with two additional participants who withdrew

consent prior to follow-up assessment). Those who completed both SSVEP and fMRI assessment did so on the same testing day, with SSVEP recordings undertaken prior to fMRI assessment.

Procedure

Participants attended an initial screening and familiarization visit, followed by baseline and post-treatment assessment sessions conducted 4 weeks apart. Participants were given an opportunity to practice each of the cognitive tasks at the screening visit, with SSVEP and fMRI assessment of functional brain activity performed at baseline and 4 weeks post-treatment. Randomization was conducted by a disinterested third party, with stratified randomization used to balance gender within each functional imaging assessment stream. The active and placebo treatments were effervescent tablets matched for color and flavor, prepared by Bayer AG (Basel, Switzerland). The active treatment, containing high doses of B vitamins, in addition to zinc, calcium, magnesium and vitamin C, is commercially available as Berocca® Performance (detailed in **Table 1** below). Participants were instructed to take one tablet daily with breakfast, dissolved in at least 200 mL of water. Participants returned after 4 weeks of supplementation having not taken a treatment on the day of their return visit. Compliance was determined by a count of returned treatments, whilst analysis of circulating levels of vitamin B6, Red Cell Folate and B12 showed increases post-treatment in the active treatment group, with significant reductions in homocysteine, confirming compliance and absorption (for details, see White et al., 2015).

TABLE 1 | Micronutrient doses of the active MVM treatment.

Nutrient	Amount (mg)	% RDA/RDI	
		Males	Females
Vitamin C	500	556 (1111)	667 (1111)
Thiamine monophosphoric acid ester chloride	18.54	1545	1685
Riboflavin (vitamin B2)	15	1154	1364
Nicotinamide (B3/niacin)	50	313	357
Vitamin B5	23	460* (383*)	460* (575*)
Vitamin B6	10	769	769
Vitamin B12	0.01	417	417
Folic Acid (Vitamin B9)	0.4	100	100
Biotin (Vitamin B7)	0.15	500*	500* (600*)
Calcium	100	10	10
Magnesium	100	25	32
Zinc	10	91 (71)	125

*% RDA (RDI) is the percentage for each micronutrient of Recommended Dietary Allowances derived from Dietary References Intakes of the Institute of Medicine, National Academy of Sciences (US) and Recommended Daily Intake from the Nutrient Reference Values of the National Health and Medical Research Council (Australia) for healthy adults in the age range 19–30. Where a second number appears in brackets, the US and Australian reference values disagree, in which case US RDA's are given first with Australian RDI's in brackets. *Indicates the use of 'Adequate Intakes' where RDA/RDI could not be determined.*

Functional MRI Acquisition

MRI scanning used a Siemens Tim Trio 3T MRI scanner equipped with a 32-channel head coil at Swinburne University of Technology, Melbourne, Australia. Functional MRI data was acquired during completion of an Inspection Time (IT) task and the RVIP task. At baseline and post-treatment assessments, the imaging protocol included a T1-weighted scan (3D MPRAGE; TR = 1900 ms, TE = 2.52 ms, flip angle = 9°, Field of View = 256 mm, 176 slices, 1 mm^3 isotropic voxels), in addition to T2*-weighted volumes obtained during completion of the two cognitive tasks using the same EPI acquisition parameters, with the first three volumes of each task run discarded (TR = 3000 ms, TE = 30 ms, flip angle = 90°, Field of View = 192 mm, 46 interleaved slices, 3 mm^3 isotropic voxels). Both IT and RVIP tasks, described below, were variants of previously published task paradigms presented using Presentation® software[1]. Participants viewed the task stimuli on a monitor through a mirror on the head coil.

Inspection Time Task Details

The IT task involved presentation of a stimulus with two vertical lines of varying lengths running perpendicular at either end of a horizontal line (white stimuli on a black background). The participant was then required to indicate which line was perceived as longer (forced choice, with two alternatives, via button box held in the right hand with an index finger (left) or middle finger (right) button press). The duration for which this stimulus remained visible, prior to the appearance of a mask (500 ms mask duration), varied from equally represented trials of 40, 60, 80, 100, and 120 ms (Waiter et al., 2008). Trials were preceded by a 600 ms fixation cross, with a variable inter-trial interval (ITI) comprising each 500 ms increment from 1880 to 3880 ms. Each ITI preceded the five stimulus durations equally, with 20 trials of each stimulus duration presented in a pseudorandom order per run of the task. Two 426-second runs (142 functional volumes), each of 100 trials, were conducted at each assessment visit.

Rapid Visual Information Processing Task Details

The RVIP task implemented was identical to that described in Neale et al. (2015). Briefly, single digits (white stimuli on a black background) were presented at a rate of 100 per minute, with the active task variant requiring participants to respond upon presentation of three consecutive odd or even digits, and a control task variant requiring detection of a single target digit not presented during the active variant ('0'). Both task variants contained target sequences at a rate of four per 30 s. The two task variants alternated in a blocked design, with each 58.5-second block preceded by a 12-second rest/instruction block, which cued participants to the upcoming task requirements. Two runs of the task were completed at each assessment visit, with each run comprising three blocks of each task variant and seven rest/instruction blocks for a total of 435 s (145 functional volumes per run).

[1]www.neurobs.com

fMRI Data Processing and Analysis

All pre-processing and participant-level statistical analysis of fMRI data were performed using SPM8 (Wellcome Trust Centre for Neuroimaging, London, UK). Data pre-processing included the following steps: the image time-series were slice-time corrected referenced to the middle slice, then realigned to the first volume acquired. The T1 structural image acquired at the corresponding treatment visit was co-registered to a mean functional image created during realignment, then normalized to MNI T1 template released with SPM8. The parameters of this T1 transformation were subsequently applied to the realigned functional images. The normalized functional images were then smoothed with an 8 mm FWHM Gaussian filter.

Participant-level modeling for the IT task explored the parametric modulation of BOLD signal as a function of stimulus duration. Correct trial events were modeled as stick functions convolved with the canonical hemodynamic response function (HRF) supplied with SPM8. These correct events were parametrically modulated with a regressor coding trial presentation duration (longest durations had the highest numbers) that was orthogonalized to the event regressor; the parametric modulator expresses how well BOLD fluctuations not explained by the average response to all stimulus onsets covaries with stimulus presentation length. Finally, motion correlated BOLD signal variance was modeled by including the motion realignment parameters as covariates of no interest.

At the group level of analysis, the parametric modulation effect showed no statistically significant effects at baseline (see Supplementary Data Section 2). In the absence of reliable task effects at baseline, this task was not pursued for potential effects of treatment.

Participant-level modeling for the RVIP task modeled active and control variants of the task using separate boxcar functions defined by the onset and duration of each task block that were convolved with the canonical HRF, with the motion parameters included as nuisance regressors. Analysis of treatment effects on RVIP task-related functional brain activity adopted a region-of-interest (ROI) approach, deriving functional ROIs from an independent sample of healthy participants matching eligibility criteria for the study. The details of the analysis generating the five ROIs used are described in Supplementary Data Section 1 (see Supplementary Table 1 and Supplementary Figure 1). The five ROIs identified by this analysis were localized to a left and right parietal cluster, a left middle frontal cluster, the left insula and a cluster comprising bilateral supplementary motor areas. Mean contrast estimates obtained from the active task minus control task contrast were extracted from the five ROIs, using the MarsBaR toolbox[2] (Brett et al., 2002). Given the demands of the control task largely match the attentional and sensorimotor demands of the active variant, this contrast largely isolates working memory demands required by the active variant of the task. These mean contrast estimates obtained at the post-treatment visit formed the input for an analysis of covariance (ANCOVA) examining the main effect of treatment group as a between-group factor, with pre-treatment baseline estimates included as a covariate using SPSS for Windows (Version 23; SPSS Inc., Chicago, IL, USA). Comparisons were thresholded for significance using False Discovery Rate correction with $q = 0.1$ in order to control for multiple comparisons across the five ROIs (Benjamini and Hochberg, 1995).

[2]http://marsbar.sourceforge.net

Steady-State Visually Evoked Potential Recordings

Two cognitive tasks, one continuous performance task and one spatial working memory task, were completed during recordings of the SSVEP. Acquisition and pre-processing of the SSVEP followed an identical procedure to that described in a recent study exploring the acute effects of MVM administration, both with and without guaraná (White et al., 2016). Briefly, recordings were acquired from 60 scalp electrodes, positioned according to the extended 10-10 system using a Quick-Caps electrode cap and Synamps[2] amplifiers with Scan 4.3 Software (Neuroscan, Inc., Abbotsford, VIC, Australia). An electrode positioned between Cz and Cpz acted as the reference, with the ground electrode positioned between Fz and Fpz. Recordings were also taken from left and right mastoids, with data subsequently re-referenced to linked mastoids off-line. The SSVEP was elicited through goggles using LED arrays emitting a 13 Hz sinusoidal flicker, which was superimposed over the visual field with half-silvered mirrors subtending a horizontal angle of 160° and vertical angle of 90°.

SSVEP Cognitive Tasks: A-X Continuous Performance Task

The details of the AX-CPT used in the present study are identical to that described in the previous investigation of the acute effects of MVM administration (White et al., 2016). The task presents single letters at a rate of approximately 34 per minute, and involves two variants: one active task in which participants respond to a target sequence of 'X' preceded by 'A' amongst a pseudo-random sequence of letters, and a control task in which participants respond to a target letter 'E' amongst a predictable repeated sequence of letters ('A' through 'E'). For both variants 200 stimuli, including 40 target responses, were presented across two recording runs of approximately 3 min each separated by a short break.

SSVEP Cognitive Task: Spatial Working Memory

The spatial working memory task adopted for the current study was based on a delayed match-to-sample paradigm with a history of use in studying the functional imaging correlates of spatial working memory (Jonides et al., 1993), which has previously been used to explore age-related changes in SSVEP response (Macpherson et al., 2014), in addition to changes associated with MVM supplementation in healthy older females (Macpherson et al., 2012). Trials presented either two or three white dots on a black background for 500 ms (encoding period), followed by presentation of a fixation cross for 3000 ms (maintenance period), after which an empty circle appeared for 1800 ms (probe). During this probe period, participants responded as to whether the location of the circle enclosed an encoded location of the dot stimuli, with a right button press indicating a match and a left button press indicating a new location. The control variant of

the task matched sensorimotor demands of the task, removing the working memory load by retaining the dot stimuli on-screen throughout the duration of each trial, such that the probe period required a response as to whether the circle stimuli either surrounded a dot (right button press) or did not enclose a dot (left button press). Active and control task variants were completed in separate recording runs, comprising 40 trials separated by a 1000 ms fixation period, half of which presented two dots during encoding and half three dots. To ensure a sufficient number of correct trials for analysis, two runs of the active task variant were completed, resulting in 80 trials for the active task per visit and a single run of 40 trials for the control variant.

SSVEP Data Processing and Analysis

All aspects of the SSVEP signal processing were identical to that described in White et al. (2016), with the 13 Hz SSVEP signal extracted from ongoing recordings using established routines implemented in in-house software (BrainSci; Silberstein, 1995b) and statistical analysis and mapping using custom MATLAB scripts (The Mathworks Inc., Natick, MA, USA). In order to control for inter-individual differences in SSVEP amplitude and phase responses, the active task variants of both AX-CPT and SWM task paradigms were normalized to the corresponding control task variant at the baseline visit. For the A-X CPT task, mean SSVEP amplitude and phase were calculated for the 250 ms period following the cue stimulus ('A'), the 1500 ms hold period following, and the 1000 ms from target appearance ('X'). For the SWM task, mean SSVEP amplitude and phase were calculated for the 500 ms encoding period, the 3000 ms maintenance period, and the 1000 ms from presentation of the probe stimulus. As only correct trials were included in analyses, one further participant was excluded from analysis of A-X CPT, due to incorrect task performance.

Both A-X CPT and SWM tasks were studied across the entire SSVEP sample at baseline to characterize the SSVEP response to task completion pre-treatment. For each of the three averaged task components, this involved direct comparison between the active and control task variant for both tasks. Subsequently, potential changes in SSVEP response from baseline to post-treatment were investigated, contrasting the active task SSVEP response at each visit for each treatment group separately. The SSVEP response is studied in terms of amplitude and phase, necessitating an alternate statistical approach to that of behavioral and fMRI data. Comparisons were conducted using Hotelling's T^2, the bivariate analog to a paired *T*-test, which tests for differences in the mean vector comprising the complex numbers quantifying the SSVEP amplitude and phase. Adjustment for multiple comparisons followed previous research to use the Steady State Topography method, in which spatial principal component analysis of SSVEP data indicating five independent components can account for over 95% of spatial variance (Silberstein and Cadusch, 1992), thus the alpha level for SSVEP analysis was adjusted to 1% (adjusted $p = 0.05/5$). In the event of significant changes, the association between these SSVEP changes and behavioral performance changes from baseline to post-treatment were also explored through correlations in order to assess any behavioral correlates of these changes (that is, the difference in SSVEP response pre- to post-treatment was correlated with the change in performance between these assessment visits).

Analysis of Behavioral Performance

Behavioral performance on cognitive tasks was measured in terms of accuracy and mean response time for the active variant of each of the four cognitive tasks. Task accuracy was operationalized as the percentage of correct responses for IT and spatial working memory tasks and the mean number correct target detections per task run in the case of the RVIP (out of 24 per run) and A-X CP tasks (out of 20 per run). To investigate potential treatment-related changes in behavioral performance, task accuracy and response times at the post-treatment visit formed the input for an analysis of covariance (ANCOVA) examining the main effect of treatment group as a between-group factor, with pre-treatment baseline estimates included as a covariate using SPSS for Windows (Version 23; SPSS Inc., Chicago, IL, USA). Criteria for significance across behavioral outcomes was set to $p < 0.05$.

Analysis Populations

The analysis population for each outcome required complete cases (55 of the 58 randomized completed follow-up assessments), with the following additional criteria for each type of outcome: (a) behavioral performance data: primary analysis on complete cases with additional exclusion of outliers identified during data-screening conducted while blind to treatment, (b) for fMRI: primary analysis on complete datasets, with follow-up sensitivity analyses excluding behavioral outliers identified during blind data-screening, (c) for SSVEP data: primary analysis was conducted on complete cases with additional exclusion of datasets with excessive artifact, as defined by an arbitrary threshold described below, with follow-up analyses excluding behavioral outliers.

Prior to unblinding, behavioral outcomes were screened for outliers, in which behavioral performance approximating chance levels was observed for five participants on the IT task, who were subsequently excluded from analysis. Poor performance was also observed for a single participant on the RVIP task, three participants on the A-X CPT, and a single participant on the Spatial Working Memory task who were excluded from analysis of these behavioral outcomes. An additional three participants were missing behavioral data for the RVIP task due to a technical fault with the response device in the scanner, and were thus missing from analysis of behavioral results. Excessive SSVEP artifact was defined by a circular statistic exceeding 0.2 in 10 or more electrodes for a given task variant (described in Silberstein, 1995a), calculated for the first 120 s of each task run. This threshold resulted in the exclusion of three datasets from the Spatial Working Memory task, and no datasets from the A-X CPT. As fMRI data was acquired from two cohorts in which testing procedures differed, these groups were analyzed separately. As previously reported, assessment of functional brain activity using SSVEP recordings during A-X CPT and Spatial Working Memory tasks significantly decreased subjective ratings of mental stamina, concentration and alertness, while

significantly increasing mental fatigue (further detailed in White et al., 2016), further emphasizing the need to consider the cohort in which fMRI was completed after SSVEP assessment separately.

RESULTS

Behavioral Performance

Behavioral performance on the cognitive tasks completed during monitoring of functional brain activity using SSVEP and fMRI methods did not significantly differ between treatment groups post-treatment, when controlling for baseline scores. Mean performance on MRI-based tasks tended to be poorer at both visits in the fatigued sample, though the two testing cohorts did not significantly differ. These results are summarized in **Table 2** below.

fMRI

Task Effects

Whole brain voxel-wise patterns of task-related activity were studied at baseline, in order to confirm that performance of the RVIP and IT tasks elicited activity consistent with previous research, also justifying the regions of interest identified from the independent sample for use in exploring treatment-related effects during RVIP task performance. The IT task failed to show reliable task effects, when exploring parametric modulation of the BOLD signal by stimulus presentation length (cf. Deary et al., 2004; Waiter et al., 2008), and as such was not pursued further for effects of treatment. The RVIP task showed results consistent with previous functional imaging studies of this task (Coull et al., 1996; Lawrence et al., 2003; Neale et al., 2015) and the ROIs defined from the independent sample, including large regions of bilateral parietal and frontal cortex. The outcomes of these analyses are further detailed in Supplementary Data Section 2 (see Supplementary Table 2 and Supplementary Figure 2).

RVIP Post-treatment Effects

Amongst fatigued participants, who underwent SSVEP assessment prior to fMRI, mean contrast estimates derived from subtracting the control RVIP task variant from the active RVIP task variant were significantly higher post-treatment in the MVM treatment group compared to placebo, when controlling for baseline. This effect survived FDR correction across three ROIs: bilateral parietal lobe ROIs and the supplementary motor area ROI. Repeating this analysis with the exclusion of a single poor-performing participant (placebo group) showed the same

TABLE 2 | Behavioral performance on cognitive tasks.

Cohort	Task/outcome	Treatment	*M* (adj)	*SE*	*F*	*p*
SSVEP	A-X CPT – Hits (/20)	Plac ($n = 18$)	18.72	0.41	0.16	0.691
		MVM ($n = 18$)	18.95	0.41		
	A-X CPT – RT	Plac ($n = 18$)	357	10.54	3.07	0.089
		MVM ($n = 18$)	331	10.54		
	Spatial Working Memory – Accuracy	Plac ($n = 20$)	76.62	1.57	0.01	0.924
		MVM ($n = 18$)	76.40	1.65		
	Spatial Working Memory – RT	Plac ($n = 20$)	631	23	1.26	0.269
		MVM ($n = 18$)	669	24		
fMRI: Fatigued	IT – Accuracy	Plac (n = 5)	92.56	2.97	2.25	0.168
		MVM ($n = 7$)	86.17	2.41		
	IT – RT	Plac ($n = 5$)	471	35	0.40	0.545
		MVM ($n = 7$)	442	29		
	RVIP – Hits (/24)	Plac (n = 6)	14.43	1.60	0.82	0.388
		MVM ($n = 6$)	16.49	1.60		
	RVIP – RT	Plac ($n = 6$)	487	24	0.12	0.735
		MVM ($n = 6$)	476	24		
fMRI: Non-fatigued	IT – Accuracy	Plac (n = 6)	94.97	0.92	0.41	0.537
		MVM ($n = 7$)	94.17	0.85		
	IT – RT	Plac (n = 6)	452	34	0.23	0.644
		MVM ($n = 7$)	430	31		
	RVIP – Hits (/24)	Plac (n = 8)	19.29	0.66	1.28	0.278
		MVM ($n = 8$)	18.21	0.66		
	RVIP – RT	Plac (n = 8)	464	20	0.20	0.663
		MVM ($n = 8$)	477	20		

Mean (and Standard Error) accuracy and response time for cognitive tasks completed during functional brain activity assessment at post-treatment assessment, adjusted for baseline performance. F- and p-values correspond to ANCOVA model testing main effect of treatment group on post-treatment performance, controlling for baseline performance. M (adj), Baseline adjusted estimated marginal means of post-treatment performance; RT, response time; Hits, mean target detections; MVM, multi-vitamin and mineral treatment group; Plac, Placebo; ANCOVA results reported for main effect of treatment group.

pattern of results, with left parietal and SMA ROIs surviving FDR correction. In contrast, no significant differences between treatment groups were observed amongst the non-fatigued cohort of participants. These outcomes are detailed in **Table 3**, while **Figure 1** depicts mean contrast estimates for fatigued and non-fatigued groups pre- and post-treatment within the three ROIs for which significant differences between treatment groups were observed.

Within the fatigued sample, the change in RVIP task-related activity from baseline to post-treatment was pursued in order to assess the relationship with changes in behavioral performance. Positive correlations were observed between the change in RVIP task performance from baseline to post-treatment, as indexed by total number of correct target detections, and change in RVIP contrast estimate from baseline to post-treatment in the Left Parietal ($r_s = 0.681$, $p = 0.015$), Right Parietal ($r_s = 0.628$, $p = 0.029$) and SMA ($r_s = 0.491$, $p = 0.105$) regions of interest. Increased functional brain activity during RVIP task completion at follow-up was found to be associated with more improved performance amongst fatigued participants. Scatterplots of the association between change in functional activity and behavioral performance are shown in **Figure 2** for the two parietal regions of interest, in which significant ($p < 0.05$) correlations were observed.

SSVEP

A-X CPT: Baseline SSVEP Task Effects

SSVEP amplitude and phase differences between active and control task variants across the sample at baseline are shown in **Figure 3**. Consistent with previous research, reduced amplitude was observed across the three periods of the task, in prefrontal and posterior sites during the Cue period, continuing across fronto-central and posterior sites during Hold and Target components of the task. SSVEP phase demonstrated modest frontal advance during the Cue period, whilst a right posterior phase lag during this period extended more broadly across Hold and Target periods. The prefrontal phase advance previously reported in combination with amplitude reduction during Hold and Target task components (cf. Silberstein et al., 1998; Silberstein et al., 2000; White et al., 2016) was less apparent in the current sample.

A-X CPT: Treatment Group SSVEP Analysis

Changes in SSVEP response from baseline to post-treatment were explored for placebo and active MVM treatment groups separately. For the placebo treatment group, there were no significant differences in SSVEP response to the A-X CPT between baseline and post-treatment visits across the three task components (shown in **Figure 4**). Trends ($p < 0.05$, not reaching threshold) were observed for a small number of parieto-occipital electrodes where amplitude reduction and phase advance across the three task components were observed at follow-up assessment. The active MVM treatment group showed significant amplitude reduction post-treatment in a single occipital site during the Hold period (shown in **Figure 5**). In addition, a cluster of frontal electrodes showed a trend ($p < 0.05$) toward decreased amplitude and phase advance. While this pattern of frontal phase advance and amplitude reduction did

TABLE 3 | Mean RVIP task-related contrast estimates post-treatment from the five regions of interest for each treatment group, adjusted for baseline, in both fatigued ($n = 16$) and non-fatigued cohorts ($n = 16$).

	ROI	Treatment	*M* (adj)	*SE*	*F*	*p*	η_p^2
Fatigued	Left Parietal	Plac	1.06	0.23	7.95	0.015 *	0.379
		MVM	1.96	0.23			
	Right Parietal	Plac	1.25	0.21	4.84	0.047 *	0.271
		MVM	1.89	0.21			
	Left Frontal	Plac	2.08	0.54	2.17	0.164	
		MVM	3.21	0.54			
	Supplementary Motor Area	Plac	1.40	0.29	7.35	0.018 *	0.361
		MVM	2.50	0.29			
	Left Insula	Plac	1.32	0.32	0.39	0.543	
		MVM	1.62	0.32			
Non-fatigued	Left Parietal	Plac	1.88	0.30	0.10	0.760	
		MVM	1.75	0.30			
	Right Parietal	Plac	1.69	0.26	0.13	0.726	
		MVM	1.56	0.26			
	Left Frontal	Plac	2.90	0.52	0.06	0.808	
		MVM	2.72	0.52			
	Supplementary Motor Area	Plac	2.39	0.44	1.22	0.290	
		MVM	1.70	0.44			
	Left Insula	Plac	1.63	0.40	0.21	0.655	
		MVM	1.37	0.40			

*ROI, region of interest; M (adj), baseline adjusted estimated marginal means; Plac, placebo group; MVM, multi-vitamin and mineral group; ANCOVA results reported for main effect of treatment group; *significant after FDR thresholding.*

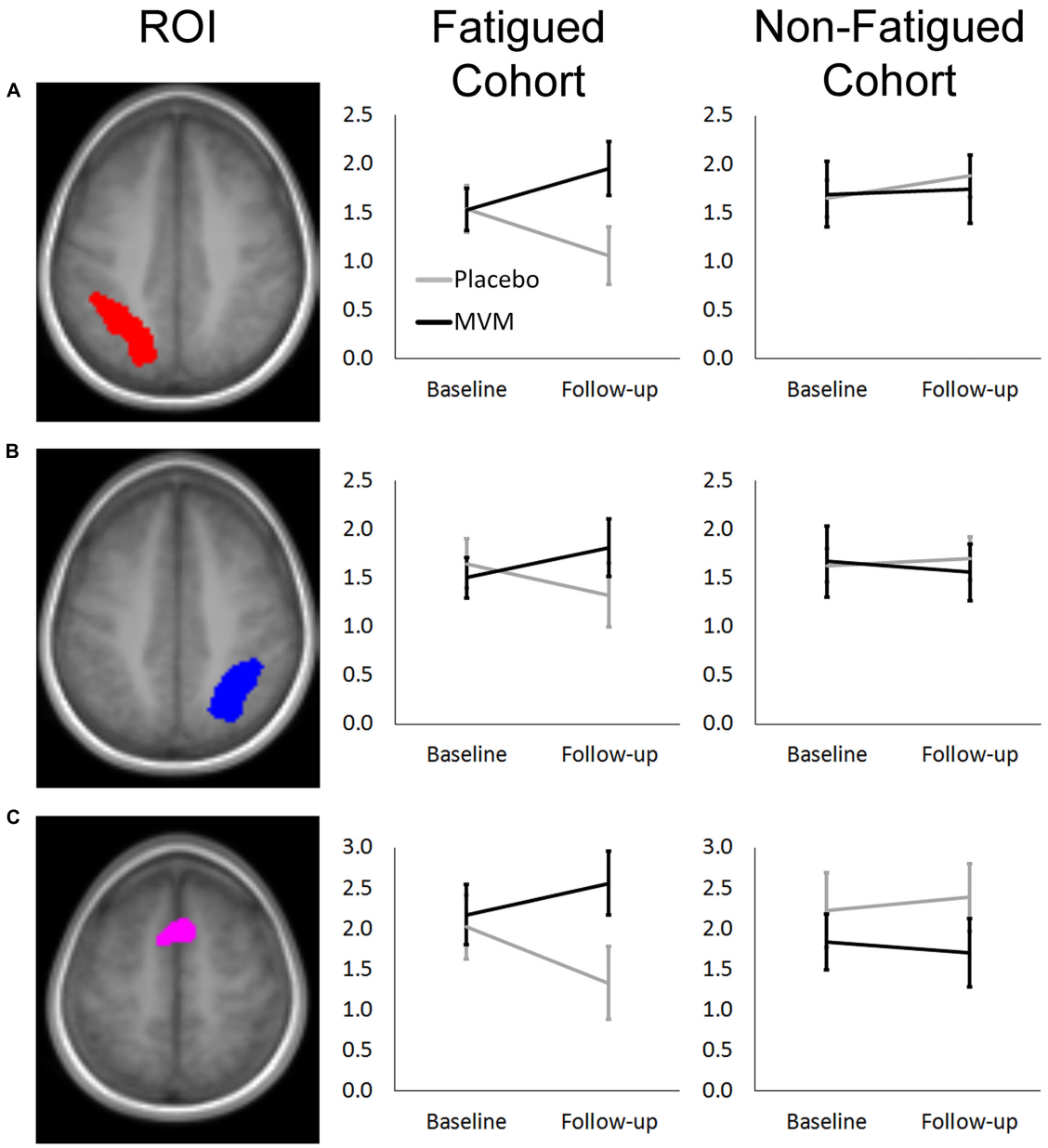

FIGURE 1 | Mean RVIP task contrast estimates extracted at baseline and post-treatment for fatigued (left) and non-fatigued (right) cohorts for (A) Left Parietal, **(B)** Right Parietal, and **(C)** SMA regions of interest, where significantly greater task-related activity was observed in active treatment group post-treatment within the fatigued cohort. Error bars ±1 SE.

not reach significance when adjusting for multiple comparisons, this effect is consistent, both in terms of task component and electrode locations, to previously reported changes in SSVEP response during completion of the A-X CPT after an acute dose of the same MVM intervention (White et al., 2016). Patterns of change and statistical significance were unchanged when sensitivity analyses were pursued, excluding behavioral outliers. There were no significant correlations between the change in behavioral performance from baseline to post-treatment and these changes in SSVEP response during A-X CPT.

Spatial Working Memory: Baseline SSVEP Task Effects

The SSVEP response to the active task variant contrasted with the control variant showed a pattern of reduced SSVEP amplitude and phase advance in prefrontal electrodes during encoding, followed by posterior phase advance during the hold period, as shown in **Figure 6**. No significant differences were observed in the retrieval segment, in the first 1000 ms following presentation of the probe, between the control and active task variants.

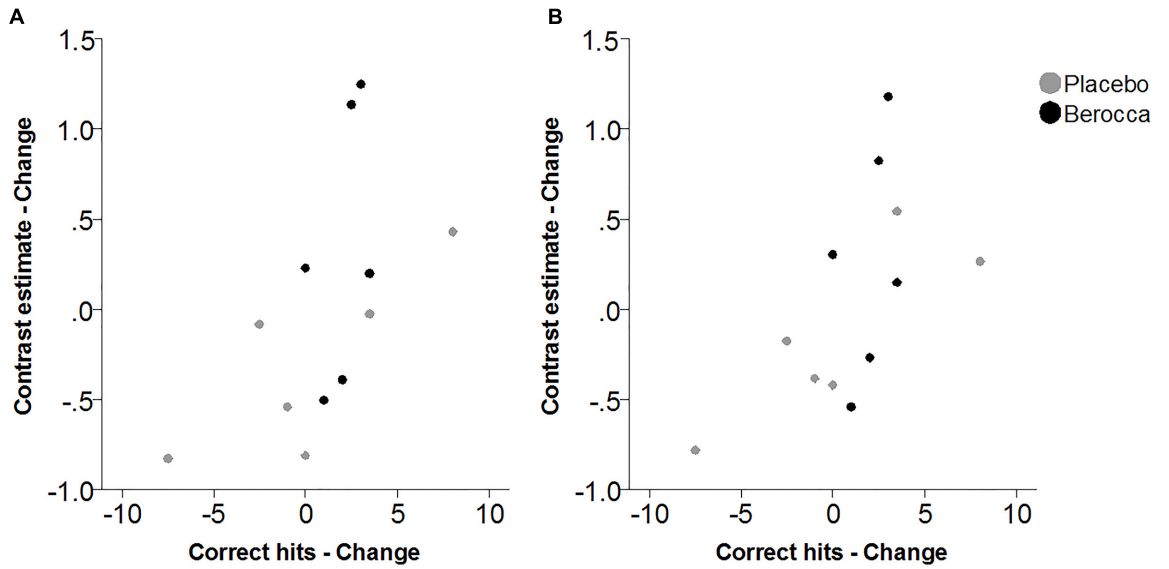

FIGURE 2 | Scatterplots of association between change in RVIP contrast estimate and change in behavioral performance from baseline to post-treatment within the fatigued cohort for (A) Left Parietal and **(B)** Right Parietal regions of interest.

Spatial Working Memory: Treatment Group SSVEP Analysis

Contrasting SSVEP responses post-treatment with baseline activity during the Spatial Working Memory task showed no significant changes in the placebo treatment group, but evidence of significant changes post-treatment in the active MVM treatment group. In the placebo group, shown in **Figure 7**, trends toward greater phase lag (increased latency) were observed in the three pre-frontal electrodes during the encoding period, in addition to a left frontal pattern of phase lag and amplitude reduction in the maintenance period, though neither reached significance when adjusting for multiple comparisons. The active MVM treatment group showed centro-parietal SSVEP phase advance (reduced latency) during the encoding segment of the Spatial Working Memory task (shown in **Figure 8**), reaching criteria for significance in two central electrodes when adjusting for multiple comparisons. This pattern of SSVEP phase advance in centro-parietal regions persisted into the maintenance period, though it did not reach criteria for significance in this time window. This change in SSVEP phase response in central electrodes during encoding was not correlated with the change in behavioral performance from baseline to post-treatment within the active MVM treatment group.

DISCUSSION

This study investigated the effects of 4 weeks daily treatment with a MVM preparation on functional brain activity, as assessed by complementary hemodynamic and electrophysiological methods. Both fMRI and SSVEP modalities revealed changes in functional brain activity following treatment with the active MVM treatment, with converging patterns of activity consistent with greater activity in overlapping centro-parietal regions during working memory task performance. Within the fMRI data, this increase in task-related BOLD response was correlated with improved behavioral performance, however, these fMRI findings were only apparent amongst the fatigued cohort of participants who had first undergone SSVEP assessment. The active MVM treatment was also associated with greater posterior SSVEP amplitude reductions post-treatment during the period between the cue and target stimuli of the A-X CPT, as well as a trend toward SSVEP amplitude reductions and phase advance across frontal regions. MVM treatment was not associated with significant changes in behavioral performance on the cognitive tasks completed during monitoring of functional brain activity. Thus, outcomes of this trial point to changes in functional brain activity associated with MVM treatment over 4 weeks. These findings, however, must be considered in light of the limited evidence of behavioral change in cognitive task performance (while acknowledging that the cognitive evaluation were primarily designed as activation tasks rather than to capture any treatment-related effects).

Changes in Functional Brain Activity during Working Memory

Changes in functional brain activity reflecting greater activity in centro-parietal regions in the MVM arm were observed during working memory tasks using both imaging methodologies. As part of the SSVEP changes, the active MVM treatment group showed SSVEP phase advance (reduced latency) across centro-parietal regions. The temporal resolution of the SSVEP method allows isolation of sub-components of the working memory task, which indicated this increase was maximal during the encoding

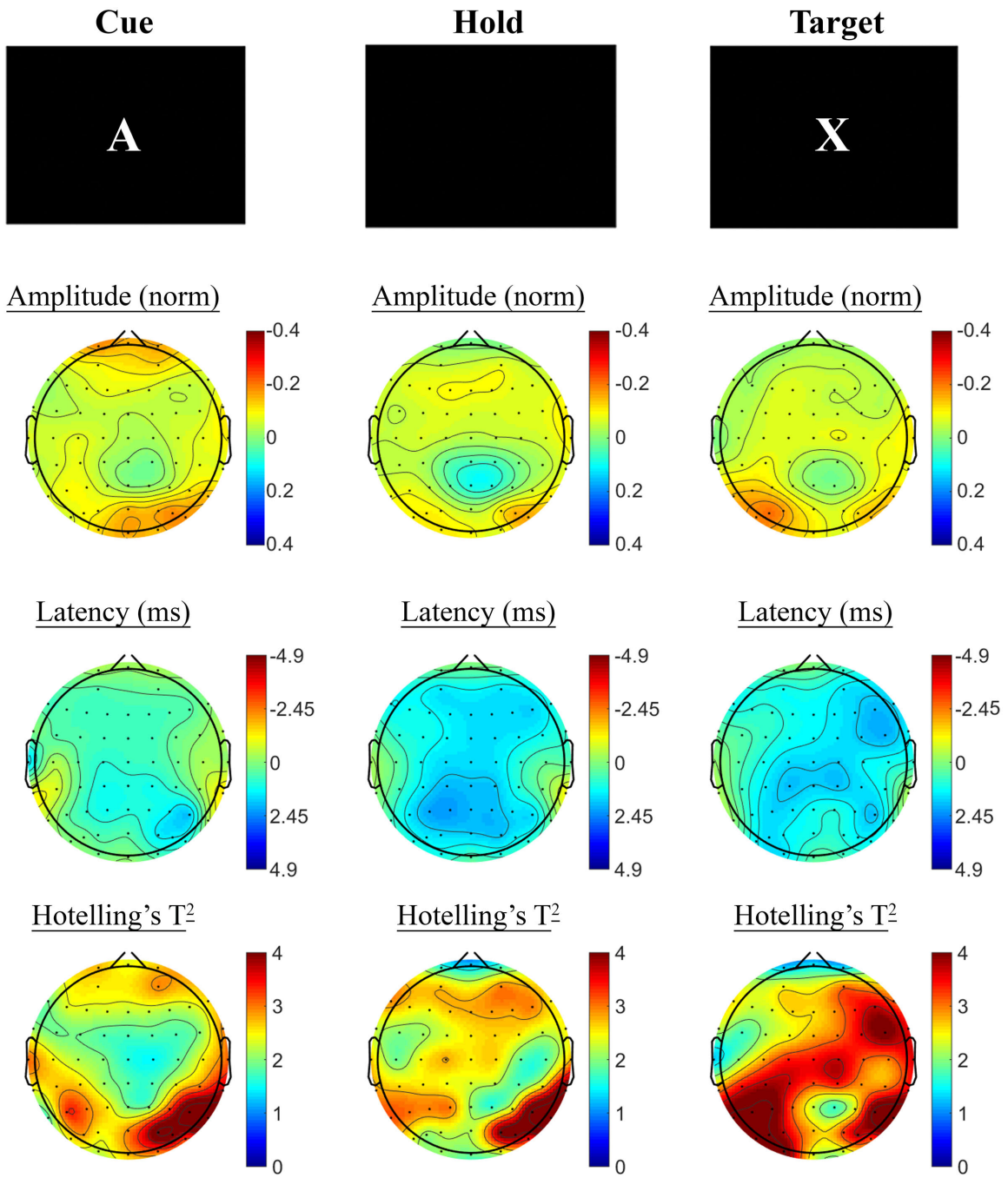

FIGURE 3 | A-X CPT SSVEP amplitude and phase differences, contrasted with the control task, at the baseline assessment across the sample. Columns show topographic maps of SSVEP amplitude (top) and phase (as latency, middle), with Hotelling's T^2 corresponding to the contrast of active and control task variants for Cue **(Left)**, Hold **(Middle)** and Target **(Right)** task segments.

period of the task. This pattern was broadly consistent with the effects observed in the fatigued cohort during the RVIP task using fMRI, where bilateral parietal and supplementary motor area regions of interest showed greater task-related BOLD in the MVM group. While these two tasks represent distinct working memory paradigms (delayed matching-to-sample versus continuous performance), the probing of treatment effects within the fMRI data used contrast images derived from the active task minus the control task variant. This allows relatively complete isolation of working memory demands during the active task. Thus these two patterns of change provide converging evidence of increased functional brain activity associated with MVM

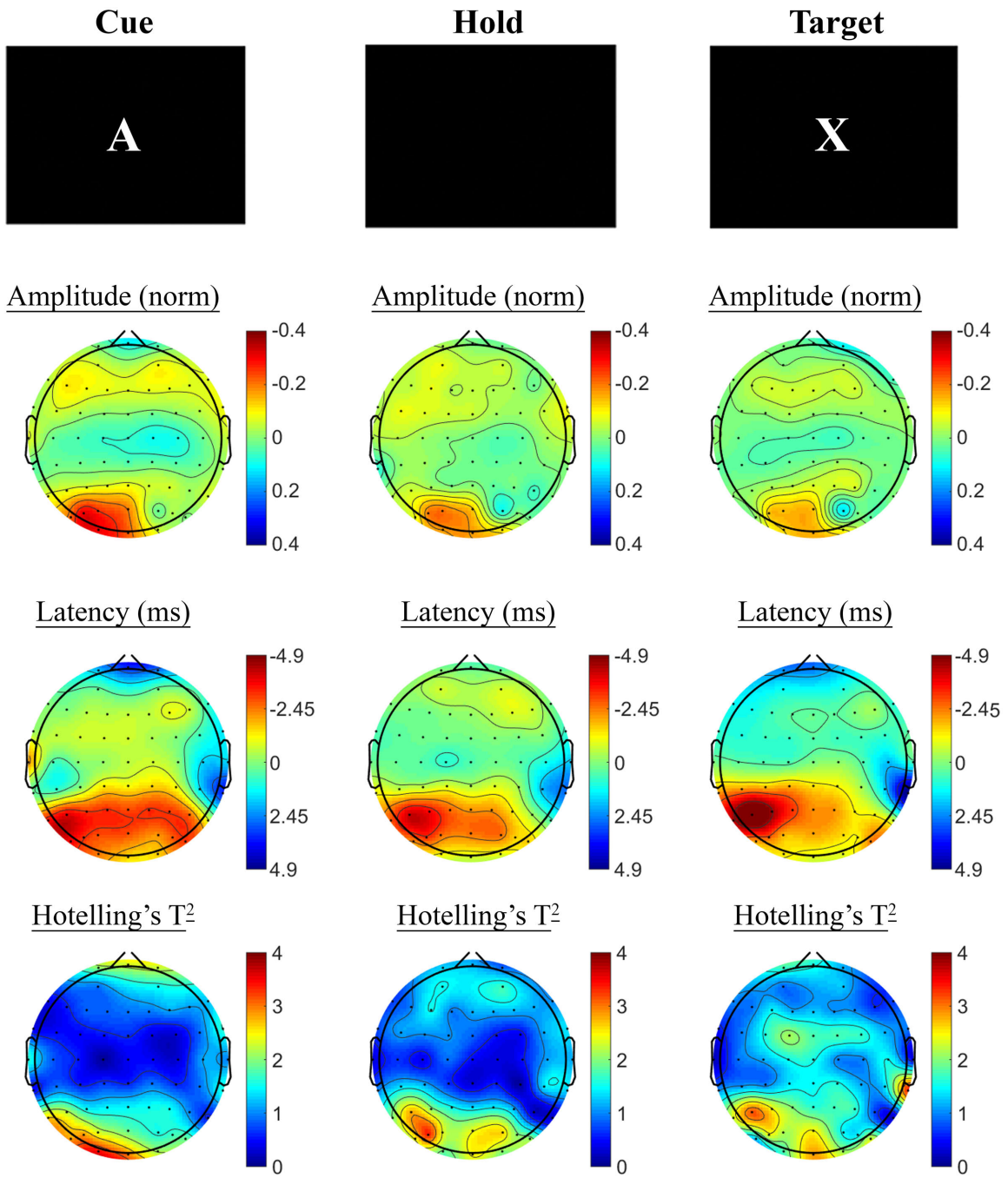

FIGURE 4 | A-X CPT SSVEP amplitude and phase changes from baseline to post-treatment in the placebo group. Columns show topographic maps of SSVEP amplitude (top) and phase (as latency, middle), with Hotelling's T^2 corresponding to the contrast of active and control task variants for Cue **(Left)**, Hold **(Middle)**, and Target **(Right)** task segments.

supplementation during actively engaged working memory processes.

Given the preliminary nature of this investigation, the source of disparate findings relating to potential treatment-related changes in task-related BOLD response between the fatigued and non-fatigued fMRI cohorts remains unclear. One possible explanation is that, amongst healthy adults, the neurocognitive benefits of this supplementation are more apparent under conditions of greater cognitive demand. Supporting such a possibility, previous work exploring cognitive changes following 4 weeks of MVM supplementation in healthy adult males demonstrated benefits, both in terms of cognitive performance

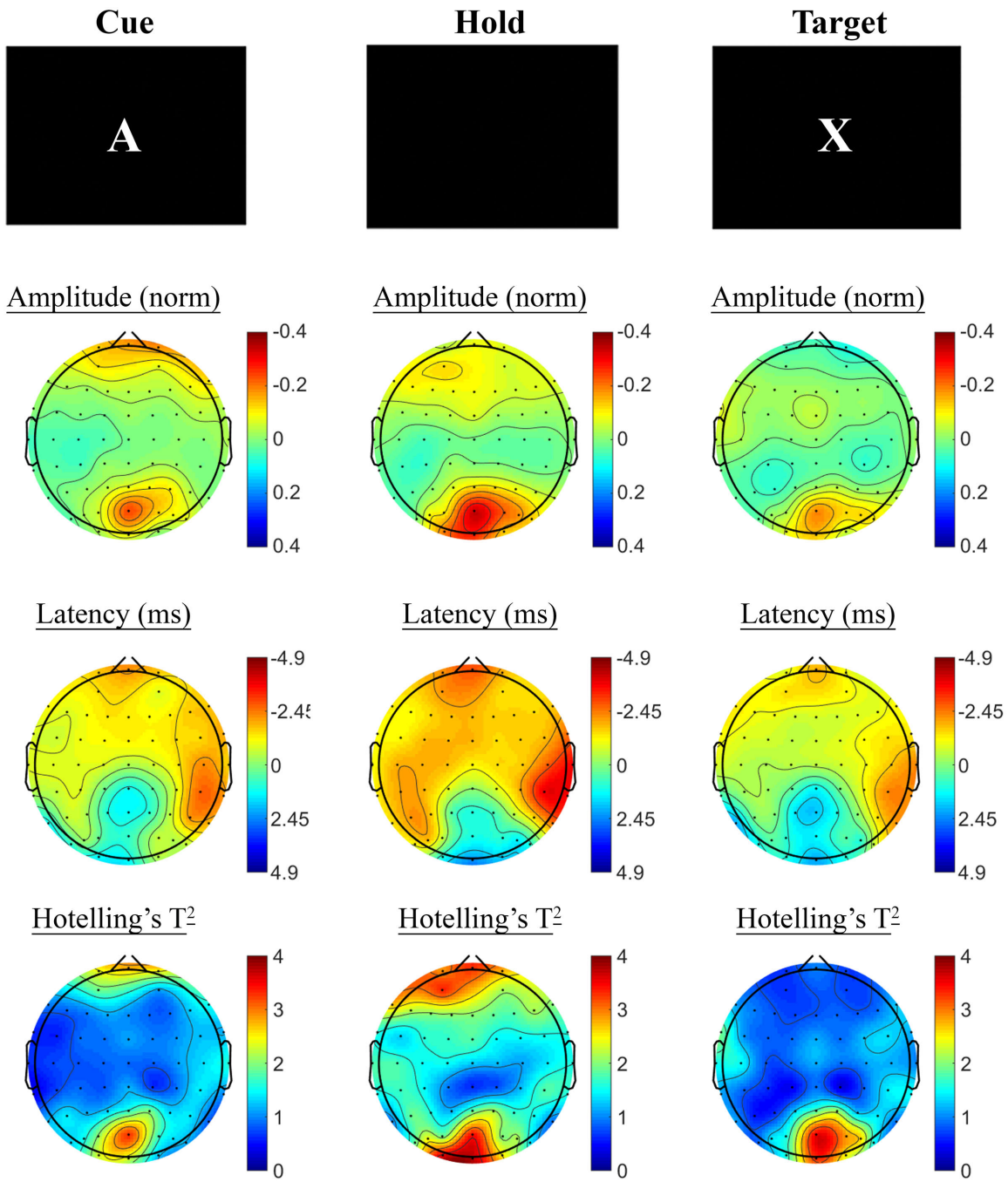

FIGURE 5 | A-X CPT SSVEP amplitude and phase changes from baseline to post-treatment in the active MVM treatment group. Columns show topographic maps of SSVEP amplitude (top) and phase (as latency, middle), with Hotelling's T^2 corresponding to the contrast of active and control task variants for Cue **(Left)**, Hold **(Middle)** and Target **(Right)** task segments.

and subjective ratings of fatigue, during completion of a 1 h cognitive demand battery (Kennedy et al., 2010). Given the relatively insensitive approach to detecting BOLD changes adopted in the current investigation (extracting mean task-related estimates of activity from broad regions of interest), it may be that robust differences are only apparent when placing the individual under greater demand. Further replication and detailed investigation of this effect is required to better understand these effects.

The observed changes in SSVEP response during completion of the spatial working memory task associated with MVM supplementation are in contrast with those of Macpherson et al.

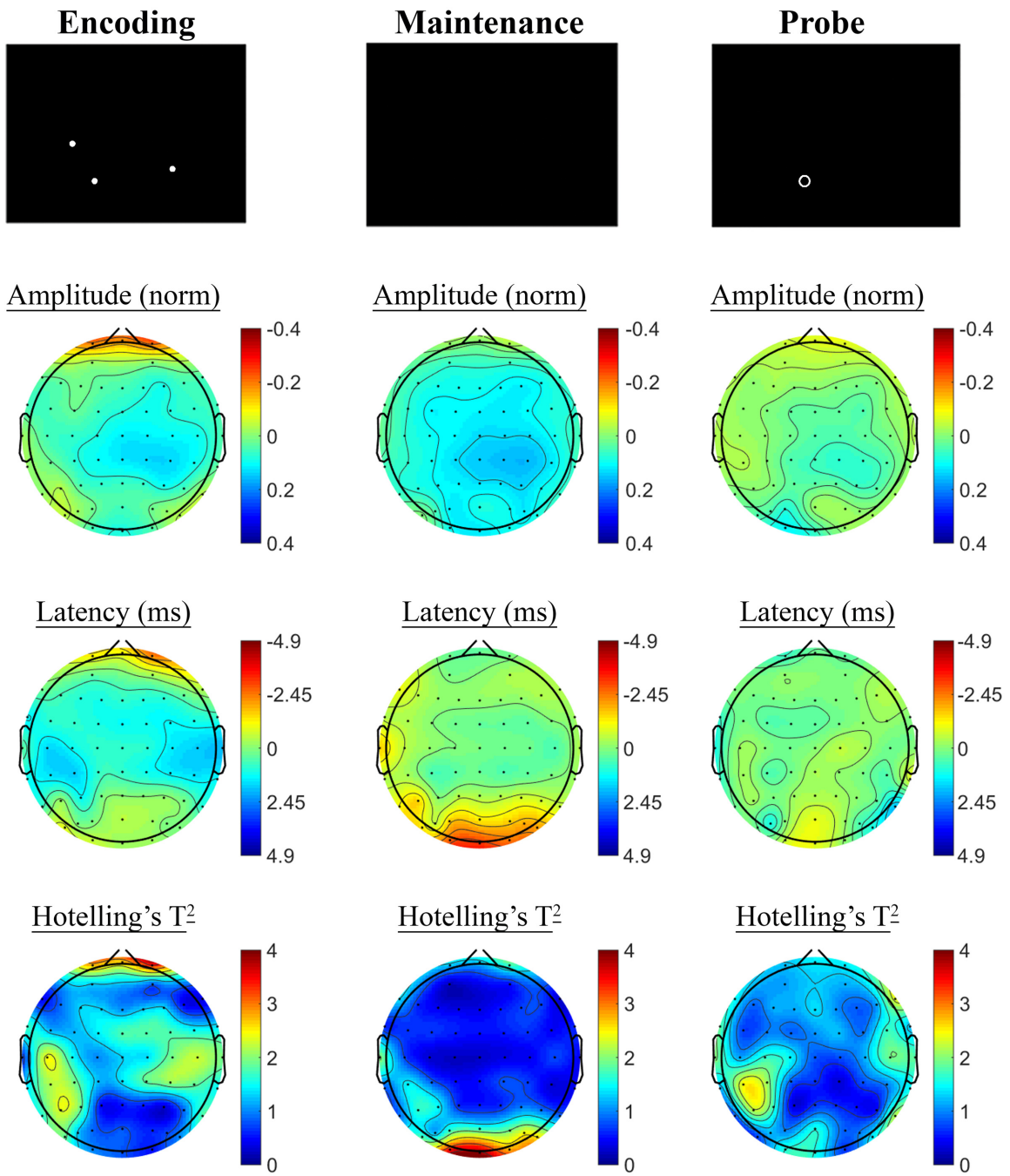

FIGURE 6 | Spatial Working Memory SSVEP amplitude and phase differences, contrasted with the control task, at the baseline assessment across the sample. Columns show topographic maps of SSVEP amplitude (top) and phase (as latency, middle), with Hotelling's T^2 corresponding to the contrast of active and control task variants for Encoding **(Left)**, Maintenance **(Middle)**, and Retrieval **(Right)** task segments.

(2012), where latency increases (phase lag) were observed in elderly women after MVM supplementation during the retrieval period of the same task used herein. However, the previous trial by Macpherson et al. (2012) studied an intervention which contained 19 plant extracts and three probiotics in addition to micronutrients. For example, the intervention described by Macpherson et al. (2012) included a relatively large dose of *Ginkgo Biloba* (1000 mg), an extract which has been shown to exert a range of complex actions *in vitro* as a GABA, glycine and 5HT receptor antagonist (Huang et al., 2003; Hawthorne et al., 2006; Thompson et al., 2011). As such, the disparate findings of Macpherson et al. (2012) and those of the present report, in

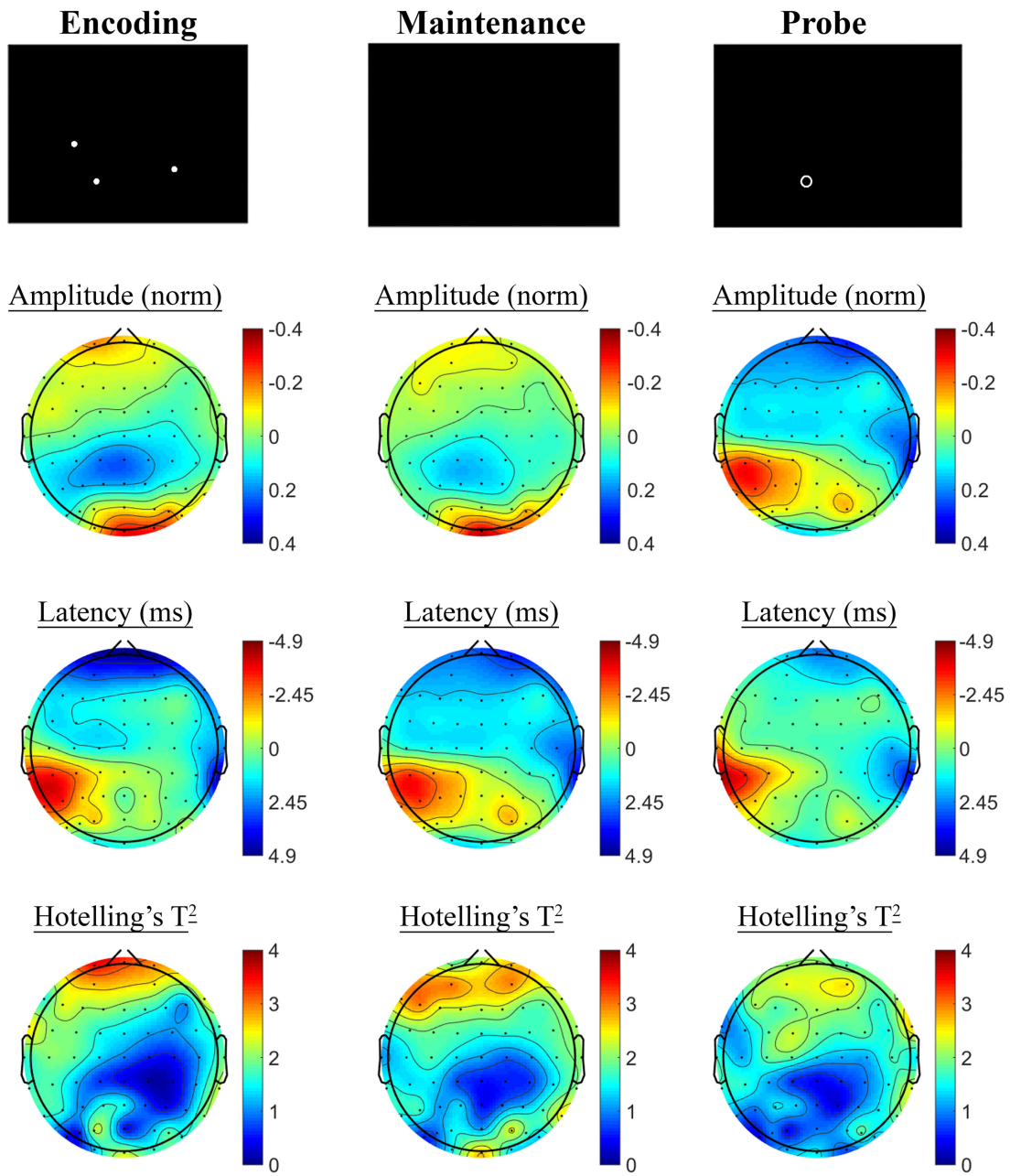

FIGURE 7 | Spatial Working Memory SSVEP amplitude and phase changes from baseline to post-treatment in the placebo treatment group. Columns show topographic maps of SSVEP amplitude (top) and phase (as latency, middle), with Hotelling's T^2 corresponding to the contrast of active and control task variants for Encoding **(Left)**, Maintenance **(Middle)** and Retrieval **(Right)** task segments.

which treatment involved only B vitamins, vitamin C, and three minerals, are likely driven by the variability in the treatment constituents.

Changes in Functional Brain Activity during Cued Attention

The active MVM treatment group also showed greater posterior SSVEP amplitude reductions post-treatment during the period between the cue and target stimuli of the A-X task, as well as a trend of reduced amplitude and phase advance across frontal regions. Previous studies to explore SSVEP correlates of attention showed decreased amplitude in posterior electrodes where task demands were increased during visual vigilance tasks (Silberstein et al., 1990), which are consistent with the pattern of results observed in the present study across all participants at baseline. This reduced SSVEP amplitude during attention tasks which require ongoing processing of visual stimuli is thought to reflect

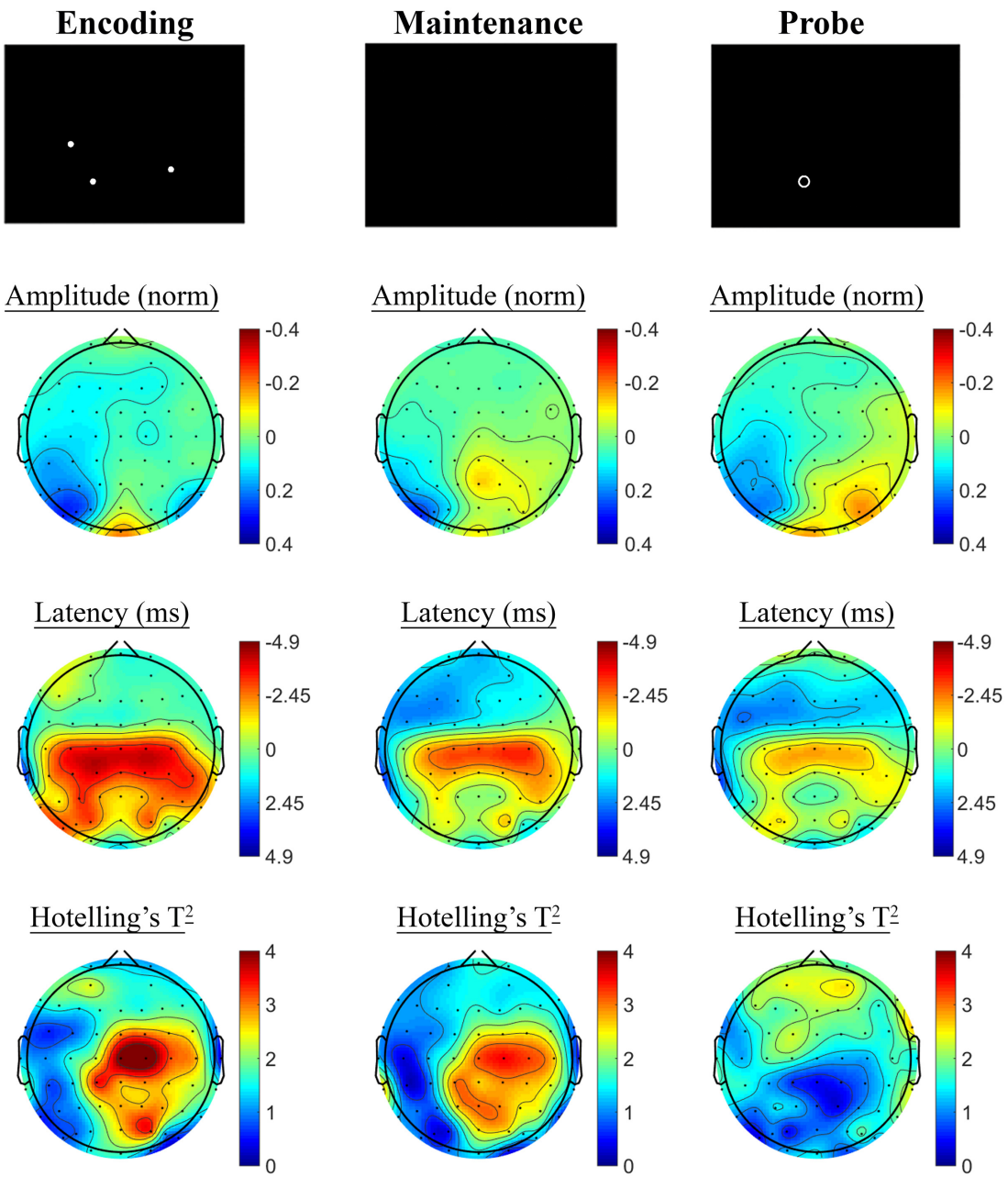

FIGURE 8 | Spatial Working Memory SSVEP amplitude and phase changes from baseline to post-treatment in the active MVM treatment group. Columns show topographic maps of SSVEP amplitude (top) and phase (as latency, middle), with Hotelling's T2 corresponding to the contrast of active and control task variants for Encoding **(Left)**, Maintenance **(Middle)** and Retrieval **(Right)** task segments.

an inhibition of the re-entrant cortico-cortical loop transmission efficiency through cortical layer I, as the sensory inputs to layer IV are enhanced with visual processing of the task demands (Silberstein et al., 2001). At this stage, it is unclear whether the reduced posterior SSVEP amplitude in this window following the cue stimuli reflects this visual processing, or a greater top-down biasing of attention to the visual stimuli following the cue in a manner paralleling the proposed role for alpha oscillations in top-down control of attention (for a review, see Foxe and Snyder, 2011). Similarly, the potential mechanism by which this amplitude was further reduced post-MVM treatment is not fully elucidated.

We have previously reported a single dose of MVM resulted in frontal SSVEP amplitude reductions and phase advance, in this period between cue and target stimuli during the A-X CPT (White et al., 2016). Whilst not reaching criteria for significance when controlling for multiple comparisons, the frontal SSVEP amplitude reduction and phase advance observed in the current

trial was consistent with this previous acute investigation off MVM treatment, in terms of the precise task window, the direction of change in both SSVEP amplitude and phase, and the spatial location of the effect. The previous study, however, observed that this pattern of change was correlated with the change in task performance, this was not the case in the current study.

Behavioral Measures of Cognitive Task Performance

Despite evidence for changes in functional brain activity, behavioral performance on the cognitive tasks completed during monitoring of functional brain activity did not significantly differ between treatment groups in parallel with these neurophysiological changes. This trial was designed as an exploratory investigation with functional brain activity (measured with SSVEP and fMRI) as the primary outcomes. As such, *a priori* sample size determination did not power the study to detect behavioral performance changes, nor were the task paradigms themselves optimized for capturing behavioral differences (rather they were operationalized primarily as activation tasks). Studies to test cognitive benefits of MVM supplementation in healthy adults have involved considerably larger samples in detecting relatively subtle cognitive effects (Haskell et al., 2010; Kennedy et al., 2010), indeed, meta-analysis suggests a standardized mean difference for immediate recall in the small to medium range (Grima et al., 2012), further emphasizing the need for considerably larger sample sizes where assessing cognitive performance changes as the primary outcome.

An alternative strategy in assessing the functional relevance of observed neurophysiological changes associated with MVM treatment has been to explore the association between the change in functional brain activity and the change in behavioral measures of cognitive task performance. In the present study, the greater RVIP task-related BOLD response observed after active MVM treatment within the fatigued cohort was correlated with performance changes. This indicates that the greater the increase in functional brain activity post-treatment, the more improved behavioral performance on the RVIP task. However, while we have previously reported associations between patterns of SSVEP response change and behavioral performance (White et al., 2016), this relationship was not observed in the current sample, despite trends toward faster response times on the A-X CPT in the MVM treatment group compared to placebo. It remains to be fully elucidated by larger scale research whether the observed neurophysiological effects of MVM administration represent changes of relevance to neurocognitive function which were subthreshold given the limitations in sample size and insensitivity of the behavioral cognitive outcomes in the present study.

Mechanisms of MVM Supplementation in Neurocognitive Function

The very nature of MVM supplementation complicates the isolation of specific mechanisms by which these interventions exert observed effects, given both the multitude of constituents and the multitude of cellular processes in which they serve as critical cofactors (Kennedy, 2016). Indeed, the complementary and interactive roles these micronutrients play in such critical cellular pathways suggest that a reductionist search to isolate a single candidate active constituent and pathway may be inappropriate (Kennedy (2016) for a thorough account of the potential advantages of multiple micronutrient administration over single nutrient preparations). Instead a more integrated view of the multiple mechanisms impacted by MVM treatment may be relevant. For example, the role of these micronutrients in energy metabolism and neurotransmitter synthesis appear as two prominent, not mutually exclusive, pathways through which MVM supplementation may exert neurocognitive effects such as the improved mood (White et al., 2015) and potential changes to functional brain activity during cognitive task engagement observed as part of the current trial.

Nonetheless, MVM formulations are not standardized in the marketplace and greater provision of one nutrient relative to another may contribute to the benefits obtained from supplementation. In the current study, the levels of B vitamins are at or above the recommended daily intakes (**Table 1**), suggesting that such treatment should redress any insufficiencies in these micronutrients (White et al., 2015). Blood markers showed that levels of key vitamins did change in this cohort (White et al., 2015), although the study was not powered to relate individual differences in nutrient status to neurocognitive or behavioral responses. Future studies may also consider the influence of pre-existing health and nutrient status of the individuals receiving the supplement. This is particularly important given evidence of inadequate intakes of a range of micronutrients associated with a Western style diet (Troesch et al., 2012).

Beyond the theoretical biochemical pathways which link micronutrient treatment with these mechanisms, experimental data also supports the possibility for manipulating these processes through micronutrient administration. Daily MVM supplementation amongst obese adult females over 26 weeks has been shown to result in increased resting energy expenditure, along with reductions in fat mass, body weight and cholesterol (Li et al., 2010). Furthermore, Kennedy et al. (2016) reported increased energy expenditure following both acute and chronic MVM administration in healthy younger adult females during completion of a cognitive battery. In support of the notion that administration of even a single B vitamin may influence neurotransmitter synthesis, there exists research in lower mammals demonstrating that administration of vitamin B6 leads to an increase in central serotonin levels (Hartvig et al., 1995; Calderon-Guzman et al., 2004).

Whilst the present work has focused on enhancing function in healthy adults, a further pathway through which a more targeted B vitamin supplementation scheme may exert neurocognitive changes in at least one at-risk population warrants discussion. Smith et al. (2010) have described a very clear candidate mechanism by which B vitamin supplementation may exert neurocognitive changes, reducing homocysteine as part of the methionine cycle, in turn minimizing gray matter atrophy and

slowing cognitive decline in older adults diagnosed with Mild Cognitive Impairment presenting with elevated homocysteine (de Jager et al., 2012; Douaud et al., 2013). Thus, while further research is required to fully characterize the physiological changes associated with MVM supplementation in both health and disease, there is evidence to suggest energy metabolism, neurotransmitter synthesis, as well as direct outcomes of one carbon transfer cycles such as reduced homocysteine, are mechanisms modifiable by micronutrient administration.

CONCLUSION

An emerging focus on optimal micronutrient intake, beyond that required to avoid clinical deficiency, questions whether otherwise healthy individuals may gain benefit from MVM supplementation. Whilst some research supports benefits to mood and possibly cognitive function, research exploring parallel physiological changes is also underway. Outcomes of the present trial provide evidence to suggest potential changes in functional brain activity following 4 weeks MVM supplementation using both hemodynamic and electrophysiological measures. In particular, converging evidence from fMRI and SSVEP recordings showed patterns of brain activity consistent with greater activity in overlapping centro-parietal regions following MVM administration during working memory. The fMRI findings were, however, only observed amongst the subset of participants undergoing this assessment under fatigue. Evidence of SSVEP changes during a cued attention task were also observed, partially consistent with a previous report after a single dose (White et al., 2016), however, these changes were not associated with behavioral performance on the task. All of the observed changes were consistent with recruitment of additional neural resources or greater excitatory processes during task engagement, that is, greater task-related BOLD response and SSVEP amplitude reductions and phase advance. Thus, the present study provides preliminary evidence to suggest daily ingestion of a high dose B vitamin micronutrient supplement over 4 weeks may increase task-related functional brain activity in healthy adults. These findings are of further relevance given that, while a healthy diet may be sufficient to maintain adequate supplies of these essential micronutrients, dietary surveys of countries which follow a modern Western dietary pattern indicate a significant proportion of the population may not meet recommendations for a range of micronutrients, including B vitamins (Troesch et al., 2012).

AUTHOR CONTRIBUTIONS

AS and DW conceived the study and had significant input into design and interpretation (aided by AP). KC and RP were largely responsible for data collection and interpretation. DW, MH and RP were responsible for data processing. All authors had input into the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnagi.2016.00288/full#supplementary-material

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Effect of Probiotic Supplementation on Cognitive Function and Metabolic Status in Alzheimer's Disease: A Randomized, Double-Blind and Controlled Trial

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Alzheimer's disease (AD) is associated with severe cognitive impairments as well as some metabolic defects. Scant studies in animal models indicate a link between probiotics and cognitive function. This randomized, double-blind, and controlled clinical trial was conducted among 60 AD patients to assess the effects of probiotic supplementation on cognitive function and metabolic status. The patients were randomly divided into two groups ($n = 30$ in each group) treating with either milk (control group) or a mixture of probiotics (probiotic group). The probiotic supplemented group took 200 ml/day probiotic milk containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Lactobacillus fermentum* (2×10^9 CFU/g for each) for 12 weeks. Mini-mental state examination (MMSE) score was recorded in all subjects before and after the treatment. Pre- and post-treatment fasting blood samples were obtained to determine the related markers. After 12 weeks intervention, compared with the control group (−5.03% ± 3.00), the probiotic treated (+27.90% ± 8.07) patients showed a significant improvement in the MMSE score ($P < 0.001$). In addition, changes in plasma malondialdehyde (−22.01% ± 4.84 vs. +2.67% ± 3.86 μmol/L, $P < 0.001$), serum high-sensitivity C-reactive protein (−17.61% ± 3.70 vs. +45.26% ± 3.50 μg/mL, $P < 0.001$), homeostasis model of assessment-estimated insulin resistance (+28.84% ± 13.34 vs. +76.95% ± 24.60, $P = 0.002$), Beta cell function (+3.45% ± 10.91 vs. +75.62% ± 23.18, $P = 0.001$), serum triglycerides (−20.29% ± 4.49 vs. −0.16% ± 5.24 mg/dL, $P = 0.003$), and quantitative insulin sensitivity check index (−1.83 ± 1.26 vs. −4.66 ± 1.70, $P = 0.006$) in the probiotic group were significantly varied compared to the control group. We found that the probiotic treatment had no considerable effect on other biomarkers of oxidative stress and inflammation, fasting plasma glucose, and other lipid profiles. Overall, the current study demonstrated that probiotic consumption for 12 weeks positively affects cognitive function and some metabolic statuses in the AD patients. Clinical Trial Registration: http://www.irct.ir/, IRCT201511305623N60.

Keywords: Alzheimer's disease, clinical trial, cognitive function, metabolic status, probiotic

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BACKGROUND

Alzheimer's disease (AD) is recognized as one of the most common forms of senile dementia (Qiu et al., 2007). AD begins with memory loss of recent events (short-term memory impairment) and finally robs the patients of their sense of self (Amemori et al., 2015). Early onset of the disease, older age, low education level, and several poor health conditions affect the prevalence rate of the disease and the degree of cognitive impairment (de Souza-Talarico et al., 2016). Increased biomarkers of oxidative stress (Furman et al., 2016), inflammation and chronic neuroinflammation are reported to be associated with many neurodegenerative disorders of central nervous system (CNS) including AD (Leszek et al., 2016). Furthermore, previous studies have demonstrated that metabolic alterations such as insulin resistance (Arrieta-Cruz and Gutierrez-Juarez, 2016), hyperglycemia (Arrieta-Cruz and Gutierrez-Juarez, 2016), and dyslipidemia (Presecki et al., 2011) are associated with the pathogenesis and development of AD. Experimental evidence indicates that alterations in micronutrients are also among the risk factors for AD (Taghizadeh et al., 2014).

The microbiota is a dynamic ecosystem which is influenced by several factors including genetics, diet, metabolism, age, geography, antibiotic treatment, and stress (Hufeldt et al., 2010; Cho et al., 2012; Drago et al., 2012). Recent evidence indicates a clear association between changes in the microbiota and cognitive behaviors (Gareau, 2014). In addition, animal studies imply on the necessity of an optimal function of what is known as the microbiome-gut-brain axis in the behavioral as well as electrophysiological aspects of brain action (Davari et al., 2013). There is a preliminary research on the effect of probiotics on the prognosis of cognition (Bhattacharjee and Lukiw, 2013). However, data on the effects of probiotics on improving cognitive disorders are scarce (Bhattacharjee and Lukiw, 2013; Davari et al., 2013). Gareau reported that intestinal dysbiosis in germ free animals (containing no microbiota), bacterial infection with an enteric pathogen and administration of probiotics can modulate cognitive behaviors including learning and memory (Gareau, 2014).

Some complications such as cognitive disorders, oxidative stress, neuroinflammation, insulin resistance, and altered lipid metabolism, which are observable in AD, are identified to be influenced by the gut flora as well as probiotics. However, no direct study has considered the gut microbiota manipulation in AD patients. Hence, this clinical trial was designed to assess if reinforcement of the intestinal microbiota via probiotic supplementation helps to improve cognitive and metabolic disorders in the AD patients.

MATERIALS AND METHODS

Trial Design

This study was a 12-week randomized, double-blind, and controlled clinical trial.

Participants

Participants included in this study were people with AD (60–95 years old) residing at the Golabchi (Kashan, Iran) and Sadeghyeh (Esfahan, Iran) Welfare Organizations between December 2015 and February 2016. The AD patients were diagnosed following the NINDS-ADRDA criteria (McKhann et al., 1984) and revised criteria from the National Institute on Aging-Alzheimer's Association (Jack et al., 2011). Patients with metabolic disorders, chronic infections and/or other clinically relevant disorders with exception of AD and consuming probiotic supplements within 6 weeks prior to the study, taking other forms of probiotics including probiotic yogurt, kefir, and other fermented foods were excluded.

Ethics Statements

This trial was performed in accordance with the Declaration of Helsinki. Informed consent was received from all patients before beginning the study. The research was approved by the ethics committee of Kashan University of Medical Sciences (KUMS) and was registered in the Iranian website for registration of clinical trials (http://www.irct.ir) IRCT201511305623N60.

Study Design

At the onset of the study, all subjects were matched for disease severity based on gender, BMI, and age. The participants were then randomly divided into two groups to receive either milk (control group, $n = 30$: 24 females and 6 males) or milk containing a mixture of probiotics (probiotic group, $n = 30$: 24 females and 6 males) for 12 weeks. The patients were requested not to change their ordinary physical activity and not to take any nutritional supplements during the 12-week trial. A trained researcher recorded dietary intakes (3-day food records) of all participants at the study baseline, the 3rd, 6th, and 9th week of the intervention and at the end of the trial. Daily macronutrient and micronutrient intakes were analyzed by nutritionist IV software (First Databank, San Bruno, CA). The nutritional questionnaire was completed by a trained researcher (the first author: E. Akbari).

Intervention

In the intervention group ($n = 30$), patients received 200 ml/day probiotic milk containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum,* and *Lactobacillus fermentum* (2×10^9 CFU/g for each) for 12 weeks. It is generally agreed that probiotic strains should be of host origin with a beneficial effect on the host, withstand into food stuff with a high count, withstand transits through intestine and colonize lumen of the tract, produce antimicrobial agents, and technologically appropriate for industrial production (Shewale et al., 2014).

Due to the lack of evidence about the appropriate dosage of probiotics for AD, we used the above-mentioned doses based on few previous studies in healthy subjects (Benton et al., 2007; Mohammadi et al., 2015). Probiotic supplements were produced by Tak Gen Zist Pharmaceutical Company (Tehran, Iran) that was approved by the Food and Drug Organization of the Ministry of Health and Medical Education.

Assessment of Anthropometric Measures

Weight and height of participants were determined in an overnight fasting status using a standard scale (Seca, Hamburg, Germany) at the onset of the study and after 12 weeks of the treatment. BMI was calculated as weight in kg divided by height in meters squared.

Assessment of Outcomes

The primary outcome measurements were Mini-Mental State Examination (MMSE) in the current study. The secondary outcome measurements were biomarkers of oxidative stress, inflammation and metabolic profiles. The MMSE was used to assess cognition in the AD subjects (Folstein et al., 1975).

Assessment of Biochemical Parameters

Twelve-hour fasting blood samples were collected by venipuncture at weeks 0 and 12 at Kashan Reference Laboratory. The blood samples were taken according to a standard protocol and immediately centrifuged (Hettich D-78532, Tuttlingen, Germany). Then, the samples were stored at −80°C until analysis. Plasma total antioxidant capacity (TAC) was measured using the ferric reducing antioxidant power method developed by Benzie and Strain (1996). Total glutathione (GSH) was assessed by the method of Beutler et al. (Beutler and Gelbart, 1985). Malondialdehyde (MDA) concentrations was evaluated using the thiobarbituric acid reactive substance method (Janero, 1990). Serum high sensitivity C-reactive protein (hs-CRP) concentrations were quantified by the use of commercial ELISA kit (LDN, Nordhorn, Germany) with the intra- and inter-assay CVs 3.5 and 5.4%, respectively. Plasma nitric oxide (NO) were quantified by the Griess method (Tatsch et al., 2011). Available kits (Pars Azmun, Tehran, Iran) were used to determine the concentrations of fasting plasma glucose (FPG), serum triglyceride (TG), total holesterol, LDL, and HDL. All inter- and intra-assay CVs for NO, TAC, GSH, MDA, FPG, and lipid profiles were lower than 5%. Circulating levels of serum insulin were assessed using ELISA kit (Monobind, California, USA) with the intra- and inter-assay CVs 3.1 and 4.9%, respectively. The homeostatic model of assessment for insulin resistance (HOMA-IR), homeostatic model assessment for B-cell function (HOMA-B) and the quantitative insulin sensitivity check index (QUICKI) were calculated according to suggested formulas (Pisprasert et al., 2013).

Statistical Methods

To determine whether or not the study variables were normally distributed, we applied the Kolmogrov-Smirnov test to the data. Analyses were conducted based on an intention-to-treat (ITT) principle. Missing values were treated based on Last-Observation-Carried-Forward method (LOCF) (Lachin, 2016). LOCF ignores whether the participant's condition was improving or deteriorating at the time of dropout, instead, it freezes outcomes at the values observed before dropout (i.e., last observation) (Lachin, 2016). For non-normally distributed variables (FPG, insulin, HOMA-IR and hs-CRP), we applied Log transformation. Independent samples Student's *t*-test was used to detect the differences in anthropometric measures as well as in macronutrient and micronutrient intakes between the two groups. For comparison of the categorical variables, Pearson Chi-square test was used. To determine the effects of probiotic milk consumption on MMSE, biomarkers of oxidative stress, inflammation, and metabolic profiles, we used independent samples Student's *t*-test. Adjustment for changes in the baseline values of biochemical parameters, age and BMI at was performed by analysis of covariance (ANCOVA) using general linear models. The *P*-value of < 0.05 were considered statistically significant. All statistical analyses used the Statistical Package for Social Science version 18 (SPSS Inc., Chicago, Illinois, USA). To calculate sample size, we used the standard formula suggested for clinical trials by considering type one error (α) of 0.05 and type two error (β) of 0.20 (power = 80%). Based on a previous study (Malaguarnera et al., 2007), we used 1.3 as SD and 1.1 as the difference in mean (d) of MMSE as key variable. Accordingly, we needed 25 persons in each group. Assuming 5 dropouts in each group, the final sample size was determined to be 30 persons per group. Randomization assignment was conducted using computer-generated random numbers. Randomization and allocation were concealed from the researchers and individuals until the final analyses were completed. The randomized allocation sequence, enrolling subjects and allocating them to interventions were conducted by a trained staff at the Welfare Organizations.

RESULTS

All participants were introduced to the MMSE cognitive test. Also the blood samples were assessed for the biomarkers status. During the study 4 out of 30 patients in the two groups were died and a total of 52 subjects [control ($n = 26$) and probiotic ($n = 26$)] completed the experiments (**Figure 1**). However, as the analysis was based on the ITT principle, all 60 patients were included in the final analysis. The between group comparisons are made considering the post-treatment changes in the baseline values.

No side effects were reported following administration with probiotic in AD patients throughout the study. Mean age, height, weight, and BMI at baseline and end of trial were not statistically different between the two groups (**Table 1**). Based on the 3-day dietary records obtained at study baseline, end of trial and throughout the trial, we found no significant difference in mean dietary macronutrient and micronutrient intakes between the two groups (Data not shown).

Cognitive Assessment

The degree of cognitive impairments in the probiotic and control groups was evaluated by the MMSE test. Twelve weeks intervention resulted in an improvement in MMSE score in the probiotic group (+27.90% ± 8.07) compared to their control counterparts (−5.03% ± 3.00). The difference between the two groups of testing was statistically significant ($P < 0.001$).

Biochemical Measurements

Twelve weeks probiotic treatment decreased the level of the factors affecting metabolism of carbohydrates. The changes in hs-CRP were −17.61% ± 3.70 and +45.26% ± 3.50 μg/ml

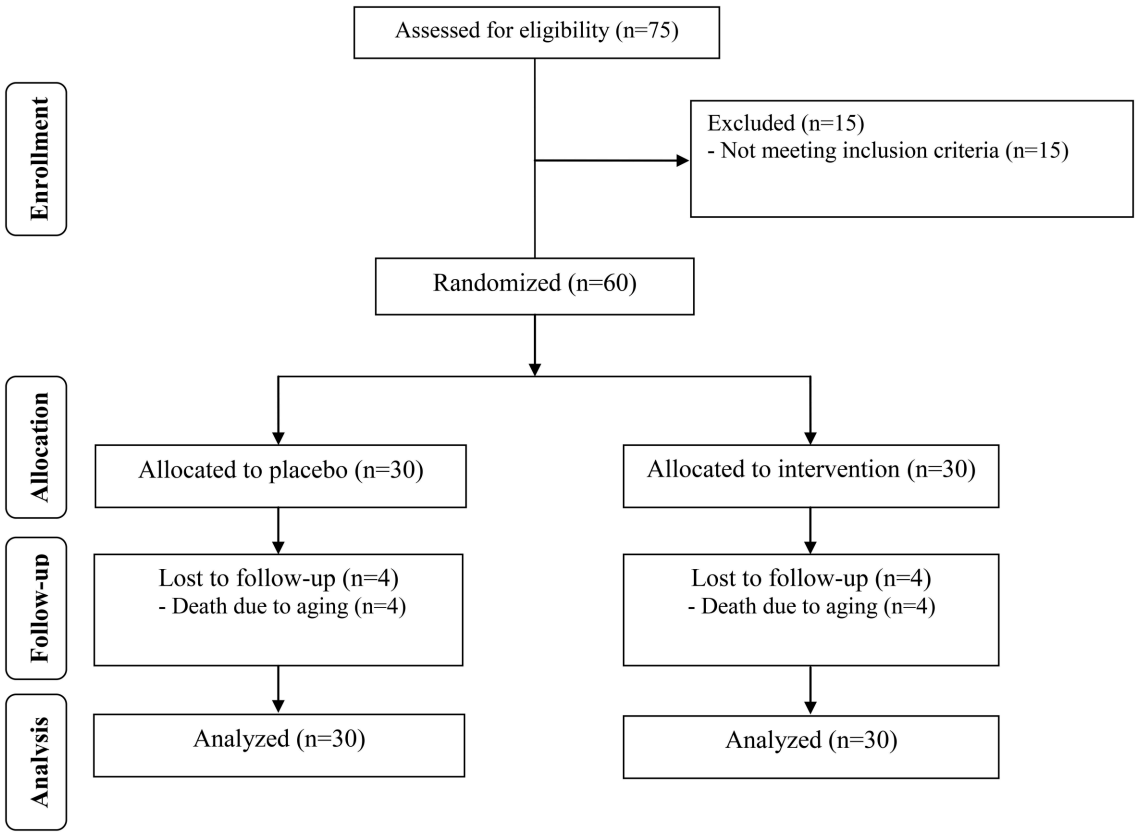

FIGURE 1 | Summary of patient flow.

in the probiotic and control groups, respectively ($P < 0.001$). HOMH-IR index decreased in the probiotic group (+28.84% ± 13.34) compared to their control counterparts (+76.95% ± 24.60, $P = 0.002$). The probiotic treatment considerably reduced the HOMA-B index (+3.45% ± 10.91) in the probiotic compared to the control (+75.62% ± 23.18) patients leading to a significant variation between the two groups ($P = 0.001$). The QUICKI level was significantly ($P = 0.006$) increased in the probiotic subjects (−1.83% ± 1.26) in comparison to their control counterparts (−4.66% ± 1.70). On the other hand, the probiotic supplementation was ineffective on the FBP; the changes are (3.41% ± 2.12) and (4.50% ± 4.39) mg/dl in the probiotic and control groups, respectively.

The probiotic supplementation differently influenced the lipid profiles. The TG level was substantially decreased ($P = 0.003$) in the probiotic patients (−20.29% ± 4.49 mg/dl) compared to the control group (−0.16% ± 5.24 mg/dl). Although the concentration of VLDL was reduced in the probiotic subjects compared to their control counterparts (−20.29% ± 4.49 vs. −0.16% ± 5.24 mg/dl, $P = 0.003$), however, the other lipid profiles (LDL, HDL and cholesterol) were insensitive to the probiotic treatment.

It should be noted that the baseline levels of serum triglycerides, VLDL, and HDL were significantly different between the two groups. Therefore, we controlled the analyses for the baseline values of biochemical parameters, age, and baseline BMI. Nevertheless, this adjustment indicated no considerable changes in our findings (Data not shown).

The probiotic treatment caused a significant decrease ($P < 0.001$) in the MDA of the probiotic group (−22.01% ± 4.84 μmol/l) in comparison to the control patients (+2.67% ± 3.86 μmol/l). We found no difference in the level of the TAC and NO between the two groups.

Table 2 explains the pre- and post-trial values in the control and probiotic patients.

DISCUSSION

The current study demonstrated that the probiotic administration for 12 weeks has favorable effects on MMSE score, MDA, hs-CRP, markers of insulin metabolism and triglycerides levels of the AD patients; however, the changes in other biomarkers of oxidative stress and inflammation, FPG and other lipid profiles are negligible. To the best of our knowledge, this study is the first evaluating the beneficial effects of probiotic supplementation on cognitive function, biomarkers of oxidative stress, inflammation and metabolic

TABLE 1 | General characteristics of the participants (firstly entered the study).

	Control group ($n = 30$)	Probiotic group ($n = 30$)	P[a]
GENDER (%)			
Male	6 (20.0)	6 (20.0)	1.00[†]
Female	24 (80.0)	24 (80.0)	
Age (y)	82.00 ± 1.69	77.67 ± 2.62	0.13
Height (cm)	157.43 ± 1.86	157.77 ± 2.03	0.90
Weight at study baseline (kg)	56.63 ± 2.21	59.03 ± 1.99	0.42
Weight at end-of-trial (kg)	56.80 ± 2.17	59.50 ± 1.98	0.36
Weight change (%)	0.37 ± 0.41	0.85 ± 0.27	0.25
BMI at study baseline (kg/m^2)	22.73 ± 0.68	23.77 ± 0.73	0.30
BMI at end-of-trial (kg/m^2)	22.81 ± 0.67	23.95 ± 0.72	0.25
BMI change (%)	0.37 ± 0.41	0.85 ± 0.27	0.31

Data are mean ± SEM.
[a] *P-values obtained from independent t-test.*
[†] *Obtained from Pearson Chi-square test.*

status in patients with AD. AD patients are predisposed to some complications including increased oxidative stress (Sultana et al., 2011), morbidity, mortality (Schelke et al., 2016), microvascular disease, dyslipidemia, and insulin resistance (Sridhar et al., 2015). Our results indicated that the probiotic treated patients showed some improvement in their MMSE scores. Studies considering the effect of probiotic supplements on brain behavioral phenomena are scant. In our previous work, we have shown that probiotics efficiently reverse the impaired spatial learning and memory as well as synaptic transmission in diabetes mellitus (Davari et al., 2013). It is demonstrated that other brain related disorders such as multiple sclerosis (Kouchaki et al., 2016) and stress are also influenced by probiotics (Liang et al., 2015). In the levels of molecular mechanism, microbiome is known to play a pronounced role in synaptic transmission. Numerous studies have shown capability of bacteria in producing neurotransmitters and neuromodulators including gamma-aminobutyric acid (GABA), norepinephrine, serotonin, dopamine, and acetylcholine (Cryan and Dinan, 2012). Further, findings from germ free animals indicated a decreased level of brain derived neurotrophic factor (BDNF), important neurotrophic factor in the neuronal growth and survival, and a reduced expression of some subunits of *N*-Methyl-D-aspartate (NMDA) receptors (Sudo et al., 2004) involved in most abundant neurotransmission in brain (Salami et al., 2000). GABA is the major inhibitory neurotransmitter in the CNS. Dysfunctions in GABA-signaling are linked to anxiety and depression, defects in synaptogenesis and cognitive impairments (Aziz et al., 2013; Mitew et al., 2013). Also the glutamatergic NMDA receptors are involved in the most important excitatory neurotransmission of brain (Talaei et al., 2016) which is engaged in the neural circuits involved in learning and memory. From these considerations it can be concluded that, at least through contributing in neurotransmitter synthesis or receptor expression, probiotics might adjust the brain activity.

Findings of the current study indicated that while the probiotic supplementation decreased the plasma MDA and the serum hs-CRP levels it was ineffective on other biomarkers of oxidative stress and inflammation. Consistent to our findings, a significant reduction in MDA levels was reported in type 2 diabetic patients after consuming probiotic yogurt (Ejtahed et al., 2012). In addition, Zarrati et al. (2014) showed that 8 weeks consumption of yogurt, enriched by *L. acidophilus*, *Bifidobacterium langum*, and *L. casei* (10^8 CFU/g each) decreased inflammatory cytokines in overweight people. In contrast, others reported no beneficial effects of probiotics on biomarkers of oxidative stress and inflammation. For example, it was reported that 6 weeks probiotic treatment caused no significant change in inflammatory factors of diabetic patients (Mazloom et al., 2013). Furthermore, 8 weeks consumption of capsules containing 10^8 CFU/g of *L. casei* by the people suffered from rheumatoid arthritis displayed no significant within- and between-group changes in MDA and TAC levels (Vaghef-Mehrabany et al., 2015). Oxidative stress is a frequently observed feature of AD, although its pathological significance is not understood (Selvarajah et al., 2011). Evidence indicates that, through production of reactive oxygen species, oxidative stress acts as vehicle for deposition and accumulation of amyloid β (Aβ) in AD (Kim et al., 2015). It has also been postulated that oxidative stress may decrease the activity of α-secretase, which promotes the expression and activity of β and γ-secretase, leading to the increased production of Aβ (Tan et al., 2013). It seems that probable anti-inflammatory role of probiotics needs further investigation to be cleared. Altogether, since the microbiota-gut-brain axis conduits some of its main actions through nerve pathways both dysbiosis and probiotic treatment could have profound effects on the CNS functions. Accumulating evidence from experimental studies supports the hypothesis that; via affecting inflammation, endocrine system, and neurotransmission; the gut microbiome takes a crucial role in the CNS function (Collins et al., 2012; Dinan and Cryan, 2013). Accordingly, it is suggested that dysfunction of the neuroendocrine system, behavior, and cognition are correlated with gut microbiota dysbiosis (Liang et al., 2015). These considerations led to establishing the term "psychobiotics" to highlight the potential effects of probiotics in treatment of mental disorders (Wall et al., 2014). Consistently, in a meta-analysis study, Kasińska and Drzewoski (2015) reported a reduced HOMA-IR and insignificant FGP in probiotic treated subjects. Mazloom et al. (2013) also reported that probiotic supplementation had no significant effect on fasting blood glucose, markers of insulin metabolism and lipid profiles. Consumption of symbiotic bread containing the heat-resistant probiotic *Lactobacillus sporogenes* (1×10^8 CFU/g) for 8 weeks also decreased the serum triglyceride and VLDL concentrations in patients with type 2 diabetes (Shakeri et al., 2014).

Emerging evidence has demonstrated that brain insulin resistance, as a key mediator in prediabetes and diabetes mellitus, may take a role in AD (Cervellati et al., 2016). Insulin resistance plays an important role in the development of cognitive impairment in primary elderly hypertensive patients (Ma et al., 2015). On the other hand, the role of lipids in the etiology and progress of AD is still unclear. Some evidence from clinical studies support the fact that abnormal cholesterol metabolism in the brain leads to progressive cognitive

TABLE 2 | Mean values of the behavioral test and the biomarkers measurements in the probiotic and control groups.

	Control group		Probiotic group		Difference between the two groups
	Baseline	End-of-trial	Baseline	End-of-trial	*P*-value[a]
MMSE (score out of 30)	8.47 ± 1.10	8.00 ± 1.08	8.67 ± 1.44	10.57 ± 1.64	< 0.001
TAC (mmol/L)	895.66 ± 25.96	915.35 ± 26.60	876.13 ± 26.48	922.42 ± 28.53	0.25
GSH (μmol/L)	390.78 ± 17.46	386.76 ± 20.33	377.26 ± 14.82	401.25 ± 16.68	0.19
MDA (μmol/L)	4.26 ± 0.30	4.32 ± 0.31	4.31 ± 0.26	3.21 ± 0.23	< 0.001
hs-CRP (μg/ml)	4.54 ± 1.30	6.59 ± 1.14	6.61 ± 1.24	5.44 ± 0.85	< 0.001
NO (imol/L)	44.76 ± 0.53	45.56 ± 0.82	43.68 ± 0.64	44.37 ± 1.14	0.93
FPG (mg/dl)	83.40 ± 2.36	86.77 ± 4.07	92.00 ± 7.92	94.13 ± 7.72	0.98
HOMA-IR	1.43 ± 0.24	2.08 ± 0.27	1.30 ± 0.13	1.60 ± 0.19	0.002
HOMA-B	25.04 ± 3.21	37.86 ± 4.64	27.36 ± 3.50	22.06 ± 2.43	0.001
QUICKI	0.38 ± 0.01	0.36 ± 0.01	0.38 ± 0.01	0.37 ± 0.01	0.006
Triglycerides (mg/dl)	84.32 ± 4.65	81.74 ± 4.76	119.60 ± 10.25	94.33 ± 10.04	0.003
VLDL (mg/dL)	16.86 ± 0.93	16.35 ± 0.95	23.92 ± 2.05	18.87 ± 2.01	0.003
LDL (mg/dl)	90.44 ± 4.58	94.34 ± 4.39	85.16 ± 4.14	90.64 ± 5.29	0.76
HDL (mg/dl)	51.27 ± 1.75	44.49 ± 1.97	45.81 ± 1.45	38.82 ± 1.35	0.93
Total cholesterol (mg/dl)	158.57 ± 5.75	155.17 ± 5.59	154.88 ± 4.91	148.32 ± 5.43	0.63
Total/ HDL-cholesterol	3.15 ± 0.12	3.62 ± 0.16	3.43 ± 0.12	3.95 ± 0.2	0.81

Data are mean ± SEM.[a] represents P-values obtained from the time × group interaction analysis. FPG, fasting plasma glucose; GSH, total glutathione; HOMA-IR, homeostasis model of assessment-estimated insulin resistance; HOMA-B, homeostasis model of assessment-estimated B cell function; hs-CRP, high-sensitivity C-reactive protein; MMSE, mini-mental state examination; MDA, malondialdehyde; NO, nitric oxide; QUICKI, quantitative insulin sensitivity check index; TAC, total antioxidant capacity.

dysfunction (Wang et al., 2014). Accordingly, high lipid levels could be one of the risk factors for AD (Reitz et al., 2010). Probiotics intake may improve markers of insulin metabolism and lipid profiles by reducing cytokines and suppressing the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (Shi et al., 2006) and gut microbiota-short chain fatty acids (SCFA)-hormone axis (Yadav et al., 2013).

The current study had some limitations. Measurement of fecal bacteria loads before and after the probiotic supplementation in the AD patients was very difficult. In addition, we assessed cognition of the AD patients based on only MMSE test. Hence, considering some other cognitive criteria could be helpful in confirming relevancy of cognition to probiotic supplementation. Also evaluation of other biomarkers of inflammation and oxidative stress including interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), catalase and superoxide dismutase (SOD) seems to be informative.

Overall, the current study demonstrated that 12 weeks consumption of probiotic in the AD patients had favorable effects on MDA, hs-CRP, markers of insulin metabolism, and serum levels of triglyceride and VLDL. However, the pobiotic treatment was inefective on other biomarkers of oxidative stress and inflammation, FPG, and other lipid profiles. Considering the MMSE data we concluded that the probiotic supplementation shows some hopeful trends that warrant further study to assess if probiotics have a clinically significant impact on the cognitive symptoms.

CLINICAL REGISTRATION

http://www.irct.ir: IRCT201511305623N60.

AUTHOR CONTRIBUTIONS

MS designed the research project. EA had principal role in performing the protocol. RD visited all patients for the MMSE tests. EK, GAH and OT assisted in performing the protocol. ZA and FB performed measurement of metabolic biomarkers. ZA analyzed the data. The manuscript was written by MS and ZA. Final edit was accomplished by MS.

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Inferior Prefrontal Cortex Mediates the Relationship between Phosphatidylcholine and Executive Functions in Healthy, Older Adults

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Objectives: This study examines the neural mechanisms that mediate the relationship between phosphatidylcholine and executive functions in cognitively intact older adults. We hypothesized that higher plasma levels of phosphatidylcholine are associated with better performance on a particular component of the executive functions, namely cognitive flexibility, and that this relationship is mediated by gray matter structure of regions within the prefrontal cortex (PFC) that have been implicated in cognitive flexibility.

Methods: We examined 72 cognitively intact adults between the ages of 65 and 75 in an observational, cross-sectional study to investigate the relationship between blood biomarkers of phosphatidylcholine, tests of cognitive flexibility (measured by the Delis–Kaplan Executive Function System Trail Making Test), and gray matter structure of regions within the PFC. A three-step mediation analysis was implemented using multivariate linear regressions and we controlled for age, sex, education, income, depression status, and body mass index.

Results: The mediation analysis revealed that gray matter thickness of one region within the PFC, the left inferior PFC (Brodmann's Area 45), mediates the relationship between phosphatidylcholine blood biomarkers and cognitive flexibility.

Conclusion: These results suggest that particular nutrients may slow or prevent age-related cognitive decline by influencing specific structures within the brain. This report demonstrates a novel structural mediation between plasma phosphatidylcholine levels and cognitive flexibility. Future work should examine the potential mechanisms underlying this mediation, including phosphatidylcholine-dependent cell membrane integrity of the inferior PFC and phosphatidylcholine-dependent cholinergic projections to the inferior PFC.

Keywords: phosphatidylcholine, inferior prefrontal cortex, executive functions, cognitive aging, nutritional cognitive neuroscience

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INTRODUCTION

A rapidly expanding older adult population has produced significant medical and economic demands for the treatment and care of individuals with age-related health disorders that continue to rise. The prevalence of Alzheimer's disease, for example, is projected to increase in the United States from 5.1 to 13.2 million by 2050, and associated healthcare expenditures are estimated to surpass one trillion dollars (Alzheimer's Association, 2013). Therefore, establishing a successful strategy to promote healthy brain aging is of great interest to public health efforts and the United States economy. Nutrition and the many bioactive substances present in the diet have been increasingly recognized as a promising target for intervention efforts to promote healthy brain aging (Zamroziewicz and Barbey, 2016). Identifying the means through which dietary intake may influence brain health will guide the development of successful dietary strategies for healthy brain aging.

Accumulating evidence suggests that phosphatidylcholine is a robust marker of age-related membrane degeneration and is associated with cognitive decline (Zeisel, 2006; Frisardi et al., 2011; Mapstone et al., 2014; Whiley et al., 2014; Norris et al., 2015; Wurtman, 2015). Phosphatidylcholine is a phospholipid that carries a choline head group (Li and Vance, 2008). Phosphatidylcholine found in the blood may be derived from dietary sources, or may be endogenously synthesized by the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway (Zeisel, 2006). Phosphatidylcholine serves a neuroprotective role by providing an essential component of neuronal membranes and a significant portion of the total choline pool, which contributes to forebrain cholinergic projections (Frisardi et al., 2011). However, the core brain regions upon which phosphatidylcholine may act are unknown. This study aims to investigate the neural structures that mediate the relationship between plasma phosphatidylcholine levels and an important aspect of cognitive aging, decline in a component of the executive functions known as cognitive flexibility.

Low plasma phosphatidylcholine levels are highly predictive of cognitive decline, and low levels of important components of phosphatidylcholine, including the long-chain polyunsaturated fatty acid docosahexaenoic acid (DHA) and choline, are predictive of age-related decline in executive functions (Beydoun et al., 2007; Bowman et al., 2012; Nurk et al., 2013; Witte et al., 2013; Naber et al., 2015). Executive functions traditionally consist of planning and execution of goal-directed behaviors, abstract reasoning, and judgment, but also reflect the efficiency with which an individual applies his or her knowledge to cope with everyday life (Stuss and Alexander, 2000; Princiotta and Devries, 2014). Within the continuum of normal aging or preclinical stages of dementia, the presence of executive dysfunction may occur without measurable deficits in general cognition. Therefore, executive dysfunction may be a robust early marker of cognitive decline (Johnson et al., 2007). Importantly, components of phosphatidylcholine, including long-chain polyunsaturated fatty acids and choline, have been associated with prefrontal cortical integrity and forebrain cholinergic projections, respectively, suggesting a link between phosphatidylcholine and the PFC-driven executive functions (Kolisnyk et al., 2013; Zamroziewicz et al., 2015). More specifically, long-chain polyunsaturated fatty acids have been shown to influence cognitive flexibility, a component of the executive functions (Bowman et al., 2013; Johnston et al., 2013; Zamroziewicz et al., 2015). Cognitive flexibility refers to the ability to adjust to new demands or rules, and can be measured using task switching paradigms (Diamond, 2013).

Executive functions are implemented within the prefrontal cortex (PFC), and particular aspects of the executive functions may be localized to specific sub-regions within the PFC (Barbey et al., 2012, 2013a,b,c, 2014a,b). Larger gray matter thickness and volume in the PFC has been associated with better performance on tasks that elicit executive functions (Kochunov et al., 2009; Burzynska et al., 2012; Tu et al., 2012; Yuan and Raz, 2014). For example, the inferior PFC has been implicated in the cognitive control of memory, including semantic retrieval, recollection of contextual details about past events, resolution of proactive interference in working memory, and task switching (Badre and Wagner, 2007). The inferior PFC is particularly susceptible to age-related cortical thinning, and age-related changes in cholinergic projections (Aron et al., 2004; Fjell et al., 2009). Integrity of the left inferior PFC has been linked to cognitive flexibility, as measured by task switching paradigms (Aron et al., 2004).

In summary, prior research indicates that: (i) phosphatidylcholine is highly predictive of age-related cognitive decline; (ii) cognitive flexibility is an early marker of cognitive decline amenable to the effects of phosphatidylcholine components; and (iii) particular regions within the PFC, such as the inferior PFC, are critical for cognitive flexibility and susceptible to age-related degeneration. Therefore, we examined the role of regions within the PFC in mediating the relationship between plasma phosphatidylcholine and cognitive flexibility in cognitively intact aging individuals.

MATERIALS AND METHODS

Participants

This cross-sectional study enrolled 122 elderly adults from Carle Foundation Hospital, a local and readily available cohort of well-characterized elderly adults. No participants were cognitively impaired, as defined by a score of lower than 26 on the Mini-Mental State Examination (Folstein et al., 1975). Participants with a diagnosis of mild cognitive impairment, dementia, psychiatric illness within the last 3 years, stroke within the past 12 months, and cancer within the last 3 years were excluded. Participants were also excluded for current chemotherapy or radiation, an inability to complete study activities, prior involvement in cognitive training or dietary intervention studies, and contraindications for magnetic resonance imaging (MRI). Of these 122 participants, 72 subjects had a complete dataset at time of data analysis,

including neuropsychological testing, MRI, and blood biomarker analysis.

Standard Protocol Approval and Participant Consent

This study was approved by the University of Illinois Institutional Review Board and the Carle Hospital Institutional Review Board and, in accordance with the stated guidelines, all participants read and signed informed consent documents.

Biomarker Acquisition and Analysis

Plasma was spiked with stable labeled internal standards of all the analytes, and extracted using the method modified from Bligh and Dyer (1959). Samples were extracted with methanol/chloroform (2:1, v/v). The mixture was vortexed and left at −20°C overnight. At the end of the extraction with methanol/chloroform, samples were centrifuged and supernatants transferred into new microcentrifuge tubes. Residues were re-extracted with methanol/chloroform/water (2:1:0.8, v/v/v). After vigorous vortexing and centrifugation, supernatants were collected and combined with the first extract. Water and chloroform were added into the resulting solutions to allow for phase separation. After centrifugation, the organic phase, which contains phosphatidylcholine, was 1:10 diluted with methanol and transferred into HPLC vials for instrumental analysis.

Quantification of the analytes was performed using liquid chromatography-stable isotope dilution-multiple reaction monitoring mass spectrometry (LC-SID-MRM/MS). Chromatographic separations were performed on an Atlantis Silica HILIC 3 μm 4.6 × 50mm column (Waters Corp, Milford, CT, USA) using a Waters ACQUITY UPLC system. The column was heated to 40°C, and the flow rate maintained at 1 mL/min. The mobile phases were: A – 10% acetonitrile/90% water with 10 mM ammonium formate and 0.125% formic acid, and B – 90% acetonitrile/10% water with 10 mM ammonium formate and 0.125% formic acid. For organic analytes, the gradient was at 5% A for 0.05 min, to 20% A in 2.95 min, to 55% A in 0.05 min, at 55% A in 0.95 min, to 5% A in 0.05 min, and at 5% A for 2.95 min. The analytes and their corresponding isotopes were monitored on a Waters TQ detector using characteristic precursor-product ion transitions. Concentrations of each analyte in the samples were determined using the peak area ratio of the analyte to its isotope. MS parameters for phosphatidylcholine were as follows: precursor at 193 m/z, product at 193 m/z. Phosphatidylcholine levels were included in analyses as a continuous variable.

Neuropsychological Tests

Executive functions were measured by the Delis–Kaplan Executive Function System (D–KEFS) Trail Making Test (Delis et al., 2006). This assessment yields a measure of the executive functions that can be isolated from underlying skills, including visual scanning, number sequencing, letter sequencing, and motor speed. In this task, participants alternate between multiple task goals (either number or letter sequencing), which elicits a specific component of the executive functions known as cognitive flexibility. The reported results from the D-KEFS Trail Making Test assess cognitive flexibility while controlling for number and letter sequencing trials and therefore provide a measure of cognitive flexibility that is not confounded by underlying cognitive abilities (i.e., number and letter sequencing) required by the task.

Volumetric Brain MRI

Volumetric analysis was performed on data from a 3D high-resolution (0.9 mm isotropic) T1-weighted scan using MPRAGE acquisition. Cortical reconstruction was performed with the Freesurfer image analysis suite, which is documented and freely available for download online[1]. The technical details of these procedures are described in prior publications (Dale and Sereno, 1993; Dale et al., 1999; Fischl et al., 1999a,b, 2001, 2002, 2004; Fischl and Dale, 2000; Fischl, 2004; Ségonne et al., 2004; Han et al., 2006; Jovicich et al., 2006; Reuter et al., 2010, 2012). All cortical reconstructions were manually checked for accuracy, as recommended by the software developers. This analysis focused on gray matter thickness in the PFC provided by Freesurfer parcellation. These regions included the superior frontal cortex, rostral middle frontal cortex, the caudal middle frontal cortex, pars opercularis, pars triangularis, pars orbitalis, lateral orbitofrontal cortex, medial orbitofrontal cortex, precentral gyrus, paracentral gyrus, frontal pole, rostral anterior cingulate cortex, and caudal anterior cingulate cortex.

Covariates

Covariates previously associated with cognitive decline (Coffey et al., 1998, 1999; Fotenos et al., 2008; Gunstad et al., 2008; Raz et al., 2010; van Tol et al., 2010) were tested, including age (continuous), gender (nominal, man/woman), education (ordinal, five fixed levels), income (ordinal, six fixed levels), body mass index (continuous, BMI), and depression status (nominal, yes/no). Although all participants had received a diagnosis of no depression at enrollment, the SF-36 Health Survey (Ware et al., 1993) revealed five participants with symptoms consistent with depression and so, in accordance with other studies, this was considered in the analysis as a covariate. PFC gray matter thickness (continuous) was also included as a covariate in mediation analyses to assess the relationship between specific regions within the PFC, plasma phosphatidylcholine, and cognitive flexibility. Covariates were included in each of the three steps of the mediation analysis.

Statistical Analyses

A formal mediation analysis was used in an effort to better understand the relationship between phosphatidylcholine levels, gray matter thickness of regions within the PFC, and cognitive flexibility using a three-step framework. The goal of the mediation analysis was to understand whether the relationship between phosphatidylcholine levels and cognitive flexibility was mediated by gray matter thickness of regions within the PFC. The primary requirement for mediation is a significant indirect mediation effect, or the effect of the independent

[1] http://surfer.nmr.mgh.harvard.edu/

variable (phosphatidylcholine) through the mediator (gray matter thickness of a PFC region) on the dependent variable (cognitive flexibility) (Zhao et al., 2010).

Statistics were performed in SPSS Statistical Packages version 23 (SPSS, Inc., Chicago, IL, USA), and mediation analyses were performed using the *indirect* macro designed for SPSS (Preacher and Hayes, 2008). Statistics were performed as follows:

(1) In the first step, a regression model was used to characterize the relationship between phosphatidylcholine levels and gray matter thickness of regions in the PFC, controlling for the covariates in Section "Covariates" (path a).
(2) In the second step, a regression model was used to characterize the relationship between phosphatidylcholine levels and cognitive flexibility, controlling for the covariates in Section "Covariates" (path c).
(3) In the third step, the *indirect* macro was used to implement the bootstrapping method to estimate mediation effects. This analysis drew 1000 bootstrapped samples with replacement from the dataset to estimate a sampling distribution for the indirect and direct mediation effects, controlling for the covariates in Section "Covariates." The indirect mediation effect refers to the pathway from phosphatidylcholine to gray matter thickness of a PFC region to cognitive flexibility (path a–b). The direct mediation effect refers to the direct pathway from phosphatidylcholine to cognitive flexibility (path c').

A statistically significant mediation that matched the hypothesized framework was indicated by: (i) an indirect mediation effect that did not include zero within 95% bias-corrected confidence intervals, and (ii) a direct mediation effect that did include zero within 95% bias-corrected confidence intervals (Zhao et al., 2010). Results are reported using unstandardized regression coefficients (β) and statistical significance (p) for each individual regression relationship, and a 95% bias-corrected confidence interval (95% CI) for the direct and indirect effects of the mediation.

RESULTS

Participant Characteristics

Participants had a mean age of 69 years and 64 percent of participants were females. Education levels were reported as follows: 1 percent of participants completed some high school, 14 percent of participants received a high school degree, 18 percent of participants completed some college, and 68 percent of participants received a college degree. Annual household income levels were reported as follows: 1 percent of participants earned less than $15,000, 3 percent of participants earned $15,000 to $25,000, 17 percent of participants earned $25,000 to $50,000, 24 percent of participants earned $50,000 to $75,000, 22 percent of participants earned $75,000 to $100,000, and 33 percent of participants earned over $100,000. The mean phosphatidylcholine level was 2101 μM. The mean D-KEFS Trail Making Test cognitive flexibility score was 8. The mean gray matter thickness of the left PFC was 2.39 mm, and mean gray matter thickness of the left inferior PFC was 2.38 mm (**Table 1**).

Mediation Results

The mediation analyses indicated that out of all regions within the PFC, gray matter thickness of only the left inferior PFC (pars triangularis, Brodmann area 45) mediated the relationship between phosphatidylcholine and cognitive flexibility, corresponding with prior work that suggests an influential role of this region. Each relationship within the mediation is described below in a stepwise fashion.

First, higher phosphatidylcholine associated with greater thickness of the left inferior PFC ($\beta = 0.001$, $p = 0.007$; **Figures 1** and **2,** path a). Second, higher phosphatidylcholine associated with better cognitive flexibility ($\beta = 0.002$, $p = 0.016$, **Figure 2**, path c). Third, the indirect pathway of meditation was significant (95% CI: 0.001 – 0.002, $\beta = 4.688$, $p = 0.047$, **Figure 2**, path a–b), but the direct pathway of mediation was insignificant (95% CI: −0.002 – 0.003, $\beta = 0.001$, $p = 0.089$, **Figure 2**, path c'). Therefore, the mediation indicated that gray matter thickness of the left inferior PFC fully mediated the relationship between phosphatidylcholine and cognitive flexibility (**Figure 2**).

DISCUSSION

This study revealed that gray matter thickness of the left inferior PFC mediates the relationship between plasma phosphatidylcholine and cognitive flexibility. The mediation

TABLE 1 | Characteristics of sample[1].

Demographics	**Total $n = 72$**
Age (mean years ± standard deviation)	69 ± 3
Female, *n*(%)	46(64)
Education, *n*(%)	1(1) some high school
	10(14) high school degree
	12(17) some college
	49(68) college degree
Income, *n*(%)	1(1) < $15,000
	2(3) $15,000 – $25,000
	12(17) $25,000 – $50,000
	17(24) $50,000 – $75,000
	16(22) $75,000 – $100,000
	24(33) > $100,000
Depression, *n*(%)	67(93) no
	5(7) yes
Plasma nutrients	**(uM ± std)**
Phosphatidylcholine	2101 ± 400
Psychometrics	**(mean ± std)**
Cognitive flexibility score	8 ± 2
Volumetric MRI (gray matter thickness)	**(mm± std))**
Left PFC	2.39 ± 0.08
Left inferior PFC	2.38 ± 0.13

[1]*Demographics reflect covariates included in analyses. Plasma nutrients, psychometrics, and volumetric MRI reflect variables of interest included in analyses.*

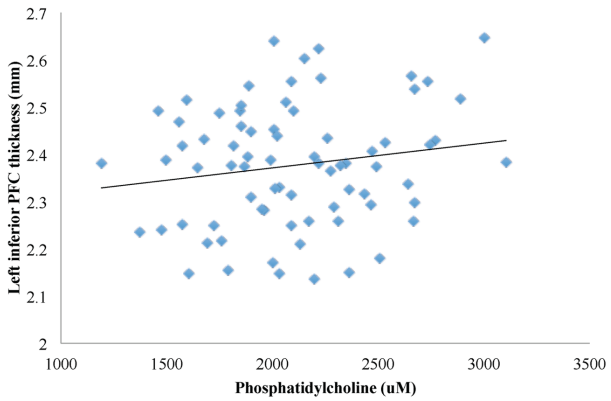

FIGURE 1 | A linear regression model was used to characterize the relationship between phosphatidylcholine levels and gray matter thickness of regions in the PFC. Phosphatidylcholine levels positively associated with gray matter thickness of the left inferior PFC ($\beta = 0.001$, $p = 0.007$).

analysis provided a novel finding that links phosphatidylcholine to gray matter integrity of a specific cortical region and a particular component of the executive functions. The individual relationships reported within the mediation, including those between phosphatidylcholine levels and left inferior PFC (**Figure 2**, path a), between phosphatidylcholine levels and cognitive flexibility (**Figure 2**, path c), and between left inferior PFC and cognitive flexibility (**Figure 2**, path b), are each substantiated by prior findings reviewed in turn below.

The first relationship demonstrated a positive association between higher phosphatidylcholine levels and greater thickness in the inferior PFC of the left hemisphere (**Figure 2**, path a). Past studies suggest that phosphatidylcholine plays a critical role in age-related changes in cortical integrity, and the inferior PFC, being a region that thins early in aging, may be particularly susceptible to these effects (Söderberg et al., 1990; Wurtman, 2015). More specifically, phosphatidylcholine may contribute to structure and function of the inferior PFC via cholinergic projections, which enhance functional activity within this region (Blusztajn et al., 1987; Berry et al., 2015). Second, higher phosphatidylcholine levels are associated with better cognitive flexibility (**Figure 2**, path c). Prior work demonstrates that higher phosphatidylcholine levels are related to slower cognitive decline, and components of phosphatidylcholine, including long-chain polyunsaturated fatty acids, such as DHA, and choline, are linked to superior performance on executive function tasks (Schaefer et al., 2006; Beydoun et al., 2007; Bowman et al., 2012; Nurk et al., 2013; Witte et al., 2013; Hartmann et al., 2014; Mapstone et al., 2014; Naber et al., 2015; Zamroziewicz et al., 2015). The indirect pathway of mediation indicated a mediatory effect of left inferior PFC gray matter thickness on the relationship between phosphatidylcholine levels and cognitive flexibility (**Figure 2**, path a–b). Previous studies indicate that greater gray matter thickness within the inferior PFC contributes to superior cognitive flexibility, and that cholinergic transmissions, originating, for example, from phosphatidylcholine-derived choline, underlie activity within the inferior PFC during tasks of cognitive control (Blusztajn et al., 1987; Burzynska et al., 2012; Berry et al., 2015). The unilateral nature of this mediation is supported by prior work, which suggests that regions within the left hemisphere may be selectively susceptible to degeneration and cognitive impairment (Chételat et al., 2005; Querbes et al., 2009; Risacher et al., 2010; Mosconi et al., 2014).

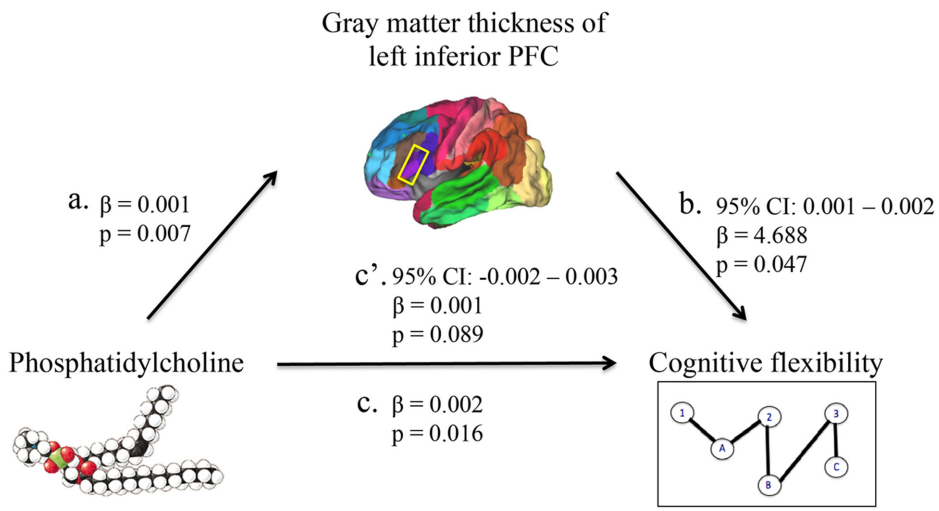

FIGURE 2 | A mediation model was used to characterize the relationship between phosphatidylcholine levels, gray matter thickness of regions in the PFC, and cognitive flexibility. Phosphatidylcholine levels positively associated with gray matter thickness of the left inferior PFC (path a). Phosphatidylcholine levels positively associated with cognitive flexibility (path c). The indirect pathway of mediation (i.e., the effect of phosphatidylcholine levels through gray matter thickness of the left inferior PFC on cognitive flexibility; path a–b) was statistically significant. The direct pathway of mediation (i.e., the effect of phosphatidylcholine levels directly on cognitive flexibility, accounting for the effect of gray matter thickness of the left inferior PFC; path c') was not statistically significant. Therefore, gray matter thickness of the left inferior PFC fully mediated the relationship between phosphatidylcholine levels and cognitive flexibility.

Prior work indicates that the underlying physiological mechanisms of the relationship between phosphatidylcholine levels, cognitive flexibility, and cortical integrity of the inferior PFC may be threefold. First, phosphatidylcholine may help slow or prevent age-related changes in cortical thickness by delivering two molecules that are critical for cortical integrity, including choline and long-chain polyunsaturated fatty acids (Söderberg et al., 1990; Cohen et al., 1995; Zeisel, 2006; Jerneren et al., 2015). Second, the delivery of long-chain polyunsaturated fatty acids may help prevent inflammation in the brain (Wall et al., 2010). Third, delivery of choline contributes to acetylcholine synthesis, a neurotransmitter that has been implicated in set-shifting performance and projects to the inferior PFC via forebrain cholinergic transmissions (Blusztajn et al., 1987; Hasselmo and Sarter, 2011; Berry et al., 2015). Importantly, phosphatidylcholine-derived choline may be a primary contributor to the brain choline pool when age-related changes in brain choline uptake reduce extracellular choline supplies (Zeisel, 2006). Future mechanistic studies are needed to confirm underlying physiological mechanisms of the relationship between phosphatidylcholine levels, cognitive flexibility, and cortical integrity of the inferior PFC.

Research at the frontiers of nutritional cognitive neuroscience seeks to integrate methods that sensitively capture variability in nutritional intake, brain aging, and cognition, and in doing so, elucidate the neural structures that mediate the relationship between nutritional status and cognitive decline. This finding contributes to a growing line of evidence which suggests that particular nutrients may slow or prevent aspects of age-related cognitive decline by influencing specific features of brain aging (Bowman et al., 2012; Zamroziewicz et al., 2015; Gu et al., 2016). In the case of phosphatidylcholine, future studies are needed to assess the origins of plasma phosphatidylcholine, and whether dietary intake or endogenous synthesis preferentially contributes to the neuroprotective effect. Another promising direction for future work is to examine the interactive effects among nutrients through the use of nutrient biomarker pattern analysis – a technique that enables an investigation of the beneficial effects of broader nutrient profiles on healthy brain aging. Ultimately, this line of research can inform clinical investigations of comprehensive and personalized approaches to nutritional intervention that takes into account dietary patterns and individual variability in nutritional status and brain health.

The strengths of the present study include: (i) the use of blood biomarkers to measure physiological status of phosphatidylcholine, (ii) the use of structural magnetic resonance imaging to measure regional cortical integrity with high spatial resolution, and (iii) the assessment of a particular component of cognitive function known to be sensitive to age-related cognitive decline, rather than a global cognitive function measure with little variability in healthy aging adults. Directions for future research include (i) replication of results in a larger sample size, (ii) implementation of a longitudinal study to examine how changes in phosphatidylcholine levels relate to changes in executive functions and integrity of the PFC, (iii) investigation of the physiological mechanisms proposed to underlie the relationship between phosphatidylcholine and PFC structure, (iv) examination of relationship between phosphatidylcholine, executive functions, and PFC integrity in other models, including animal models and clinical populations, (v) elucidation of the relationship between phosphatidylcholine in plasma and cerebrospinal fluid, and (vi) examination of the origins of phosphatidylcholine in blood, as plasma phosphatidylcholine may be derived from the diet or *de novo* synthesis by the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway (Zeisel, 2006).

AUTHOR CONTRIBUTIONS

MZ is the primary author of this manuscript. CZ contributed to drafting and editing of the manuscript. AB is the primary investigator and contributed to drafting and editing of the manuscript.

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frontiers in Aging Neuroscience

REVIEW
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Effects of Physical Exercise Combined with Nutritional Supplements on Aging Brain Related Structures and Functions: A Systematic Review

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Age-related decline in gray and white brain matter goes together with cognitive depletion. To influence cognitive functioning in elderly, several types of physical exercise and nutritional intervention have been performed. This paper systematically reviews the potential additive and complementary effects of nutrition/nutritional supplements and physical exercise on cognition. The search strategy was developed for EMBASE, Medline, PubMed, Cochrane, CINAHL, and PsycInfo databases and focused on the research question: "Is the combination of physical exercise with nutrition/nutritional supplementation more effective than nutrition/nutritional supplementation or physical exercise alone in effecting on brain structure, metabolism, and/or function?" Both mammalian and human studies were included. In humans, randomized controlled trials that evaluated the effects of nutrition/nutritional supplements and physical exercise on cognitive functioning and associated parameters in healthy elderly (>65 years) were included. The systematic search included English and German language literature without any limitation of publication date. The search strategy yielded a total of 3129 references of which 67 studies met the inclusion criteria; 43 human and 24 mammalian, mainly rodent, studies. Three out of 43 human studies investigated a nutrition/physical exercise combination and reported no additive effects. In rodent studies, additive effects were found for docosahexaenoic acid supplementation when combined with physical exercise. Although feasible combinations of physical exercise/nutritional supplements are available for influencing the brain, only a few studies evaluated which possible combinations of nutrition/nutritional supplementation and physical exercise might have an effect on brain structure, metabolism and/or function. The reason for no clear effects of combinatory approaches in humans might be explained by the misfit between the combinations of nutritional methods with the physical interventions in the sense that they were not selected on sharing of similar neuronal mechanisms. Based on the results from this systematic review, future human studies should focus on the combined effect of docosahexaenoic acid supplementation and physical exercise that contains elements of (motor) learning.

Keywords: nutritional supplementation, nutrition, physical exercise, brain function, brain metabolism, aging

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INTRODUCTION

Thirty percent of people aged 65 and older living in the community experience at least one fall per year, and this proportion increases markedly with age (Tromp et al., 2001). The elevated incidence of falls in elderly is only one of the many physical dysfunctions that may be encountered with advanced age (Iosa et al., 2014). Elderly experience a reduction in walking speed, an increased variability in step timing, a decline in gait stability, and are compromised in their learning ability (Cai et al., 2014; Iosa et al., 2014). These reductions in movement functionality often develop already in midlife (Tomey and Sowers, 2009) and have been described as age-related deteriorations in physical functioning (Pichierri et al., 2011). Physical functioning can be defined as the ability to conduct activities that are required for independent living and that may affect quality of life, such as walking and climbing stairs (Painter et al., 1999; Pichierri et al., 2011). Limitations in physical functioning have been associated with depression, increased risk for falls and injuries, reduced quality of life, increased health care costs, and mortality (Tomey and Sowers, 2009). In the near future, the number of elderly people suffering from physical dysfunctions will increase due to demographic changes (Kluge et al., 2014).

Various factors have been proposed causing the decline in physical functioning in the older population. Loss of muscle mass, strength, and degradation of joints have been associated with gait instability and falls (Mithal et al., 2013; Iosa et al., 2014). In addition to reductions in the musculoskeletal system, impairments in vision, reaction time, and balance play an important role (Iosa et al., 2014). However, worsening of the sensorimotor system is probably not the only cause explaining deteriorations in physical functioning: reduction of cognitive functions is believed to play a significant role as well (Cai et al., 2014; Iosa et al., 2014). Cognitive functions are "...any mental process that involves symbolic operations—e.g., perception, memory, creation of imagery, and thinking..." (Concise Dictionary of Modern Medicine, 2002).

A decrease in cognitive performance in old age is predominant in most individuals. Aging associated cognitive decline has a prevalence rate of 28% for people from 65 to 84 years (Scafato et al., 2010). Another 17% of the population investigated ($n = 4785$) showed objective evidence of cognitive decline without cognitive complaints, which sums up to a total of 45% of people showing some kind of cognitive impairment without dementia. Cognitive aging is characterized by mental decline (Cotman et al., 2007), memory impairments (Gunning-Dixon et al., 2009; van Praag, 2009; Cai et al., 2014), decreased learning ability (van Praag, 2009; Cai et al., 2014), greater anxiety and poorer attention (Cai et al., 2014), slower processing speed (Gunning-Dixon et al., 2009; Clouston et al., 2013; Cai et al., 2014), and reduction of executive skills (Gunning-Dixon et al., 2009). Other studies showed that healthy participants and participants with cognitive impairment or subclinical cerebrovascular lesions had reduced gait stability and postural control during dual-task walking suggesting that cognitive abilities affect gait performance (Pichierri et al., 2011; Choi et al., 2012; Iosa et al., 2014).

Studies investigating cognitive aging support the idea that neuroanatomical changes, such as loss of brain tissue and cortical disconnections, might explain the poorer cognitive performance of healthy elderly (Colcombe et al., 2003; Resnick et al., 2003; Raz et al., 2005; Gunning-Dixon et al., 2009). Age-dependent structural changes include loss of gray matter volume (frontal and temporal lobes), vulnerability of prefrontal white matter, loss of microstructural white matter integrity, and decrease in hippocampus and cerebellum volumes (Raz et al., 2005; Gunning-Dixon et al., 2009). For example, frontal lobe white matter has been proposed to mediate the association of age and performance in tasks assessing executive skills and memory (Brickman et al., 2006). Structural alterations might partially account for observed age-dependent declines in cognition (Gunning-Dixon et al., 2009). In addition to the neuroanatomical changes, neurochemical processes change during the course of aging (Mora, 2013). In rodents, for example, an age-dependent decrease of neurotrophic factors, such as the brain-derived neurotrophic factor (BDNF), might contribute to age-related cognitive impairments (Gooney et al., 2004; Adlard et al., 2005; Mora et al., 2007).

Since cognitive decline potentially threatens independence and quality of life of older adults, prevention and treatment of cognitive impairment in the elderly has assumed increasing importance (Williams and Kemper, 2010). Two factors that may positively effect on cognition are physical activity (Gomez-Pinilla and Hillman, 2013) and nutritional supplementation (Gómez-Pinilla, 2008; **Figure 1**). Physical exercise has been described to be the most effective way to maintain a healthy body and mind (van Praag, 2009). Physical exercise lowers blood pressure, increases sensitivity to insulin, contributes to weight loss, delays age-related cognitive decline, improves learning and memory, and reduces the risk of neurodegeneration (Shephard and Balady, 1999; Cotman and Berchtold, 2002). The proposed mechanisms by which physical exercise affects cognition revolve around changes in neurotransmitters, neurotrophins, and vasculature (Cotman et al., 2007). Neurogenesis in the hippocampus is associated with improved cognition, and the strongest neurogenic stimulus seems to be physical exercise (van Praag, 2009). Moreover, physical exercise appears to affect properties of dendritic spines, to enhance long term potentiation, to influence brain vasculature through the actions of insulin like growth factor (IGF) and vascular endothelial growth factor, and to affect BDNF which plays an essential role in synaptic plasticity and cell genesis, growth, and survival (van Praag, 2009).

Nutrition and nutritional supplements may also exhibit positive effects on brain health (van Praag, 2009). Studies showed that caloric restriction (CR) and nutritional supplements such as fish oil, teas, fruits, folate, spices, and vitamins have the potential to positively effect on cognitive functioning (Gómez-Pinilla, 2008). Investigations on the effects of nutrition on brain function have usually focused on neuroprotective properties of nutritional supplements (van Praag, 2009). Recent studies focused on underlying mechanisms like neuronal signaling (van Praag, 2009). In fact, nutritional

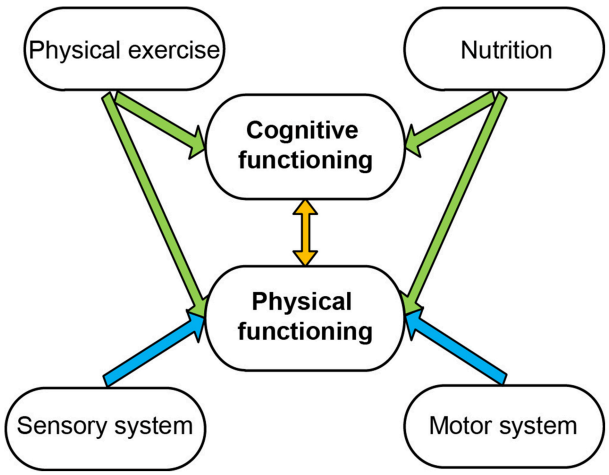

FIGURE 1 | Interaction of cognitive and physical functioning. Physical and cognitive functioning influences each other (orange arrow). In turn, both can be influenced by physical exercise and nutrition (green arrows). The motor and sensory system regulates physical functioning (blue arrows).

supplementation and CR seem to affect similar cellular and molecular pathways as physical exercise (van Praag, 2009).

From the foregoing, the assumption that physical exercise and nutrition could have additive effects on brain structures and functions that may result in greater benefits on cognition for combinatory interventions seems justified (Gómez-Pinilla, 2011). "Additive" means when two interventions are combined intendedly (physical exercise and nutrition are an integral part of one intervention) to enhance effects and "complementary" in case each intervention stands by itself. Recent studies indicate that exercise is capable of boosting the health effects of certain diets and that selected dietary factors may have the capacity to complement the effects of exercise (Gómez-Pinilla, 2011). However, existing reviews on these effects are either narrative, or, when being performed systematically, are limited in the sense that they focus on the isolated effect of either physical exercise or nutrition (Gómez-Pinilla, 2008; van Praag, 2009; Voss et al., 2013). Which combination of selected dietary factors possibly best should be added to physical exercise for additive effects of exercise on cognition in humans remains, therefore, indefinite. To the best of our knowledge, a systematic review focusing on the possible additive effects of physical exercise and nutrition/nutritional supplementation on the elderly brain has not been performed. Therefore, a systematic review was performed on the effect of combined physical and nutritional interventions with the aim of clarifying the relationship between the type of combined intervention and the effects of such an intervention on brain related structure and function in both mammalian and human studies. The following research question guided this systematic review: "Is the combination of physical exercise with nutritional supplementation more effective than nutritional supplementation or physical exercise alone in effecting on brain structure, metabolism, and/or function"?

METHOD

Data Sources and Searches

A search strategy was developed in collaboration with a librarian from the Medical Library of the University of Zurich. The search period covered all years from the inception to October, 2015, and included EMBASE, Medline, PubMed, Cochrane, CINAHL, and PsycInfo. Searches were undertaken using MeSH headings and text words including the following main terms for the population: *aged, elder, placental mammals, human, rat, mouse, mice, mammal, mammalia*; for nutritional intervention: *diet supplementation, diet therapy, protein intake, dietary intake, diet, protein, nutrient, mineral, vitamin, supplementation, supplement, additive, intake, therapy, treatment*; for physical exercise: *resistance training, physical, activity, exercise, fitness, strength, training,* and for the outcome of interest: *cognition, executive function, memory, nervous system development, nerve cell plasticity, angiogenesis, neurogenesis, synaptogenesis, neuroplasticity, brain structure, spine density, function, structure, neurotransmitter, vascular endothelial growth factor, insulin like growth factor, brain, nerve*. Furthermore, the bibliographies of all eligible articles and related reviews, as well as recent conference proceedings, were checked through hand searching. To ensure the clarity and transparency of reporting, the PRISMA guidelines (Moher et al., 2010) were followed.

Selection Criteria

Both studies with mammalians and humans were considered for this review. From mammalian research knowledge about the effects on molecular, cellular, and neural circuit levels and how these may impact cognitive function can be gained (Voss et al., 2013; Gutchess, 2014). Higher-level cognition effects and influences on macro- and systems-level change in the central nervous system can be evaluated in human studies (Voss et al., 2013; Gutchess, 2014). The search strategy included "elderly over the age of 65 years" and "older mammalians." Interventions that focused on physical exercise and nutritional supplementation or the combination of both were considered. Study outcomes were determined on brain structure, -function, and -metabolism levels. Randomized controlled trials (RCT), the most rigorous way of determining whether a cause-effect relation exists between treatment and outcome (Sibbald and Roland, 1998), were primarily included. Because well-designed observational studies have been shown to provide results similar to randomized controlled trials (Song and Chung, 2010) case control trials were also included. In addition, reviews on our topic written in English or German with no year restriction were considered for discussion.

Selection Process

The first step was the removal of duplicate citations. Afterwards two reviewers (JS, AS) determined which studies should be included by independently screening of title, abstract, and keywords. A priori set inclusion and exclusion criteria were applied to the articles (**Table 1**). An article was eligible, if the investigator examined complementary or additive effects of physical exercise and nutritional interventions on cognitive

TABLE 1 | List of inclusion and exclusion criteria.

Area	Inclusion criteria	Exclusion criteria
Population	Older (>65 years) adults and old mammalians	Patients with neurodegenerative diseases
Intervention	Nutritional supplementation, brain food, physical exercise, exercise trainings, physical activity	Pharmacological interventions
Outcome	Neurogenesis, synaptic plasticity, brain structure, spine density, angiogenesis, growth factors, neurotransmitter, neurotrophins, cognitive function	Physical benefits
Study type	Randomized controlled and case control trails	Methodological, theoretical, review, and discussion papers
Language	English and German	All other languages
Year	All years	–

functions in humans and mammalians and/or associated brain parameters in mammalians. Only longitudinal studies were included that carried out an intervention. Interventions that considered aerobe, strength, and/or coordination training were defined as physical exercise. Nutritional interventions were those that considered nutritional supplementation. Studies using pharmacological supplementation or that focused on physical outcomes were excluded. Subsequently, the results from the screening were discussed to exclude any differences in the inclusion decisions. Full text reading of the remaining literature yielded the final list of papers. Studies were included assessing brain structure (e.g., gray and white matter), brain metabolism (e.g., neurotrophic factors), and brain function (e.g., cognitive test batteries) in healthy elderly.

Data Extraction and Data Synthesis

The included studies were sub-divided into human and mammalian studies. Each of these classes was then further subdivided into three groups: Physical exercise, nutritional intervention, and studies that investigated a combination of physical exercise and nutritional intervention. In this way, comparisons between different treatments were easier to track. A purpose adjusted individualized data extraction form from Wright et al. (2007) was used to collect data from single studies. The extraction of the data included (1) reference information: author and date; (2) characteristics of study population: number of participants, gender, age, genetics (mammalians); (3) characteristics of physical exercise intervention: type of exercise, frequency, and duration; (4) characteristics of nutritional intervention: diet or nutritional supplement, amount of intake, and duration; (5) characteristics of outcomes: outcome measures and results. The data is presented in the results section as a descriptive summary of the studies and their results. Furthermore, a qualitative synthesis of the studies was executed. A meta-analysis was not performed due to the high heterogeneity of intervention types and outcome variables among the studies.

Quality Appraisal

Quality evaluation of the studies was done by reporting potential sources of bias (Harris et al., 2013). For critical quality appraisal, the purpose-adjusted Downs and Black checklist for randomized and non-randomized studies of health care interventions was used (Downs and Black, 1998). The quality checklist consisted of 27 items having a theoretical maximum score of 32 points. The checklist scored 5 different domains: the quality of reporting (10 items, maximum 11 points), the external validity (3 items, maximum 3 points), internal validity —bias (7 items, maximum 7 points), internal validity—confounding (selection bias; 6 items, maximum 6 points), and power (1 item with maximum 5 points). A summary of the set criteria (20 for human and 13 for mammalian) for quality assessments that were used is displayed in the Supplementary Table 1. The quality evaluation procedure was done independently by two reviewers (JS and AS), as previously advised (Wright et al., 1995; Harris et al., 2013). The level of agreement was assessed with Cohen's kappa analysis on all items of the checklist. Landis and Koch's benchmark for assessing agreement ranges from almost perfect (0.81–1.0), substantial (0.61–0.8), moderate (0.41–0.6), fair (0.21–0.4), slight (0.0–0.2), and poor (<0; Landis and Koch, 1977). Disagreements were resolved by consensus or by consulting a third reviewer.

RESULTS

Study Selection

The database search resulted in a total number of 3129 studies. The selection process is illustrated in **Figure 2**. After the removal of duplicates ($n = 431$) and the screening process, 75 studies were left for full text reading. During full text reading, the cited human studies in the reference lists that perhaps would be relevant for the review were kept track leading to an additional 40 papers selected for full text reading. Finally, following full text reading, 67 articles were included in the systematic review. For the mammalian studies, the included studies were performed with rodents only.

Study Characteristics

Characteristics of the Rodent Studies

The total number of mice and rats in the 24 included studies was 1396 (median: 48 rodents per study, range 11–200). The studies examined C57BL/6 mice (Lee et al., 2002; Van der Borght et al., 2007; Kuhla et al., 2013), Wistar rats (Ito et al., 2009; Khabour et al., 2010, 2013; Jacotte-Simancas et al., 2013; Rachetti et al., 2013; Cechella et al., 2014a,b), Fischer 344 rats (Markowska, 1999), Long Evans rats (Young et al., 2007), Sprague-Dawley rats (Lee et al., 2000; Hansalik et al., 2006; Strasser et al., 2006; Wu et al., 2008; Chytrova et al., 2010; Gomez-Pinilla and Ying, 2010; Noble et al., 2014), F344xBN hybrid rats (Adams et al., 2008; Fitting et al., 2008; Carter et al., 2009), and BALB/cJ mice (Bhattacharya et al., 2015). At the beginning of the interventions, all rodents were disease free. The age of the rodents varied from a few months to a few years. The articles were studies that evaluated the effects of physical exercise (Van der Borght et al., 2007; Mustroph et al., 2012; Noble et al., 2014), of nutritional intervention (Markowska, 1999;

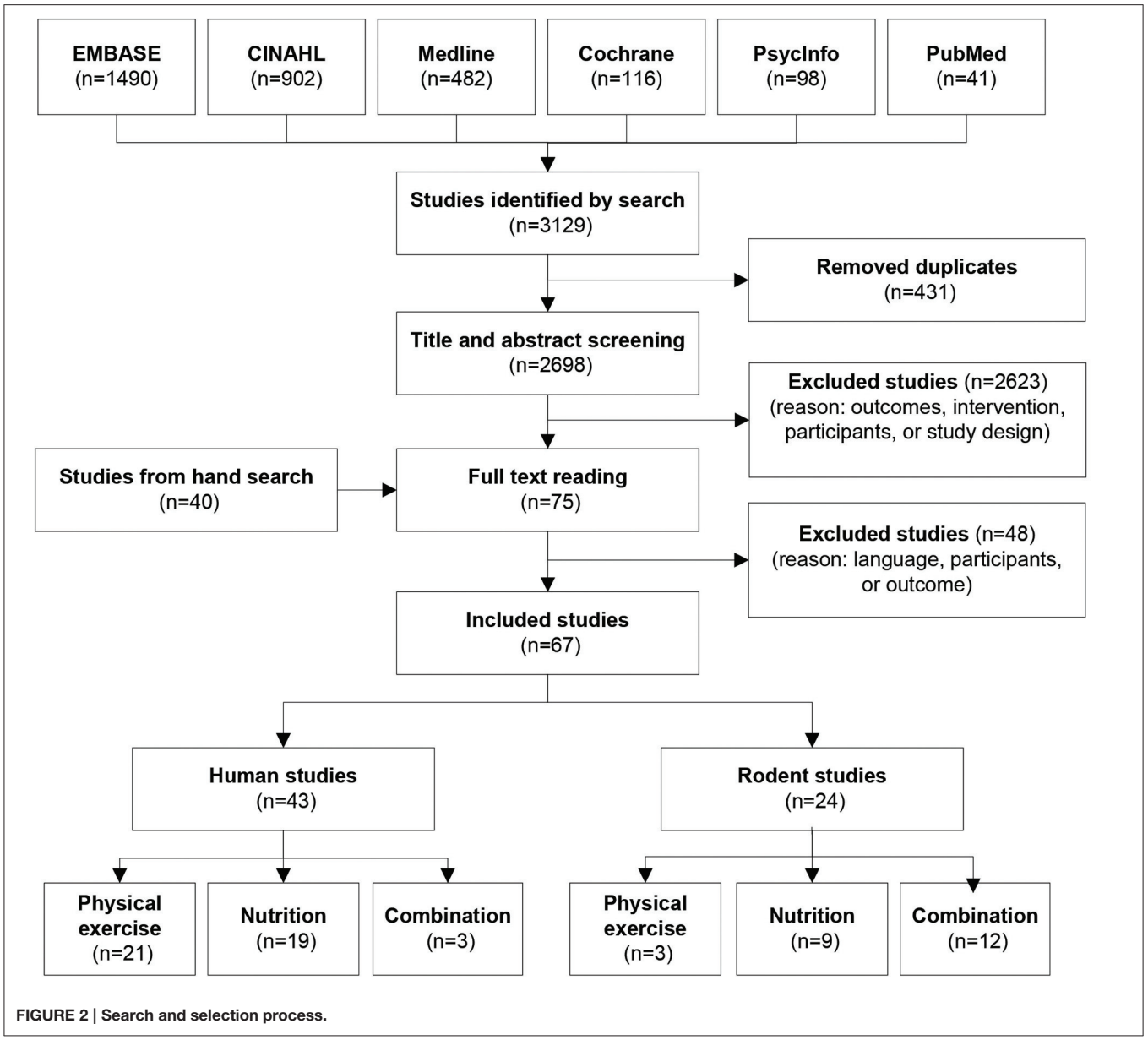

FIGURE 2 | Search and selection process.

Lee et al., 2000, 2002; Young et al., 2007; Adams et al., 2008; Fitting et al., 2008; Carter et al., 2009; Ito et al., 2009; Kuhla et al., 2013), or used a combination of physical exercise and nutritional intervention (Hansalik et al., 2006; Strasser et al., 2006; Wu et al., 2008; Chytrova et al., 2010; Gomez-Pinilla and Ying, 2010; Khabour et al., 2010; Jacotte-Simancas et al., 2013; Khabour et al., 2013; Rachetti et al., 2013; Cechella et al., 2014a,b; Bhattacharya et al., 2015). Physical exercise was always an aerobic type of exercise (running or swimming) performed five times per week for 20–60 min over a period of 2 weeks to 13 months, while nutritional interventions consisted of either dietary supplementation (taurin, niacin, amino acid, selenium, fatty acid, and epinephrine) or caloric restriction. Caloric restriction was included as a part of nutritional supplementation in the sense of nutritional depletion. A summary of measured outcomes in rodents is given in **Table 2**. The outcome categories that were assessed were behavior, neurogenesis, neurotrophins, synaptic proteins, cell signaling proteins, metabolic homeostasis proteins, and measures of oxidative stress.

Characteristics of the Human Studies

The total number of participants in the 43 included human subject studies was 19,757. This number includes the participants from large supplementation studies such as the Women's Health Initiative WHI ($n = 1420$), the Physicians Health Study II PHSII ($n = 5947$), the Women's Health Study WHS ($n = 6377$), and the Age-Related Eye Disease Study AREDS ($n = 2166$). The number of participants decreased to 3847 (median: 58 participants per study, range 12–910) without the aforementioned studies. At the time of recruitment, all participants were healthy elderly

TABLE 2 | Study outcomes measured in included rodent studies.

Category	Outcomes	Details
Behavior	Behavioral tests	• Measurement of learning, memory, motor skill, and anxiety like behavior
Neurogenesis	Ki-67 staining	• Cellular marker for proliferation
	Doublecortin staining	• Marker for neurogenesis
	Immunohistochemistry	• Determination of phenotype of newly generated cells ○ GFAP: astrocyte protein ○ Neuronal nuclear marker ○ MAP2a: mature neuron-specific protein
Neurotrophins	BDNF-, NT-3-, trkB-, and trkC- mRNAs	• BDNF and NT-3: neurotrophins • TrkB and trkC: their high-affinity receptors
Synaptic proteins	NMDA receptor subunits: NR1, NR2A, and NR2B	• Glutamate receptor • Important for synaptic plasticity and memory
	AMPA receptor subunits: GluR1 and GluR2	• Non-NMDA glutamate receptor • Involved in plasticity and synaptic transmission
	Synaptophysin	• Involved in synaptic transmission
	STX-1 and STX-3	• Plasma membrane syntaxins • Present in synaptic membranes and in neuronal growth cones
	GAP-43	• Growth associated protein
	Synapsin	• Involvement in neurotransmitter release, axonal elongation, and maintenance of synaptic contacts
Cell signaling	CaMKII	• Signaling system • Important in learning and memory
	CREB staining	• Cellular transcription factor • Involvement in learning and memory
	Akt protein determination	• Involvement in cell signaling (cell proliferation)
Metabolic homeostasis	Glucocorticoids receptor, 11-beta-HSD1, ghrelin receptor, leptin receptor, p-AMPK, and SIRT1	• Molecular systems that play dual roles on metabolism and synaptic plasticity
Oxidative stress	Amount of oxidized proteins	• Measurement of oxidative stress

11-beta-HSD, 11-beta-hydroxysteroid-dehydrogenase; AMPA, Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BDNF, Brain-derived neurotrophic factor; BrdU, Bromodeoxyuridine; CaMKII, Calmodulin-dependent protein kinase II; CREB, Cyclic adenosine monophosphate response element-binding protein; GAP-43, Growth associated protein 43; GFAP, Glial fibrillary acid protein; GluR 1/2, Glutamate receptor 1/2; MAP2a, Microtubule-associated protein 2a; mRNA, Messenger ribonucleic acid; NMDA, N-methyl-D-aspartate; NR1/2A/2B, N-methyl-D-aspartate receptor 1/2A/2B; NT-3, Neuriphin-3; p-AMPK, Phospho-adenosine monophosphate-activated protein kinase; SIRT1, Sirtuin 1; STX-1/-3, Syntaxin 1/3; trkB/C, Tropomyosin receptor kinase B/C.

living in a nursing home or at home and having a mean age around 65 years or higher. The studies evaluated the effects of physical exercise (Dustman et al., 1984; Blumenthal et al., 1991; Hill et al., 1993; Moul et al., 1995; Perrig-Chiello et al., 1998; Kramer et al., 1999; Baum et al., 2003; Bastone Ade and Jacob Filho, 2004; Colcombe et al., 2004, 2006; Ozkaya et al., 2005; Zlomanczuk et al., 2006; Cassilhas et al., 2007; Brown et al., 2009; Kamijo et al., 2009; Liu-Ambrose et al., 2010; Muscari et al., 2010; Erickson et al., 2011; Ruscheweyh et al., 2011; Voelcker-Rehage et al., 2011; Niemann et al., 2014), of nutritional intervention (Deijen et al., 1992; Smith A. et al., 1999; Smith A. P. et al., 1999; Cockle et al., 2000; Yaffe et al., 2004; Wolters et al., 2005; Kang et al., 2006; Grodstein et al., 2007; Malaguarnera et al., 2007; McMorris et al., 2007; McNeill et al., 2007; Summers et al., 2010; Presley et al., 2011; Macpherson et al., 2012; Rossom et al., 2012; Yasuno et al., 2012; Grodstein et al., 2013; Kelly et al., 2013; Szcześniak et al., 2014), or a combination of physical exercise and nutritional intervention (Cetin et al., 2010; Alves et al., 2013; van de Rest et al., 2014). Physical exercise in the human studies consisted of either strength or aerobic training that was usually done three times per week for 1 h over a period of 4–12 months. Nutritional intervention included (multi)vitamin supplementation, amino acids, nitrate enriched diets, creatine, fatty acid, or protein supplementation. Small nutritional supplementation studies were done over a period of several months, while large nutritional supplementation trials were done for several years. Many different outcomes have been measured in the human trials (**Table 3**). The focus of the majority of the articles was on cognitive tests. Briefly, cognitive tests were administered to the participants to assess general cognitive status, memory, executive function (EF), attention, intelligence, and sensorimotor performance. Measurements concerning the brain included brain volumes, brain activity, and metabolites concentrations in the brain. In addition, blood samples were used

TABLE 3 | Study outcomes measured in included human studies.

Category	Outcome	Details
Cognitive function	Cognitive tests	Tests for general cognitive functioning, memory, executive functions, intelligence, attention, and sensorimotor performance
Brain structure	Whole brain volume and regional brain volumes	MRI: voxel based morphometry
Brain activity	Electroencephalography: event-related potentials	During a cognitive task or a sensory stimulus (sensory evoked potential)
	Cerebral blood flow	Determined from MRI
	Apparent diffusion coefficients of white and gray matter	Acquired using an eight channel SENSE head coil
	Functional MRI: Cortical recruitment	Functional MRI during a cognitive task
Blood markers	Serum IGF-1, BDNF, dopamine, epinephrine and granulocyte colony-stimulating factor levels, and total antioxidant capacity	Blood samples (cephalic vein)
Metabolism	N-acetyl aspartate, creatine, choline, and myo-Inositol brain concentrations	^{1}H magnetic resonance spectroscopy

BDNF, Brain-derived neurotrophic factor; IGF-1, Insulin like growth factor 1; MRI, Magnetic resonance imaging.

to measure serum BDNF, IGF-1, and neurotransmitter/hormone levels.

Effects of Physical Exercise and Nutritional Intervention in Rodents

The detailed results of the rodent combinatory studies are listed in **Table 4**. The detailed results of the rodent studies examining physical exercise or nutritional intervention are listed in the Supplementary Table 4. Ten studies evaluated the effects of physical exercise and nutritional supplementation on behavioral tests (Hansalik et al., 2006; Wu et al., 2008; Chytrova et al., 2010; Khabour et al., 2010, 2013; Jacotte-Simancas et al., 2013; Rachetti et al., 2013; Cechella et al., 2014a,b; Bhattacharya et al., 2015). Three out of these ten studies found additive effects of physical exercise and nutritional supplementation on Morris Water Maze (MWM; Wu et al., 2008) and object recognition task (Cechella et al., 2014a,b). Cechella et al. (2014a,b) found the benefits in 24 months old rats but not in 12 months old rats. However, the other seven studies found no additive beneficial effects on cognitive performance (Hansalik et al., 2006; Chytrova et al., 2010; Khabour et al., 2010, 2013; Jacotte-Simancas et al., 2013; Rachetti et al., 2013; Bhattacharya et al., 2015). Chytrova et al. (2010) and Rachetti et al. (2013) found that both physical exercise and nutritional supplementation resulted in improved learning or memory. However, Jacotte-Simancas et al. (2013) and Khabour et al. (2010, 2013) found that only exercised groups improved spatial learning and memory. On the other hand, Hansalik et al. (2006) found no improvements in the MWM for any of the intervention groups.

Four articles assessed BDNF levels, three of them in the hippocampus (Wu et al., 2008; Khabour et al., 2010, 2013) and one in the cerebral parietotemporal cortex (Strasser et al., 2006). One study reported an additive effect (Wu et al., 2008), two studies found increased BDNF levels in the physical exercise group only (Khabour et al., 2010, 2013), and one study found no increased levels in any group. Moreover, two studies investigated the combinatory effect on synaptic proteins and both studies reported additive effects (Wu et al., 2008; Chytrova et al., 2010). In addition, three studies measured cyclic adenosine monophosphate response element-binding protein (CREB), Akt, or calmodulin-dependent protein kinase II (CaMKII) concentrations in the hippocampus (Wu et al., 2008; Cechella et al., 2014a,b). Wu et al. (2008) found that the combinatory intervention had higher cell signaling protein levels. However, Cechella et al. (2014a,b) measured increased CREB levels in the physical exercise or in the nutritional intervention group. Akt levels were only increased in the physical exercise group. Gomez-Pinilla and Ying found an inconsistent pattern of change in leptin and ghrelin receptor protein levels, phosphor-adenosine monophosphate-activated protein kinase, sirtuin 1, glucocorticoid receptor, and 11beta-hydroxysteroid dehydrogenase type 1 levels in the hypothalamus and in the hippocampus in rats (Gomez-Pinilla and Ying, 2010). At least, one study showed a positive effect of the combination group related to oxidative stress (decreased carbonyl levels; Wu et al., 2008).

Effects of Physical Exercise and Nutritional Intervention in Humans

The detailed results of the human studies that combined physical exercise with a nutritional intervention are listed in **Table 5**. The detailed results of the human studies examining physical exercise or nutritional intervention in isolation are listed in the Supplementary Table 5. Three studies evaluated the combined effects of physical exercise and nutritional supplements on cognitive functions (Cetin et al., 2010; Alves et al., 2013; van de Rest et al., 2014). None of these studies found additive effects of physical exercise and nutritional supplementation. Alves et al. investigated the effects of resistance training with creatine supplementation and found no change for any of the cognitive tests (Alves et al., 2013). Van de Rest et al. executed a similar protocol but with a protein drink (van de Rest et al., 2014). The results indicated that

TABLE 4 | Included rodent studies combining physical exercise and nutritional intervention.

Study	Subjects	Intervention	Groups	Outcome measure	Results
Bhattacharya et al., 2015	*N* = 91; male BALB/cJ mice Age: 10 weeks	Running wheel 1.49 mg of EGCG per g diet 3.34 mg of B-ALA per g diet	Exe, sed, B-ALA: exe or sed, EGCG: exe or sed, B-ALA + EGCG: exe or sed *N* = 11–12 per group	Fear conditioning (contextual and cued)	Exe increased duration of freezing (contextual) Exe approx. 4-fold greater duration of freezing behavior than sed (cued) No effect of diet or interaction between diet and exercise
				BrdU staining	Exe increased total number of BrdU+ cells in the granule layer of the denate gyrus approx. 4 fold over sed No effect of diet or interaction between diet and exercise
Cechella et al., 2014a	Male wistar rats	Swim training: 20 min, 5×/week 1 ppm of diphenyl diselenide (selenium) 5 weeks	Exe (I), selenium (II), exe + selenium (III), adult control (IV), aged control (V) *N* = 4–6 per groups	ORT	Short term memory: (I)+(II) improved compared to (V), (III) better than all other groups Long term memory: (II)+(III) better than control groups
				OLT	(II) better than aged control, (I) + (III) better than control groups
				CREB and Akt in hippocampus	pAkt/ Akt: same for (I)+(III)+(IV)+(V), (I) higher than control groups pCREB/ CREB: (I)+(II)+(III) better than aged control
Cechella et al., 2014b	*N* = 30; male wistar rats	Swim training: 20 min, 5×/week 1 ppm of diphenyl diselenide (selenium) 5 weeks	Exe (I), selenium (II), exe + selenium (III), adult control (IV), aged control (V) *N* = 4–6 per groups	ORT	Short term memory: (I)+(II)+(III) better than control groups, (III) shows the best results Long term memory: (I)+(II)+(III) better than aged control, (I) better than adult control
				OLT	(I)+(II)+(IIII) better than aged control
				CREB in hippocampus	pCREB/CREB: (I)+(II) better than control groups
Chytrova et al., 2010	*N* = 24; adult male Sprague-Dawley rats	Running wheel DHA enriched diet 12 days	RD + sed (I), DHA + sed (II), RD + exe (III), DHA + exe (IV) *N* = 6 per group	Synaptic proteins in hippocampus	NR2B: sig. increase for (II)+(III)+(IV), greatest effect for (IV) compared to (III) STX-3 and GAP-43: sig increase for (II)+(III)+(IV), greatest effect for (IV)
				MWM	Latency: (II)+(III)+(IV) decreased compared to (I)
Gomez-Pinilla and Ying, 2010	*N* = 24; male Sprague-Dawley rats	Running wheel DHA enriched diet 2 weeks	RD + sed (I), DHA + sed (II), RD + exe (III), DHA + exe (IV) *N* = 6 per group	Hip and Hyp dissection	Leptin: *Hyp*: increase for (II)+(III)+(IV), greatest for (IV), *Hip*: increase for (II) Ghrelin: *Hyp*: increase for (III), *Hip*: increase for (II)+(III) p-AMPK: *Hyp*: decrease for (II)+(III)+(IV), *Hip*: increase for (II)+(III)+(IV) SIRT: *Hyp*: increase for (II)+(III)+(IV), greatest for (IV), *Hip*: increase for (II)+(III)+(IV) Glucocorticoid receptor: *Hyp*: decrease for (II), increase for (III), *Hip*: increase for (II)+(III)+(IV) 11-beta-HSD1: *Hyp*: increase for (II), *Hip*: increase for (II)+(III)+(IV)
Hansalik et al., 2006	*N* = 200; male Sprague-Dawley rats	Running wheel Treadmill: 20 min, 5×/week CR 13 months	Baseline (age: 5 months) (I), exe (TM) (II), exe (RW) + CR (III), sed + CR (IV), sed1 (one rat, one cage) (V), sed4 (four rats, one cage) (VI)	MWM	Learning and short term memory: no effects comparing various intervention groups at age 10 and 18 months

(Continued)

TABLE 4 | Continued

Study	Subjects	Intervention	Groups	Outcome measure	Results
Jacotte-Simancas et al., 2013	*N* = 62; male wistar rats Age: 2 months	Running wheel Epinephrine: 0.01 or 0.05 mg/kg	Sed (I), sed + 0.01 ep (II), sed + 0.05 ep (III), exe (IV), exe + 0.01 ep (V), exe + 0.05 ep (VI)	Barnes maze	Distance: (IV)+(V)+(VI) sig. shorter than (I)+(II)+(III) Latency: no sig. results
Khabour et al., 2010	Male wistar rats Age: 5 months	Voluntary exercise CR 6 weeks	Sed (I), CR (II), exe (III), exe + CR (IV) *N* = 10–13 per group	RAWM Hippocampal BDNF	Spatial learning and memory formation: (IV)+(III) enhanced compared to (I)+(II), no effect of CR BDNF: (IV)+(III) sig. higher levels compared to (I)+(II), no effect of CR
Khabour et al., 2013	*N* = 92; young wistar male rats	Swimming: 60 min, 5×/week CR 6 weeks	Sed (I), CR (II), exe (III), exe + CR (IV) *N* = 15 per group	RAWM Hippocampal BDNF	Spatial learning and memory formation: (IV)+(III) enhanced learning/memory compared to (I)+(II), no effect of CR (IV)+(III) sig. higher levels compared to (I)+(II) no effect of CR
Rachetti et al., 2013	*N* = 45; adult wistar rats	Treadmill: 30 min, 5×/week (until age of 27 days) Fish oil capsules Length: prenatal to 10 months	Exe (I), exe + fish (II), control (III), control + fish (VI) *N* = 11–12 per group	Open field test ORT Plus maze discriminative avoidance task	(VI) decrement in location during 2nd exposure compared to the other groups *Test session*: all groups explored sig. more the novel object compared to familiar object, *Re-test session*: (II) explored sig. more the novel object compared to familiar object (I)+(II) discriminated the aversive from non-aversive arms and spent sig. less time in aversive arm
Strasser et al., 2006	Male Sprague-Dawley rats	Running wheel Treadmill: 20 min, 5×/week CR 13 months	Baseline (age: 5 months) (I), exe (TM) (II), exe (RW) + CR (III), sed + CR (IV), sed1 (one rat, one cage) (V), sed4 (four rats, one cage) (VI)	BDNF in parietotemporal cortex	Decrease for (V), increase for (VI), highest values for (VI)
Wu et al., 2008	*N* = 24; Sprague-Dawley rats	Running wheel DHA enriched diet 12 days	RD + sed (I), DHA + sed (II), RD + exe (III), DHA + exe (IV)	MWM BDNF, Synapsin I, CREB, Akt, CaMKII Oxidative proteins	Latency: (II)+(III)+(IV) shorter than (I), (IV) shorter than (II)+(III) (II)+(III) increased values and even more in (IV) (II)+(III) reduced oxidized protein and even more in (IV)

The studies are reported by subjects, intervention, groups, outcome measure, and results. 11betaHSD1, 11-beta-hydroxysteroid-dehydrogenase type 1; B-ALA, Beta-alanine; BDNF, Brain-derived neurotrophic factor; BrdU, Bromodeoxyuridine; CaMKII, Calmodulin-dependent protein kinase II; CR, Calorie restriction; CREB, Cyclic adenosine monophosphate response element-binding protein; DHA, Docosahexaenoic acid; EGCG, Epigallocatechin gallate; Ep, Epinephrine; Exe, Exercise; GAP-43, Growth associated protein 43; Hip, Hippocampus; Hyp, Hypothalamus; MWM, Morris Water Maze; NR2B, N-methyl-D-aspartate Receptor 2B; OLT, Object Location Test; ORT, Object Recognition Test; p-AMPK, Phospho-adenosine monophosphate-activated protein kinase; pCREB, Phosphorylated cyclic adenosine monophosphate response element-binding protein; RAWM, Radial Arm Water Maze; RD, Restricted diet; RW, Running wheel; Sed, Sedentary; SIRT, Sirtuin; STX, Syntaxins; TM, Treadmill.

physical exercise in combination with protein and the group performing physical exercise only showed improvements in different cognitive domains. No interaction effect was found between the treatments. Cetin et al. looked at the effects of endurance exercise with vitamin E supplementation (Cetin et al., 2010). They found positive effects for the exercise group and no effect for the vitamin E supplementation for electroencephalography recordings. In addition, they found no changes in the antioxidant capacity for any intervention group (Cetin et al., 2010).

Quality Evaluation

The agreement on study quality criteria between the two reviewers was substantial with an estimated Kappa value of 0.65 (95% confidence interval between 0.63 and 0.67). The percentage of agreement between the two raters was 85.15% for the human and rodent studies. The results of the physical exercise, nutrition, and combination intervention of human and rodent studies are summarized in the Supplementary Tables 2, 3.

Rodent Studies

None of the 24 studies reached the maximum possible score of 13 points. The quality scores ranged from a minimum of 9 points to a maximum of 12. The mean quality score was 10.29 points (range: 9–12 points), the median value was 10.5 points and the mode was 11 points. The mean score for reporting was 5.04 points (maximum: 6 points; range: 4–6 points), for internal validity (bias) 4.08 points (maximum: 5 points; range: 4–5 points), for internal validity (bias) 0.58 (maximum: 1 points; range: 0–1 points), and for power 0.58 (maximum: 1 points; range: 0–1 points). The mean score for physical exercise studies was 11 points (maximum: 12 points; range: 10–12 points), for nutritional supplementation studies was 9.77 points (maximum: 11 points; range: 9–11 points), and for combination studies was 10.5 points (maximum: 12; range: 9–12 points).

Human Studies

One study from the total 43 studies reached the maximum possible score of 21 points (Rossom et al., 2012). The average score was 14.12 points ranging from a minimum of 6 to a maximum of 21 points. The median value was 14 points and the mode was 15 points. The mean score for reporting was 7.21 points (maximum: 9 points; range: 3–9 points), for external validity 0.65 points (maximum: 2; range: 0–2 points), for internal validity (bias) 4.02 (maximum: 5 points; range: 2–5 points), for internal validity (confounding) 1.88 (maximum: 4 points; range: 0–4 points), and for power 0.35 (maximum: 1 points; range: 0–1 points). The mean score for physical exercise studies was 13.29 points (maximum: 20 points; range: 6–20 points), for nutritional supplementation studies was 14.68 points (maximum: 21 points; range: 10–21 points), and for combination studies was 16.33 points (maximum: 19; range: 15–19 points).

DISCUSSION

Summary

The aim of this systematic review was to evaluate whether the combination of physical exercise and nutritional supplementation has greater benefits (additive effects) on brain structure and function than their separate administrations. Studies measured cognitive functioning with the help of behavioral tests and associated parameters including metabolic and structural neuronal changes on brain level. In human trials, the combination of physical exercise and nutritional supplementation did not lead to any additive effects. In rodents, four studies showed additive effects on different outcomes (Wu et al., 2008; Chytrova et al., 2010; Cechella et al., 2014a,b). Wu et al. showed additive effects on behavioral level, on BDNF level, on synaptic protein levels, and on oxidative stress using a combination of running exercise and docosahexaenoic acid (DHA) supplementation (Wu et al., 2008). Moreover, Chytrova et al. (2010) found additive effects on synaptic protein levels using a combination of running and DHA supplementation. Cechella et al. found additive effects on behavioral level using a combination of swim training and selenium (Cechella et al., 2014a,b).

The search strategy led to the identification of many articles with different brain function related outcomes. However, the studies that used a combination approach had a poor-moderate quality and were rather heterogenic which, in turn, led to difficulties comparing the results and performing a meta-analysis. The limited availability of high-quality prospective studies that used a combined approach warrants further targeted future research investigating the effects of combined approaches on the brain. Based on our findings we will discuss and suggest directions for future research related to combined interventions with physical exercise and nutritional supplements. Through this review it became apparent that isolated interventions of either physical exercise or nutrition were able to effect on brain in both mammals and humans. However, combinations of these components were not having an effect. It appears that many of the included studies have been using a "complementary" approach where the administration of physical exercise and nutritional components were not combined with the intention to cause an effect on similar mechanisms. No studies explicitly used an "additive" approach with the aim to enhance effects of the combined physical-exercise-&-nutrition-approach because both components shared similar mechanisms. For elucidating this seemingly contradiction we continue this discussion by considering the effects of studies applying either physical exercise or nutritional supplementations with the aim to effect on brain. Practices of various interventions are discussed together with the underlying mechanisms that theoretically would explain the effects of the used intervention components.

Physical Exercise Interventions

Results from the three studies that looked at the effects of physical exercise on cognition in rodents showed better performances in learning and memory abilities. This finding is in agreement with existing literature reviews suggesting that physical exercise

TABLE 5 | Included human studies combining physical exercise and nutritional intervention.

Study	Subjects	Intervention	Groups	Outcomes	Results
Alves et al., 2013	*N* = 56; women Age range: 60–80 years	Strength exercise 3 sets for 7 exercise, 2× week Creatine: 5 g/day 24 weeks	Creatine (I), exe (II), creatine + exe (III), non creatine + non exercise (IV) *N* = 14 per group	MMSE, Stroop test, TMT, Digit span test, delay recall test	No sig. diff. for any of the variables
Cetin et al., 2010	*N* = 57; sedentary Age range: 69.6–73.1 years	Aerobic exercise 90 min, 3× week Vitamin E 6 months	Exe (I), vitamin (II), exe + vitamin (III), non exe + non vitamin (IV) *N* = 14–15 per group	EEG (auditory oddball paradigm)	P3 amplitude: no diff. P3 latency: (I), (II), (III) shorter latency compared to Pre-treatment and (I)+(II) shorter latency compared to control
				Plasma total antioxidant capacity	No diff. to control group or within a group after 6 months
van de Rest et al., 2014	*N* = 127; frail and Pre-frail Mean age: 79 ± 8 years	Strength exercise 2× week Protein shake: twice daily 24 weeks	Exe + protein (I), exe + placebo (II), non exe+ protein (III), non exe + placebo (IV) *N* = 62 (exe), 65 (non-exe)	Word learning test, Digit Span Task, TMT A&B, Stroop Color-Word Test, Verbal Fluency Test Finger Pre-cuing task	(I) vs. (III): improvement: information processing speed (II) vs. (IV): improvement: attention, working memory Reaction time: improved over time in all groups
				Interaction effects exe-protein	No sig. interaction on any of the cognitive domains

The studies are reported by subjects, intervention, groups, outcome measure, and results. EEG, Electroencephalography; Exe, Exercise; MMSE, Mini Mental Status Examination; TMT A/B, Trail Making Test A/B.

in rodents benefits cognitive functioning (van Praag, 2009; Gomez-Pinilla and Hillman, 2013). Interestingly, other studies showed that the physical exercise component, especially forced physical exercise, is able to activate neuronal brain metabolism (O'Callaghan et al., 2007; Kinni et al., 2011). Furthermore, providing opportunities for physical exercise is the critical element of environmental enrichment explaining the influence on neurogenesis (Kobilo et al., 2011). The mechanisms by which physical exercise improves cognition are, however, not yet fully understood. In the last couple of years, research seems to support the idea that physical exercise affects cellular and molecular systems associated with synaptic plasticity and energy metabolism (Gomez-Pinilla and Hillman, 2013). BDNF plays an essential role through the interaction with energy metabolism and growth factors (IGF-1) to influence downstream effectors mediating synaptic plasticity and neurogenesis, especially in the hippocampus (Gomez-Pinilla and Hillman, 2013). The hippocampus is an important area for learning and memory (Gomez-Pinilla and Hillman, 2013). In this systematic review, two studies found increased neurogenesis in the dentate gyrus of running mice (Van der Borght et al., 2007; Mustroph et al., 2012). In the hippocampus of running mice, Van der Borght et al. (2007) found increased phosphorylated CREB levels, a downstream effector of BDNF (Gómez-Pinilla, 2011).

In humans, aerobic exercise improved general cognitive functioning, EF, perceptual speed, and to some extent also memory. A meta-analytic review by Smith et al. found that aerobic exercise led to modest improvements in attention and processing speed, EF, and memory but did not affect working memory (Smith et al., 2010). In contrast, a systematic review by van Uffelen et al. found only weak evidence for better cognition after physical exercise; only 5 out of 15 studies showed significant improvements on some measures of cognition (van Uffelen et al., 2008). In addition, a meta-analysis by Colcombe et al. investigated the effects of fitness interventions on cognitive functions (Colcombe and Kramer, 2003). They showed that the largest benefits of physical exercise appear to be on EF. Hence, results from this systematic review and other reviews seem to support the idea that physical exercise has the most reliable effects on EF. Moreover, one might speculate that improvements in tests measuring general cognitive functioning might have been originated from improvements in subtests assessing EF. Although we do not know the sub-scores of the Mini Mental State Examination (MMSE), the study by Moul et al. using the Ross Information Processing Assessment to assess general cognitive functioning supports this hypothesis (Moul et al., 1995). The improved total score resulted from improvements in two attentional demanding tasks (organization and auditory processing). Differentiating the types of physical exercise, three studies found positive effects on memory examined following strength training. Two studies used strength training (Perrig-Chiello et al., 1998; Cassilhas et al., 2007) and one study combined strength training with endurance exercise (Zlomanczuk et al., 2006). Moreover, resistance training improved general cognitive functioning (Baum et al., 2003) and EF (Cassilhas et al., 2007; Liu-Ambrose et al., 2010). A review by Chang et al. on resistance exercise and cognition suggests that resistance exercise improves cognitive functions including information processing speed, attention, memory formation, and EF (Chang et al., 2012). Interestingly, participants who underwent coordination training improved in perceptual speed tasks (Voelcker-Rehage et al., 2011; Niemann et al., 2014).

The evaluation of neurogenesis and brain growth factors concentration is limited in human studies. In the studies included in our systematic review, the authors used magnetic resonance imaging techniques to assess brain volumes. Erickson et al. found that aerobic type of exercise was able to increase the hippocampus volume (Erickson et al., 2011). The results support the idea that physical exercise induces neurogenesis in the hippocampus. Furthermore, positive effects of aerobic type of exercise on brain volumes were found in the anterior cingulate cortex, the supplementary motor area, the right inferior frontal gyrus, the left superior temporal gyrus, and the anterior white matter (Colcombe et al., 2006; Ruscheweyh et al., 2011). The brain regions have been associated with critical cognitive processes (prefrontal cortex) and memory (temporal lobes; Colcombe et al., 2006). Moreover, the prefrontal cortex is considered to play an important role in EF (Funahashi, 2001) and has been shown to be susceptible to aging (Kamijo et al., 2009). In fact, Raz et al. argue that the brain regions that are late to mature (i.e., frontal regions) are also the most vulnerable to cognitive decline (Raz et al., 2005). In addition, cognitive aging seems to affect mainly tasks that require substantial mental effort and novel stimuli, such as EF, whereas semantic knowledge appears to be well preserved (Gunning-Dixon et al., 2009). On the other hand, strength training decreased whole brain volume (Liu-Ambrose et al., 2010). This finding seems paradoxical, since decreased brain volume is usually associated with impaired function (Carlson et al., 2008). However, Liu-Ambrose and colleagues concluded that this phenomenon needs to be further investigated (Liu-Ambrose et al., 2010). Interestingly, participants who underwent coordination training increased globus pallidus and caudate volumes (Voelcker-Rehage et al., 2011; Niemann et al., 2014). The two sub-regions of the basal ganglia are involved in prefrontal cognitive processes such as planning and working memory (Middleton and Strick, 1994). Since the dorsal part of basal ganglia is involved in motor learning (Niemann et al., 2014), it is not surprising that coordination training affected this area of the brain. This fact would also support the improvement in the perceptual speed task.

In an event-related potential study, latency of the P3 component has been attributed to information processing speed, attention, and working memory (Kügler et al., 1993). Ozkaya et al. found better early sensory processing for the strength but not for their endurance training group (Ozkaya et al., 2005). Kamijo et al. investigated the effects of one bout of aerobic exercise and found improved P3 latencies (Kamijo et al., 2009). Two studies used functional magnetic resonance imaging to evaluate brain activation during a flanker task (Colcombe et al., 2004; Voelcker-Rehage et al., 2011), and they showed greater activity in attentional control areas and reduced activity in the anterior cingulate cortex (ACC) that could be attributed to more efficient information processing (Voelcker-Rehage et al., 2011). Colcombe et al. argued that the successful completion of the incongruent flanker task requires activation of the frontal and parietal circuitry involved in spatial attention and a decreased activation of the ACC involved in response conflict (Colcombe et al., 2004).

With respect to blood markers, no increased serum BDNF or catecholamine levels were found after aerobic exercise. Erickson et al. found no significant changes in BDNF levels, but they found that changes in BDNF levels correlate with changes in hippocampal volume (Erickson et al., 2011). However, Ruscheweyh et al. also found no significant increase in BDNF levels after physical exercise (Ruscheweyh et al., 2011). The authors argued that the absence of increased BDNF levels in the blood could be due to two reasons: [1] levels could tailor off after approximately 1 month of training, [2] or cerebral BDNF levels might have been a better measure than blood levels to measure the impact of physical exercise on this parameter, although BDNF has been shown to pass the blood brain barrier. However, one study showed that resistance training for 6 months increased serum IGF-1 levels (Cassilhas et al., 2007), and this increase correlated with cognitive performance. The results support the hypothesis that IGF-1 plays an important role in cognition. Thus, more studies are needed to clarify the effect of aerobic exercise and resistance exercise on serum IGF-1 levels and on BDNF levels, respectively.

Overall, the results suggest that different types of physical exercise affect different cognitive domains through different mechanism. This is in line with previous research showing differing effects in the brain based on different exercise approaches. Where aerobic training increases activation in the sensorimotor network, coordination training leads to a higher activation of the visuospatial network (Voelcker-Rehage et al., 2011), and strength training has the potential to change the hemodynamic activity of brain regions associated with response inhibition processes (Liu-Ambrose et al., 2012). This is an indication that the types of physical training are likely to have task specific effects on the brain. Hence, combining aerobic exercise, strength training, and coordination training might be more beneficial for cognitive functioning than performing just one type of physical exercise. This kind of combined intervention was also suggested by Kramer et al. (1999). A recent study indeed demonstrated that a multicomponent simultaneous cognitive-physical training program was able to boost particularly EFs (including shifting attention and working memory) in healthy older adults compared to an exclusively physical multicomponent program (Eggenberger et al., 2015a), and that depending on the type of cognitive-physical training program applied differential training specific adaptations in brain function related walking parameters may be observed (Eggenberger et al., 2015b). However, it seems important that the principles of exercise training are consistently followed and accurately reported for physical exercise interventions (Ammann et al., 2014). Application of physical exercise principles (specificity, overload, progression, initial values, reversibility, and diminishing returns) ensures that the dose and type of physical exercise is planned to maximize the benefits for the recipients (Ammann et al., 2014). Such information was difficult to derive from the majority of studies included in this systematic review. It can be hypothesized that the lack of effect of physical exercise on the brain in some of the reports is partly due to not considering the quantity and quality of the exercise needed to trigger responses in the brain.

Nutritional Supplementation

In rodent studies, four out of five studies showed positive effects on learning, and only one study showed positive effects on memory using CR. However, these results have to be interpreted with caution. First of all, CR has also been shown to have negative effects on cognition. CR impairs memory assessed with object recognition (Carter et al., 2009) and increases anxiety like behavior, probably due to increased cortisol levels (Kuhla et al., 2013). Moreover, the study of Carter et al. evaluated whether the beneficial effects of CR are attributed to increased physical activity (Carter et al., 2009). They showed that CR rats had significantly higher activity levels than ad *libitum* (AL) fed rats. Moreover, the distance to reach the platform in the MWM task, which is not confounded by fitness, was the same in AL and CR rats. However, Kuhla et al. found improvements in spatial learning and working memory in CR rats that moved less than AL rats (Kuhla et al., 2013). Hence, the question whether CR has beneficial effects on cognition remains controversial. CR has often been used as an intervention because excess calorie intake might reduce synaptic plasticity through increased oxidative stress and subsequent cell damage (Gómez-Pinilla, 2008). In mice and rats, Lee et al. found that CR enhances neurogenesis by increasing survival of newly generated cells but not proliferation (Lee et al., 2000, 2002). This finding is interesting because physical exercise and CR appear to control different mechanisms; physical exercise increased newly generated cells in the hippocampus whereas CR promoted survival of cells in the hippocampus. The hypothesis that physical exercise is the strongest neurogenic stimulus is also supported by a review by van Praag on exercise and the brain (van Praag, 2009). If physical exercise and nutritional supplementation act differently on neurogenesis, their combined effects on neurogenesis could provoke additive results. However, a study by van Praag et al. showed that running increased both cell proliferation and survival in the hippocampus of mice (van Praag et al., 1999). Furthermore, the confounding effect of physical activity in CR mice or rats cannot be fully excluded (Lee et al., 2002). In addition to neurogenesis, Lee et al. showed increased BDNF (Lee et al., 2000, 2002) and NT-3 (Lee et al., 2002) levels in the hippocampus after CR (Lee et al., 2002). They argued that this might mediate the positive effects of CR on neurogenesis (Lee et al., 2002). These findings are in line with a review by Gomez-Pinilla on the effects of nutrients on brain function and a study by Duan et al. on CR (Duan et al., 2001; Gómez-Pinilla, 2008). Both studies suggested that CR increases BDNF levels and that this might mediate the effects on synaptic plasticity. Moreover, the authors illustrated that CR in rats was able to stabilize the decrease in key synaptic protein levels occurring with age. Again, these synaptic proteins are thought to be associated with synaptic plasticity in the hippocampus (Adams et al., 2008).

Studies that evaluated nutritional supplements showed no benefits for learning or memory using taurine or niacin (Young et al., 2007; Ito et al., 2009). For example, Young et al. showed that niacin supplementation worsened spatial learning ability, probably due to increased brain nicotinamide adenine dinucleotide and cyclic adenosine diphosphate ribose levels that facilitate long term depression and impair long term potentiation (Young et al., 2007). In addition, epinephrine supplementation did not lead to significant improvements in learning and memory (Jacotte-Simancas et al., 2013). On the other hand, DHA and diphenyl diselenide supplementation resulted in improved learning and memory (Wu et al., 2008; Chytrova et al., 2010; Gomez-Pinilla and Ying, 2010; Rachetti et al., 2013; Cechella et al., 2014a,b). The finding is in agreement with a review by Su that illustrated the positive effects of DHA on learning and memory performance in rodents (Su, 2010). Results from this systematic review suggest that the effects of the diphenyl diselenide supplementation on learning and memory involve CREB phosphorylation without altering the levels of Akt (Cechella et al., 2014b).

In humans, vitamin and multivitamin supplementation did not seem to positively affect scores of cognitive tests. No evidence was found for EF, processing speed, attention, or intelligence. A systematic review and meta-analysis by Grima et al. on the effects of multivitamin supplementation on cognitive performance revealed minimal benefits after vitamin supplementation (Grima et al., 2012). They showed that only immediate free recall memory seemed to profit from vitamin supplementation but not the other cognitive domains. On the other hand, cross sectional studies show associations between vitamin status and cognitive functioning. For example, a recent systematic review showed that low vitamin D status is associated with lower outcomes in cognitive tests (van der Schaft et al., 2013). In addition, Cockle et al. list many other studies that showed associations between vitamin status and cognitive functioning, for example vitamin B12 and memory or folate and spatial copying ability (Cockle et al., 2000). Smith et al. found no improvements in cognitive functions after multivitamin supplementation. However, a subgroup analysis revealed that individuals with low baseline levels of vitamin C improved in cognition after the supplementation (Smith A. P. et al., 1999). We think that the discrepancy between cross sectional and interventional studies arises because studies in our systematic review investigated the possible causal effects of supplementation in healthy individuals without known vitamin deficiencies.

The other supplementation studies showed different effects on cognitive functioning in humans. L-carnitine or anserine plus carnosine improved MMSE scores. It is noteworthy that improvements were only seen in very old people. For example, Malaguarnera et al. (2007) investigated the effects of L-carnitine on centenarians and Szcześniak et al. (2014) found improved MMSE scores after anserine and carnosine supplementation only in people aged 81–94 but not in those aged 65–80 (Malaguarnera et al., 2007; Chytrova et al., 2010). In addition, participants from the study by Malaguarnera et al. (2007) had very low baseline scores of the MMSE, averaging 16.5 points. Furthermore, fish oil together with lycopene and gingko biloba improved general cognitive functioning, memory, and processing speed and attention (Yasuno et al., 2012). Other studies that investigated the effects of n-3 polyunsaturated fatty acid on cognitive functions found no positive effects on cognition (Rogers et al., 2008; Quinn et al., 2010). McMorris et al. found that creatine supplementation improved memory scores, but the study quality

was rather low (McMorris et al., 2007). A higher ranked study (15 points) combined creatine with resistance exercise and reported no improvement in memory, EF, or MMSE in the creatine group (Alves et al., 2013). In addition, NO_3 supplementation showed no beneficial effects on cognition (Kelly et al., 2013). The inconsistent results of this systematic review limit the strength of the evidence that supports the intake of supplements on cognition.

Our search strategy detected no studies that evaluated the effects of nutritional supplementation on brain volumes or neurotrophin blood levels in humans due to the fact that the proposed mechanisms of supplements, especially of vitamin supplementation, usually involve antioxidant properties. Antioxidant foods have been claimed to favor cognition because of the susceptibility of the brain to oxidative damage (Gómez-Pinilla, 2008). However, in our systematic review few antioxidant supplements had positive effects on cognition. Reasons could be that participants were too healthy (Cockle et al., 2000; Kelly et al., 2013), In addition, antioxidant supplementation might protect against the deleterious effects of diets rich in saturated fats and sugars which have been shown to increase oxidative stress and decrease hippocampal BDNF levels (Gómez-Pinilla, 2011). Overall, results from vitamin studies seem to support the idea that vitamin supplementation is beneficial for cognition only in participants with low baseline vitamin status.

Two studies hypothesized that nitrate would be converted to nitric oxide that results in a vasodilation and consequently increases blood flow to the brain (Presley et al., 2011; Kelly et al., 2013). However, Kelly et al. found no positive effect on any of the measured outcomes (cognitive tests, apparent diffusion coefficients, and brain metabolite concentrations; Kelly et al., 2013). Furthermore, Presley et al. found no differences in global perfusion (Presley et al., 2011). Both studies used very short intervention periods of two to two-and-a-half days. Hence, studies with longer NO_3 supplementation periods seem necessary to evaluate long term effects on brain perfusion and cognition (Kelly et al., 2013).

The search strategy did not yield studies that investigated the effects of CR in elderly humans. However, hand searching yielded a study that showed beneficial effects of CR (30% reduction) on memory performance in healthy elderly (Witte et al., 2009). Higher synaptic plasticity and stimulation of neurofacilitatory pathways might be due to improved insulin sensitivity and reduced inflammatory activity.

Combination of Physical Exercise and Nutritional Supplementation

In rodent studies, DHA or diphenyl diselenide in combination with physical exercise evoked additive effects (Wu et al., 2008; Chytrova et al., 2010; Cechella et al., 2014a,b). DHA is a dietary omega-3 fatty acid and has the potential to affect synaptic plasticity and cognition (Gómez-Pinilla, 2008, 2011). A review on brain foods described why DHA is important for cognition and brain heath: DHA constitutes more than 30% of phospholipids of plasma membranes of neurons, and thus plays a crucial role for synaptic function (Gómez-Pinilla, 2008). More importantly, DHA can affect molecules such as BDNF and IGF-1 which in turn can activate signaling systems such as mitogen-activated protein kinase, CaMKII, and phosphoinositide 3-kinase/Akt/mammalian target of rapamycin (Gómez-Pinilla, 2008). Therefore, DHA seems to have the potential to facilitate synaptic transmission, to modulate synaptic plasticity and cognitive function, and to support long term potentiation which is associated with learning and memory (Gómez-Pinilla, 2008). Interestingly, the effects of physical exercise seem to depend on similar mechanisms involving BDNF mediated synaptic plasticity and energy homeostasis (Gómez-Pinilla, 2011). Thus, DHA supplementation could complement the actions of physical exercise resulting in an effective strategy to counteract cognitive decline (Gómez-Pinilla, 2011). In both studies, the combination had greater effects on hippocampal BDNF levels, synaptic protein and signaling molecules levels, on proteins involved in metabolic homeostasis, and on oxidative stress (Wu et al., 2008; Chytrova et al., 2010). However, the positive effects were only seen in two studies (Wu et al., 2008; Chytrova et al., 2010) which used an identical design: 12 days of 1.25% DHA supplementation with or without free access to a running wheel on 24 Sprague-Dawley rats. Both studies using diphenyl diselenide and swimming expected increased CREB level as mediator for improved memory (Cechella et al., 2014a,b). As the CREB levels did not change, the question about the underlying neurobiological mechanism remains to be elucidated.

In humans, none of the three studies that combined physical exercise and nutritional supplementation showed additive effects (Cetin et al., 2010; Alves et al., 2013; van de Rest et al., 2014). The reason for no additive effects might be that the combination of intervention components was not explicitly selected based on a shared mechanism and, therefore, evoked complementary effects at best. This means that the chosen single components (physical exercise or nutritional intervention) of the combined administration act not on the same neurobiological cascade to produce additive effects.

To evoke possible additive effects, three items seemingly should be taken into account: [1] training principles to ensure quality and quantity of the exercise component, [2] dose and duration of diet or nutritional supplementation, and [3] the selected nutritional component(s) and physical exercise should act on the same neurobiological cascade. The results can be interpreted in the sense that so far there seems to be a mismatch in many studies between the exercise program offered and the nutritional supplements given. It seems reasonable to assume that the nutritional supplements should be selected based on the theoretical effect they have on the brain; e.g., they preferably should share similar mechanisms with exercise (Gómez-Pinilla, 2011) and, thus, theoretically have the potential to complement the action of exercise. Possible additive effects might be achieved, if both components (physical exercise and nutrition) act complementary on the same molecular mechanisms. Other influencing factors are the genetic component and the living environment that are very individual in humans, but more or less identical in rodents. In humans, the individual genetic variability influences individual response to nutritional intervention (Dauncey, 2015).

Strengths and Limitations

Review

The standards of reporting animal experiments lag behind those of human RCTs (Muhlhausler et al., 2013), which is a potential concern for bias. Furthermore, publication bias related to overstatement of efficacy may negatively affect the interpretation of animal studies (Sena et al., 2010). A further limitation relates to the focus on older animals and humans. The precise correlation between the age of rodents and humans is subject of debate implying, when age is an important factor, differences between animals and humans should be taken into consideration (Sengupta, 2013).

For the human trials, the majority of included studies resulted from the reference list search. Hence, we cannot guarantee that all studies examining nutritional supplementation and physical exercise on cognition in healthy elderly are included in this systematic review. Moreover, we performed no gray literature search, and thus cannot guarantee that there was no publication bias. Another limitation is that the included studies are very heterogeneous regarding included participants, interventional design, and outcomes. This heterogeneity hindered a meta-analytical approach which would have been a more objective way to quantify the results.

Individual Studies

Generally, the included studies in this systematic review were of good quality. In the quality evaluation of rodent studies, the question assessing power had a low average score because it was often not possible to be determined (the results were displayed in graphs and not tabulated). Question 25 addressing confounding averaged only 0.58 points due to insufficient information regarding the number of rodents that were used for the analysis. The small differences in scores between studies might be explained through the very similar study designs. However, in human studies quality scores varied much more, probably due to heterogeneous study designs. Moreover, it is important to keep in mind that quality scores rely upon the quality of reporting rather than the quality of the actual study conduct (Harris et al., 2013).

Furthermore, the number of cognitive tests used in human studies is huge, and almost every study used different tests. Moreover, it was common that some authors used the same specific test but evaluated different cognitive domains. In other words, no standardized way is present to evaluate cognitive functioning. In addition, it can be argued that the applied tests were not always appropriate causing a suspected misfit between the targets of the intervention and the used (un)specific outcome tests. For example, many authors used the MMSE to evaluate general cognitive functioning. However, the MMSE is a diagnostic tool not designed to measure change or improvement and might, therefore, suffer from ceiling effects (Summers et al., 2010). In animals, the MWM was often used to evaluate learning and memory. However, Fitting et al. suggested that the improvements in latency after CR were due to preservation of motor function and not due to cognition (Fitting et al., 2008). In addition, Jacotte-Simancas et al. argued that exercised animals might perform better than sedentary animals in the MWM because they cope better with the physical effort and stress generated by the task (Jacotte-Simancas et al., 2013). Hence, motor fitness could be a confounding factor for cognitive performance evaluation in the MWM. An additional possible limitation of the studies with rodents relates to the gender distribution of the investigated animals. The fast majority of the animal studies identified through the systematic literature search were carried out on male animals only. From human studies we know that some brain related impairments affect women more than men; e.g., sex disparity in stroke prevalence persists with women being more affected then men (Towfighi et al., 2011). It seems important that future studies test interventions in both sexes.

Conclusion

In healthy elderly humans, no additive effects were identified for nutritional supplementation and physical exercise. In rodents, DHA and physical exercise or selenium and physical exercise resulted in additive effects on learning and on neurobiological measures. The main interventions that resulted in improved cognition or associated parameters were aerobic type of exercise, strength training, coordination training, CR, and DHA supplementation. It can, thus, be speculated that a combination of these interventions might provide better cognitive outcomes than just their sole administration. More research is needed examining the possible additive effects of physical exercise and nutritional intervention in humans. This systematic review reveals that applications of targeted exercise in combination with nutritional supplements with the aim to effect on the brain are still at a fledgling stage. There are, however, interesting first results in rodent studies that encourage further work in this field and which hold promise for utilizing the combined exercise-nutrition approach as a therapeutic tool.

Future Direction

A central element of successful cognitive rehabilitation for older adults should be the design of interventions that either re-activate disused or damaged brain regions, or that compensates for decline in parts of the brain through the activation of compensatory neural reserves (Hogan, 2005). Based on the results of the systematic review, we would design further combinatory studies as follows: Based on the findings of this review the nutritional supplements should be selected such that they share similar mechanisms with exercise and, thus, theoretically have the potential to support the action of exercise. Physical exercise would base on a combination of aerobic and strength exercise that also includes a cognitive component. Considering the cognitive part, previous research suggests a focus on executive functioning processes including enriched environments that provide physical activities with decision-making opportunities because these are believed to be able to facilitate the development of both motor performance and brain functions (Yan and Zhou, 2009). The use of virtual reality environments for virtual augmented exercise has recently been proposed as having the potential to increase exercise behavior in older adults in combination with the potential to influence cognitive abilities (de Bruin et al., 2010). At present there is

evidence that specific types of video games are able altering brain structure (Shams et al., 2015) and function (Eggenberger et al., 2016) and, when added to a multicomponent exercise program, improve certain aspects of cognitive functioning (Eggenberger et al., 2015a,b). Future research should develop, implement, and evaluate for example virtual reality based training scenarios that allows the combination of aerobic and strength exercises together with cognition. Moreover, video games allow the implementation of FITT (Frequency, Intensity, Type, and Time) training principles to ensure that the dose and type of physical exercise is planned to maximize the benefits for the recipients. For nutrition, a diet including omega-3 fatty acid, is assumed to have the potential to affect synaptic plasticity and cognition. A previous study performed in humans showed beneficial effects on cognitive functioning and memory (Witte et al., 2014). Furthermore, one study investigated the effects of CR in healthy elderly humans (Witte et al., 2009). In rodents, CR showed positive effects on brain function, but CR studies should be controlled for the confounding factor of increased physical activity.

In future studies, authors should agree upon a standardized set of tests in order to compare the results between studies, since there is a myriad of tasks that have been proposed to evaluate cognitive functioning. However, physical activity and nutrition are closely linked together, and positive effects of physical exercise might be confounded by better nutrition. Controlling for this factor appears necessary, if one wants to evaluate the additive effects of physical exercise and nutrition on cognition.

AUTHOR CONTRIBUTIONS

AS, KB, and JS developed the research question under the lead of PW and ED. The concept and design part was established by AS, KB, and JS while PW and ED acted as methodological councils. AS, KB, and JS did articles acquisition as well as analysis and interpretation of the articles which was edited and improved by PW and ED. AS and JS produced an early version of the manuscript. KB, PW, and ED substantially revised the manuscript to bring it to its current version. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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EPA/DHA and Vitamin A Supplementation Improves Spatial Memory and Alleviates the Age-related Decrease in Hippocampal RXRγ and Kinase Expression in Rats

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Studies suggest that eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and vitamin A are critical to delay aged-related cognitive decline. These nutrients regulate gene expression in the brain by binding to nuclear receptors such as the retinoid X receptors (RXRs) and the retinoic acid receptors (RARs). Moreover, EPA/DHA and retinoids activate notably kinase signaling pathways such as AKT or MAPK, which includes ERK1/2. This suggests that these nutrients may modulate brain function in a similar way. Therefore, we investigated in middle-aged rats the behavioral and molecular effects of supplementations with EPA/DHA and vitamin A alone or combined. 18-month-old rats exhibited reference and working memory deficits in the Morris water maze, associated with a decrease in serum vitamin A and hippocampal EPA/DHA contents. RARα, RXRβ, and RXRγ mRNA expression and CAMKII, AKT, ERK1/2 expression were decreased in the hippocampus of middle-aged rats. A combined EPA/DHA and vitamin A supplementation had a beneficial additive effect on reference memory but not in working memory in middle-aged rats, associated with an alleviation of the age-related decrease in RXRγ, CAMKII, AKT, and ERK1 expression in the hippocampus. This study provides a new combined nutritional strategy to delay brain aging.

Keywords: n-3 long-chain PUFA, vitamin A, spatial memory, hippocampus, kinases, retinoid receptors

Abbreviations: AA, arachidonic acid; AKT, protein kinase B; CAMKII, calcium/calmodulin-dependent kinase II; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; n-3 LC-PUFA, n-3 long-chain polyunsaturated fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PPAR, peroxisome proliferator-activated receptor; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor.

INTRODUCTION

Brain aging is associated with multiple morphological and biochemical changes leading to cognitive decline such as learning and memory impairments (Lister and Barnes, 2009). Nutrition is one of the multiple environmental factors which can contribute to successful aging and may modulate mental health (Parletta et al., 2013). Among the dietary nutrients most closely associated with optimal brain functioning, two n-3 LC-PUFAs, namely DHA and EPA, are particularly involved in the maintenance of cognitive functions during aging (Su, 2010; Joffre et al., 2014). The ability of these n-3 LC-PUFAs to modulate brain functions depends on their accretion level in the brain, closely related to the dietary intake and age. Indeed, aging is associated with a decrease in brain n-3 LC-PUFA contents (Labrousse et al., 2012). More specifically, an age-related decrease in DHA content in the hippocampus has been observed (Favrelière et al., 2003; Dyall et al., 2007). Moreover, we have recently shown that an EPA-DHA supplementation can reverse the age-related decrease in brain DHA content (Letondor et al., 2014). Such a supplementation has already been shown to improve memory performance in aged animals (for review, see Alfos, 2014).

Besides, brain aging is associated with an hypoactivation of the RA signaling pathway which could be involved in age-related memory impairments (Mingaud et al., 2008; Dyall et al., 2010; Bonhomme et al., 2014). RA, the active metabolite of vitamin A, plays a key role in the regulation of synaptic plasticity and in learning and memory in adults (Lane and Bailey, 2005). Indeed, it has been shown that the administration of RA improves reference and relational memory performance of aged animals by restoring the expression of retinoid nuclear receptors (RAR and RXR) and of synaptic plasticity markers in the hippocampus (Etchamendy et al., 2001). Similarly, Dyall et al. (2010) have shown that the age-related decrease in RARα, RXRα, and RXRβ protein levels in the rat forebrain and in the CA1 and dentate gyrus of the hippocampus are reversed by a 12 weeks EPA/DHA supplementation. Moreover, we have recently shown that a mid-life vitamin A supplementation during 4 months prevents spatial memory decline in 17-month-old rats and improves the dendritic arborisation of newborn immature neurons by inducing an increase in the intracellular availability of RA (Touyarot et al., 2013).

Retinoids and n-3 LC-PUFAs may modulate cerebral plasticity and memory by regulating gene expression through nuclear receptors that function as ligand-controlled transcription factors (Lane and Bailey, 2005; Su, 2010). Indeed, DHA and RA can bind to nuclear receptors, such as the PPARs, the RARs, and the RXRs (Evans and Mangelsdorf, 2014). Several studies highlighted multiple levels of interactions between the fatty acid and the retinoid signaling pathways. On the one hand, it has been shown that RXR is the obligatory heterodimerization partner of RARs and PPARs, suggesting that RXRs play a key role in both retinoid- and n-3 PUFA-mediated signaling pathways (van Neerven et al., 2008). On the other hand, *in vitro* studies have shown that fatty acids and particularly DHA can bind and activate RXRs (de Urquiza et al., 2000; Lengqvist et al., 2004) and that RA can bind to the PPARs (Shaw et al., 2003; Schug et al., 2007), implying interactions at the nuclear level between DHA and RA for binding to their receptors.

Moreover, RA and n-3 LC-PUFAs have additional extra-nuclear and non-transcriptional effects that activate kinase signaling pathways such as, AKT or the MAPK, which includes ERK1/2, thus influencing gene expression through phosphorylation processes (Masia et al., 2007; Rao et al., 2007; Al Tanoury et al., 2013). These signaling pathways are involved in the modulation of cerebral plasticity and thus in learning and memory processes (Giese and Mizuno, 2013). It has been shown that the ERK2 mRNA expression is impaired in the rat hippocampus during aging (Simonyi et al., 2003). A disruption of the AKT signaling pathway was also recently highlighted in a mouse model of accelerated-senescence (Armbrecht et al., 2014). Other kinases such as the CAMKII involved in synaptic plasticity (Ma et al., 2015) seem to be modulated by both n-3 PUFAs and RA. Indeed, although the transcriptional regulation of CAMKII depends on retinoids (Chen and Kelly, 1996), it has been shown that DHA treatment normalizes the CAMKII expression in the hippocampus of rats after a traumatic brain injury (Wu et al., 2011).

Altogether these data indicate that there is a close relationship between the n-3 LC-PUFA and the retinoid signaling pathways with both intra- and extra-nuclear interactions, suggesting that these nutrients may act together to modulate synaptic plasticity processes and memory altered during aging.

The present study therefore evaluates in middle-aged rats the potential synergetic behavioral and neurobiological effects of nutritional supplementation with EPA-DHA and vitamin A. For this purpose, spatial reference memory and working memory were assessed in the Morris water maze. To specify the molecular mechanisms mediated by the dietary supplementations on memory processes, we measured mRNA expression of RXRs and RARs and kinases CAMKII, AKT, and ERK1/2 and their protein levels in the hippocampus.

MATERIALS AND METHODS

Animals and Diets

The study was conducted according to the INRA Quality Reference System and to the directive 2010/63/UE of the European Parliament and of the Council on the protection of animals used for scientific purposes. The protocols were approved by the French Ministry for Higher Education and Research and the Animal Care and Use Committee of Bordeaux (n°. 5012051-A).

3-week-old and 13-month-old male Wistar rats were purchased from Janvier (France) and maintained under standard housing conditions in a temperature- (22 ± 1°C) and humidity-controlled room (40%) with a 12-h light/dark cycle. All the animals were fed and given water *ad libitum*. After 1 week of acclimatization to the housing conditions with a standard chow, the 13-month-old rats were randomly divided in four groups (n = 9–10 per group): the first group received a control diet (middle-aged control group), the second group received an EPA/DHA-enriched diet (middle-aged EPA/DHA group), the

third received a vitamin A-supplemented diet (middle-aged Vit A group) and the fourth received an EPA/DHA + vitamin A-enriched diet (middle-aged EPA/DHA + Vit A group). The 3-week-old rats ($n = 10$) received exclusively the control diet (adult control group). All the diets were given for 21 weeks therefore adult rats were 6-month-old and middle-aged rats were 18-month-old at the end of the experiment.

The control and vitamin A diets were free from LC-PUFA and consisted of a mix of peanut, rapeseed and sunflower oils (60/25/15, by weight) added to a fat-free diet containing a standard amount of 5 IU/g diet of vitamin A (control group) or enriched with 45 IU/g diet of vitamin A (Vit A group; UPAE-INRA Jouy-en-Josas, France). The EPA/DHA supplemented diet was a mix of fish, rapeseed and sunflower oils (50/20/30, by weight) added to the fat-free diet (EPA/DHA group) or the fat-free diet enriched in vitamin A (EPA/DHA + Vit A group). The composition of the different diets is detailed in **Table 1**. The fatty acid composition of the diets was assessed by gas chromatography as previously described (Buaud et al., 2010). Rats had a maximal daily intake of 395 mg/kg body weight EPA and 403 mg/kg body weight DHA. Fish oil was aliquoted in glass bottles under nitrogen and stored at 4°C for 21 weeks. The diets were freshly prepared every 2 days and stored at 4°C until their use. Food was changed daily between 5:00 and 7:00 p.m. and any left-over food was discarded. At the end of the 21-weeks feeding period, the adult rats (6-month-old) and the middle-aged rats (18-month-old) were anesthetized with isoflurane and rapidly decapitated. Blood was collected from the sectioned jugular vein. Brains were quickly removed, the whole hippocampus was dissected bilaterally and stored at −80°C until further analysis.

TABLE 1 | Composition of experimental diets.

	Control	Vit A	EPA/DHA	EPA/DHA+ Vit A
Ingredients ***(% of total provided energy)***				
Lipids	11.2	11.2	11.2	11.2
Saturated	1.6	1.6	1.0	1.0
Monounsaturated	6.9	6.9	3.4	3.4
Polyunsaturated	2.7	2.7	6.8	6.8
Proteins	18.0	18.0	18.0	18.0
Carbohydrates	69.8	69.8	69.8	69.8
Energy (kJ/g of diet)	16.7	16.7	16.7	16.7
Vitamin A (IU/g of diet)	5.0	45	5.0	45
Fatty acid composition ***(g/100 g of diet)***				
16:0	0.4	0.4	0.2	0.2
18:0	0.2	0.2	0.2	0.2
18:1n-9	3.0	3.0	1.6	1.6
18:2n-6	1.1	1.1	1.2	1.2
20:4n-6	0.0	0.0	0.1	0.1
22:5n-6	0.0	0.0	0.0	0.0
18:3n-3	0.1	0.1	0.1	0.1
20:5n-3 (EPA)	0.0	0.0	0.7	0.7
22:5n-3	0.0	0.0	0.1	0.1
22:6n-3 (DHA)	0.0	0.0	0.7	0.7
Total SFAs	0.6	0.6	0.4	0.4
Total MUFAs	3.0	3.0	1.6	1.6
Total PUFAs	1.2	1.2	2.9	2.9
Total n-6 PUFAs	1.1	1.1	1.3	1.3
Total n-3 PUFAs	0.1	0.1	1.6	1.6
18:2n-6/18:3n-3	11	11	12	12
n-6 PUFA/n-3 PUFA	11	11	0.8	0.8

MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids.

Behavioral Testing

Reference memory and working memory were tested in a Morris water maze (180 cm diameter, 60 cm high) filled with water (21–22°C) made opaque with non-toxic white paint. Before the learning phase, animals were habituated to the pool without any platform 60 s/day for 2 days. The principle of this test is based on the capacity to memorize and to develop a spatial map of the extra-maze cues to find an escape platform hidden 2 cm below the surface of the water. For each trial, the distance swum, the speed and the latency to reach the platform were measured with a computerized tracking system (Videotrack, Viewpoint, Lyon, France).

Spatial Learning and Reference Memory (Place Version)

Spatial reference memory was evaluated according to the protocol of Bonnet et al. (2008) modified as follow. Rats were trained for four consecutive trials a day (90 s with an inter-trial of 30 s, starting from different points in a randomized order every day) for nine consecutive days (learning phase). The distance swum to reach the platform should decrease over testing sessions (days) as the rats learn the location of the platform. 24 h after the last training day (day 10), the probe test was performed by placing the rats for 60 s in the pool without the platform. Reference memory was evaluated by measuring the percentage of time spent in the quadrant where the platform was during the learning phase (target quadrant).

Working Memory (Matching-to-Place Version)

Working memory was evaluated according to the protocol of Wainwright et al. (1999) modified as follow. 48 h after the probe test, rats were tested in the matching-to-place version of the Morris water maze. In each testing session, the rats received a pair of trials in which the start position was varied pseudo-randomly, but the platform remained at the same place. However, in contrast to the reference memory task, the location of the platform is changed everyday for 6 days. On the first 3 days, animals were tested with an inter-trial interval (ITI) of 30 s and an ITI of 2 min was applied on the last 3 days. In this version of the task, each of the testing session can be considered as a separate "problem" in which the first trial is a search trial and the second trial is a test trial which highlights the ability to remember the immediately preceding location of the platform in the first trial.

Cued Learning Version

24 h after the end of the working memory phase, rats were tested (one session with four trials) to find a cued visible platform. Differences in performance on this task would be indicative

of potential alterations in the sensory, motor or motivational attributes of the animals.

Measurement of Serum Retinol Concentration

Blood collected during the sacrifice was immediately spun at 1,500 g for 15 min. The supernatant was removed and snap frozen on dry ice. Serum retinol was assessed by HPLC according to the method previously described by Leclercq and Bourgeay-Causse (1981).

Hippocampal Phosphatidylcholine and Phosphatidylethanolamine Analysis

Total lipids from the other half of the hippocampi were extracted and PC and phosphatidylethanolamine (PE) were isolated from other lipid classes by thin layer chromatography as previously described (Letondor et al., 2014). PC and PE total fatty acids were transmethylated and analyzed in a blinded fashion by gas chromatography as previously described (Letondor et al., 2014). Results are expressed as percentage of PC and PE total fatty acids.

mRNA Expression Analysis by Real-time PCR

The other half of the dissected hippocampi was homogenized in 1 ml of Trizol reagent (Invitrogen, France) and total RNAs were extracted according to the manufacturer's instructions. The organic phase containing proteins was stored at −20°C for subsequent Western blot analysis. The concentration of the purified RNA was measured by spectrophotometry at 260 nm using a Nanodrop ND-1000 (Labtech, France). The integrity of RNA samples was assessed using the RNA 6000 Nano LabChip kit in combination with the 2100 Bioanalyzer (Agilent Technologies, France). RNAs were reverse transcribed in complementary DNAs using Improm II reverse transcriptase (Promega, France) according to the manufacturer's instructions. Real-time PCR were run on a LigthCycler 480 thermal cycler using the SYBR Green I Master kit (Roche Diagnostics, Mannheim, Germany) as previously described (Bonnet et al., 2008). The forward and reverse primer sequences used are shown in **Table 2**. The specificity and identity of the amplified products were verified by the melting curve analysis showing a single melting peak after amplification and by sequencing the amplified PCR products using the Big Dye Terminator v1.1 and an ABI3130 sequencer (Applied Biosystems). Data analysis was performed using the Roche's E-method of relative quantification, which uses standard curve derived efficiencies, of the LightCycler 480 1.5 version software. In this study we used the β2-microglobulin (BMG) housekeeping gene as the reference gene since its expression level was unaffected by our experimental conditions. The results are expressed as the target/reference ratio divided by the target/reference ratio of the calibrator.

TABLE 2 | Primers used for quantitative RT-PCR.

Gene name	Nucleotide sequence	Product length (bp)
BMG	F: 5′-GCCCAACTTCCTCAACTGCTACG-3′	180
	R: 5′-GCATATACATCGGTCTCGGTGGG-3′	
RARα	F: 5′-GCCTCGATTCTACAAGCCTTGC-3′	107
	R: 5′-GGATACTGCGTCGGAAGAAGC-3′	
RARβ	F: 5′-CAGCTGGGTAAATACACCACGAA-3′	227
	R: 5′-GGGGTATACCTGGTACAAATTCTGA-3′	
RARγ	F: 5′-GCCCTAAGGCTTTATGCCCGG-3′	104
	R: 5′-GCTCCCTTGGTGCTGATGCCC-3′	
RXRα	F: 5′-GCTGGTGTCGAAGATGCGTGAC-3′	171
	R:5′-GGGTACTTGTGTTTGCAGTACG-3′	
RXRβ	F: 5′-TGGGAACAGGGAGAATGTGG-3′	129
	R: 5′-CTGGAAAGCGACTTTATGTGCAAG-3′	
RXRγ	F: 5′-GGAAAGACCTCATCTACACG-3′	123
	R: 5′-CAGCTTCCCTCTTCATGCCC-3′	
ERK1	F: 5′-TCCCCTTGACCTGAGTGATGAG-3′	102
	R: 5′-CCATTCCAGAACCGTCTACCAGA-3′	
ERK2	F: 5′-CGTCTCAGCTTACCCACTCTTGA-3′	109
	R: 5′-TGCAGGAGAACTCTCTGGACTG-3′	
CREB	F: 5′-GTTCAAGCCCAGCCACAGATT-3′	84
	R: 5′-GGTTACAGTGGGAGCAGATGAC-3′	
CAMKII	F: 5′-TGCACAGACAGGAGACCGTGGAC-3′	122
	R: 5′-GTTTCCTCCACTCTTCCCTCCGG-3′	
AKT	F: 5′-TGAGCGCGTGTTTTCAGAGG -3′	131
	R: 5′-CCTTGTCCAGCATGAGGTTCTC -3′	

Sequences are shown for forward (F) and reverse (R) primers.

Western Blot Analysis

Total proteins from half hippocampi were extracted from the Trizol fraction previously recovered from the RNA extraction step according to the manufacturer's instructions slightly modified by Simoes et al. (2013). Briefly, the tube containing the organic phase was centrifuged at 12,000 *g* for 15 min at 4°C, and the remaining supernatant was removed and discarded. DNA was precipitated by addition of 100% ethanol and centrifuged at 2,000 *g* for 5 min at 4°C. Proteins in the phenol-ethanol supernatant were precipitated by addition of isopropanol and centrifuged at 12,000 *g* for 10 min at 4°C. The protein pellet was next washed three times with 0.3 M guanidine hydrochloride in 95% ethanol. After the final wash and centrifugation at 7,500 *g* for 5 min at 4°C, proteins were precipitated by addition of 100% ethanol followed by a final centrifugation at 7,500 *g* for 5 min at 4°C. The protein pellet was solubilized by sonication in a 1:1 solution of 1% SDS and 8 M urea in Tris-HCl 1 M, pH 8.0. The samples were centrifuged at 3,000 *g* for 10 min at 4°C, to sediment insoluble material and the supernatant containing the solubilized proteins was stored at −80°C. Protein concentration was determined using the MicroBC Assay protein quantitation kit. Western blot analysis was performed as previously described by Boucheron et al. (2006) with slight modifications. Briefly, aliquots containing 40 μg of total proteins were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to immobilon polyvinyllidene difluoride membranes (Millipore, Billerica, MA, USA). Blots were incubated overnight at 4°C with primary rabbit anti-actin (diluted 1:5000, Sigma, France), anti-AKT, anti-phospho-AKT, anti p44/42 MAPK (ERK1/2) or anti-phospho-p44/42 MAPK (phospho-ERK1/2), diluted 1:1000 (Cell Signaling

Technology, Danvers, MA, USA). After washing, the blots were incubated with appropriated horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch, Westgrove, PA, USA). Following several washings, the bands were developed using the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Science, Waltham, MA, USA) and quantified by measuring chemiluminescence with an image analysis system (Syngene, Frederick, MD, USA). The relative levels of proteins in middle-aged control and supplemented rats were expressed as a percentage of the same proteins in adult rats. The constant level of actin was verified and found to be identical in all groups (**Figure 6A**).

Statistical Analysis

Results are expressed as mean ± standard error of the mean (mean ± SEM). Statistical analyses were performed with StatView 5.0 software. For reference memory, data were analyzed by a one-way ANOVA or repeated measures ANOVA (for learning phase) followed by a Fischer PLSD *post hoc* test and a Student's *t*-test to compare with chance level. For working memory data were analyzed by a two-way (group × trial) ANOVA followed by a paired Student's *t*-test to compare trials within groups. *P* values < 0.05 were considered to be statistically significant.

RESULTS

Spatial Learning and Reference Memory

During the learning phase, rats were trained in the Morris water maze over 9 days with four trials per day to find a submerged platform. The swimming speed during this phase was significantly different between middle-aged and adult rats (adult control: 22.3 ± 0.3 cm/s, middle-aged control: 18.6 ± 0.4 cm/s, middle-aged Vit A: 20.6 ± 0.4 cm/s, middle-aged EPA/DHA: 19.7 ± 0.4 cm/s, middle-aged EPA/DHA + Vit A: 19.6 ± 0.4 cm/s; $F_{(4,38)} = 3.87$; $p < 0.01$), therefore we analyzed the mean distance swum to reach the platform over the 9 days of training (**Figure 1A**). The distance to reach the escape platform decreased along the days with a main effect of days ($F_{(8,304)} = 35.863$; $p < 0.0001$), meaning that all rats learned this task. However, we did not observe any group effect on learning ($F_{(4,38)} = 1.310$; $p = 0.284$).

During the probe test, we measured the percentage of time spent in the target quadrant from where the platform was removed. The analysis of the probe test over the 60 s did not reveal any group effect (adult control: 46.4 ± 2.8%, middle-aged control: 35.8 ± 5.1%, middle-aged Vit A: 34.2 ± 4.4%, middle-aged EPA/DHA: 32.2 ± 3.8%, middle-aged EPA/DHA + Vit A: 36.9 ± 2.2%; $F_{(4,38)} = 2.25$; $p < 0.08$; **Figure 1B**). However, only adult control rats and middle-aged rats supplemented with EPA/DHA + Vit A spent significantly more than 25% (chance level) of the total time in the target quadrant ($t = 7.472$, $p < 0.0001$ and $t = 5.501$, $p < 0.001$, respectively). Then, analyzing more precisely the first 20 s (**Figure 1C**) which is more relevant to avoid a possible motivational extinction (Blokland et al., 2004), a significant group effect was observed on the time spent in the target quadrant (adult control: 52.7 ± 3.8%, middle-aged control: 34 ± 4.6%, middle-aged Vit A: 39 ± 5.4%, middle-aged EPA/DHA: 23.3 ± 4.8%, middle-aged EPA/DHA + Vit A: 43.6 ± 4.8%; $F_{(4,38)} = 4.54$; $p < 0.01$). Indeed, middle-aged control and supplemented rats with only EPA/DHA or vitamin A exhibited lower memory performance than the adult control rats. In contrast, the performance of the EPA/DHA + Vit A supplemented rats was not different from the adult control rats and even better than that of the group supplemented only with EPA/DHA ($p = 0.026$). However, the performance of the rats receiving the combined supplementation was not significantly improved over that receiving vitamin A alone ($p = 0.5$). When comparing the time spent in the target quadrant over the first 20 s with the chance level, the analysis revealed that only three groups of rats: adult control ($p < 0.0001$), middle-aged Vit A ($p < 0.05$) and middle-aged EPA/DHA + Vit A ($p < 0.01$) spent more time in the target quadrant, suggesting that only these groups remembered the location of the platform.

Spatial Working Memory

In the matching-to-place version of the Morris water maze, on the test trial (T2) rats need to remember the location of the platform learned during the previous search trial (T1) that occurred 30 s or 2 min before. For an ITI of 30 s (**Figure 2A**), a two-way ANOVA on mean distance swum in T1 and T2 revealed no group effect ($F_{(4,76)} = 1.59$; $p = 0.18$) but a main effect of trial ($F_{(1,76)} = 15.8$; $p < 0.001$). Thus the distance swum for T2 was shorter than for T1 when data was collapsed across the group, suggesting that all groups remembered the location of the hidden platform learned at the previous trial. However, the two-way ANOVA also revealed a group × trial interaction ($F_{(4,76)} = 2.9$; $p < 0.05$). Therefore, when comparing T1 with T2, adult control rats and middle-aged rats supplemented with EPA/DHA exhibited distance swum in T2 significantly shorter than in T1 (paired *t*-test, $t = 3.59$, $p = 0.005$ and $t = 4.27$ $p = 0.04$, respectively), revealing better working memory performance in middle-aged rats supplemented with EPA/DHA. With a 2 min ITI (**Figure 2B**), there was no group effect ($F_{(4,76)} = 0.9$; $p = 0.45$) and the trial effect was just significant ($F_{(1,76)} = 4.00$; $p = 0.05$). The two-way ANOVA revealed a group × trial interaction ($F_{(4,76)} = 2.6$; $p < 0.05$) however, only the adult control rats swum a shorter distance during T2 than during T1, indicating that only adult control rats remembered the location of the platform (paired *t*-test, $t = 2.97$, $p < 0.05$). The EPA/DHA supplementation did not improve working memory in these conditions (paired *t*-test, $t = 0.75$, $p = 0.47$).

Cued Learning

The distances swum to find the visible platform were not statistically significant different between the five different groups of rats tested in the cued version of the Morris water maze ($F_{(4,38)} = 1.68$; $p = 0.17$), indicating no difference in physical capabilities (**Figure 1D**).

Nutritional Status of the Rats

Serum Retinol Concentration

In order to assess the vitamin A status of middle-aged rats and the impact of vitamin A supplementation, serum

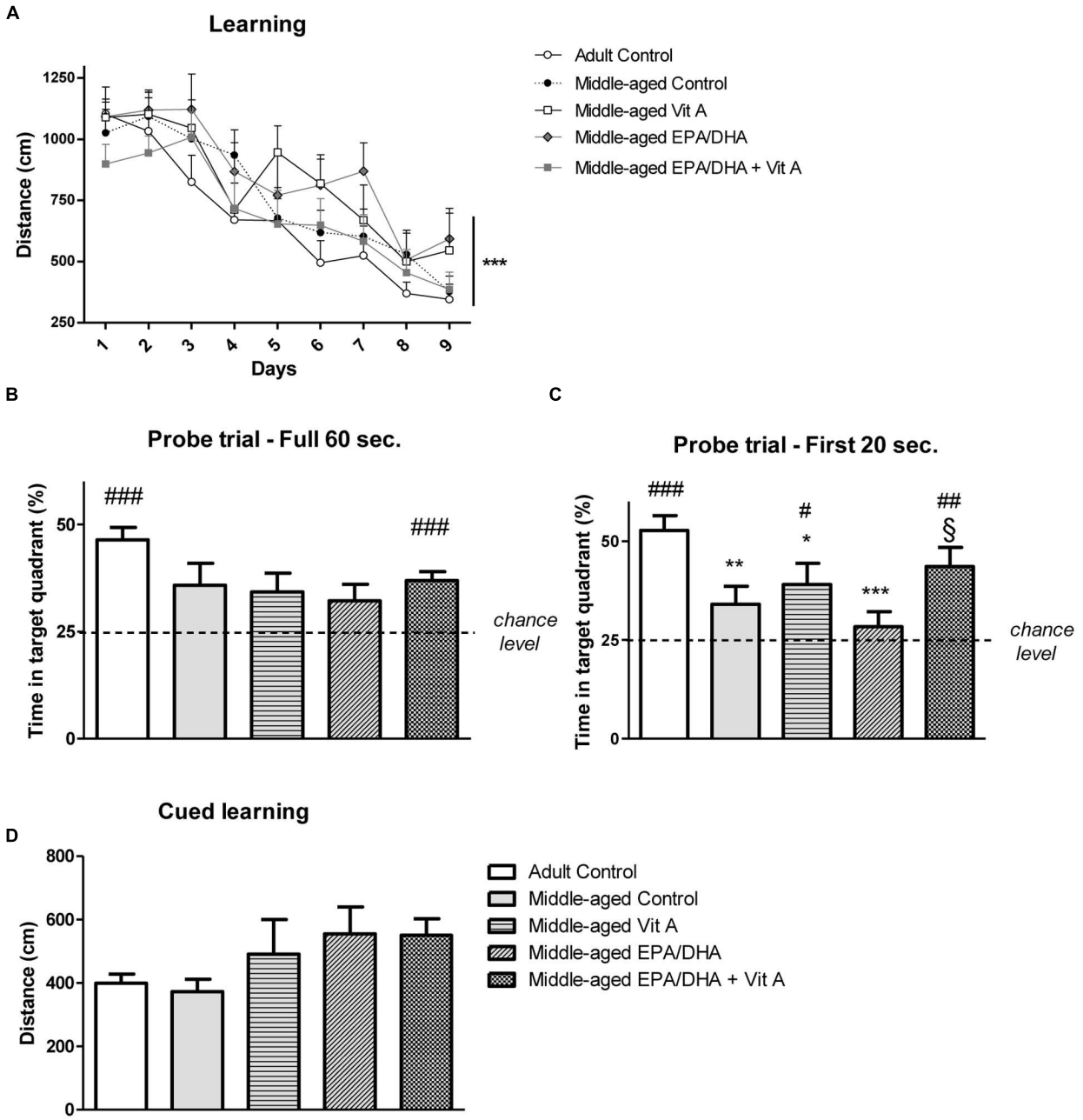

FIGURE 1 | Effect of age and diet (EPA/DHA, Vit A, or EPA/DHA + Vit A) on spatial learning, reference memory and cued learning in the Morris water maze. Figures show **(A)** the mean distance swum during acquisition to reach a submerged platform located at the same position over 9 days with four trials per day; **(B,C)** Percentage of time spent in the target quadrant with the platform removed in the probe test over **(B)** 60 s or **(C)** 20 s; **(D)** the mean distance swum during the cued learning. Values are mean ± SEM, $n = 8$–10 rats per group. Data were analyzed by **(A)** repeated measures ANOVA or **(B–D)** one-way ANOVA followed by the Fischer PLSD *post hoc* test. Signs indicate values different from **(A)** day 1 or **(B,C)** from Adult control: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ and from middle-aged EPA/DHA: §$p < 0.05$; or compared with chance level by a Student's one group *t*-test: #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$.

retinol concentrations were assessed (**Figure 3**). Our results showed a significant difference in retinol levels between groups ($F_{(4,38)} = 20.76$; $p < 0.0001$). Indeed, *post hoc* analysis revealed that middle-aged control group displayed lower serum retinol levels compared to the adult control group (−32%, $p < 0.0001$). Neither a vitamin A supplementation, nor an EPA/DHA supplementation, nor the combined supplementation were able to alleviate the age-related serum retinol decrease (−29%, $p < 0.0001$, −41%, $p < 0.0001$, and −46%, $p < 0.0001$, respectively). Interestingly, rats that received the EPA/DHA + Vit A diet displayed lower levels of retinol compared to middle-aged control rats ($p = 0.024$). The EPA/DHA group displayed an

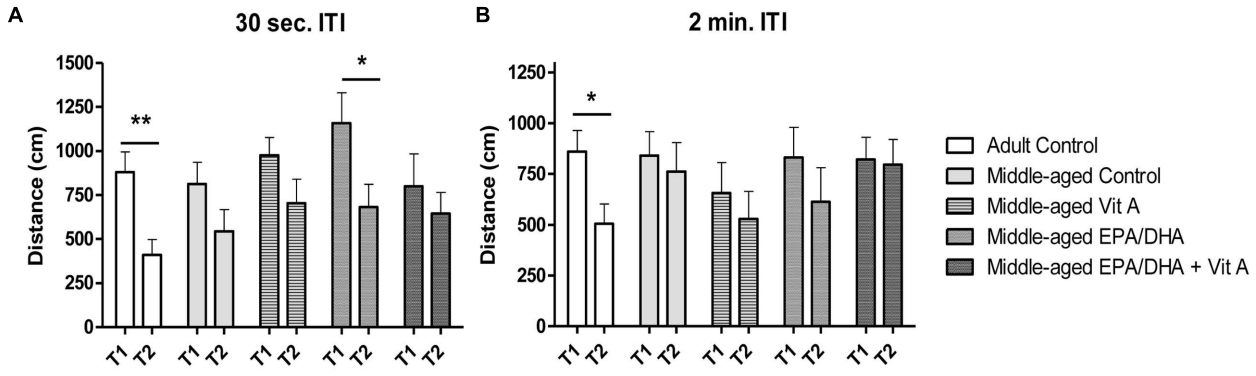

FIGURE 2 | Effect of age and diet (EPA/DHA, Vit A, or EPA/DHA + Vit A) on spatial working memory (matching-to-place version) in the Morris water maze. Figures show **(A)** the mean distance swum to reach a submerged platform over 3 days in the first trial (T1) followed by a second trial (T2) with an inter-trial interval (ITI) of 30 s; **(B)** the mean distance swum to reach a submerged platform over 3 days in the T1 and T2 with an ITI of 2 min. The platform was moved to a different location each day. Values are mean ± SEM, $n = 8$–10 rats per group. Data were analyzed by Student's paired *t*-test: different from T1: $*p < 0.05$; $**p < 0.01$.

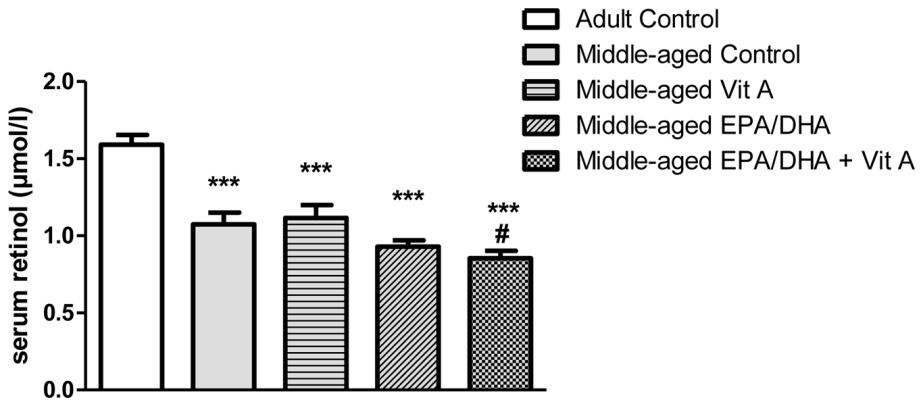

FIGURE 3 | Effect of age and diet (EPA/DHA, Vit A, or EPA/DHA + Vit A) on serum retinol concentrations. Values are mean ± SEM, $n = 8$–10 rats per group. Data were analyzed by a one-way ANOVA followed by the Fischer PLSD *post hoc* test. Signs indicate values different from Adult control: $***p < 0.001$ and from middle-aged control: $\#p < 0.05$.

intermediate level of serum retinol between the vitamin A and the EPA/DHA + Vit A supplemented rats and differed only from the adult control rats.

Lipid Status in the Hippocampus

The results of the fatty acid composition of the hippocampal PC and PE are reported in **Tables 3** and **4** respectively. Aging was associated with a decrease in total n-3 LC-PUFAs only in hippocampal PC (−16%; $p < 0.05$) not in the PE, due to a decrease in DHA level (−17%; $p < 0.05$), leading to a slight elevation of the n-6 PUFA/n-3 PUFA ratio (+8%, $p < 0.05$) in middle-aged control group compared to the adult control group. Furthermore, our results showed a higher level of total monounsaturated fatty acids (MUFAs) in middle-aged control rats compared to adult control rats both in hippocampal PC and PE (+6 and +14%, respectively; $p < 0.05$). Dietary supplementation was associated with a similar increase in DHA levels in middle-aged EPA/DHA and in middle-aged EPA/DHA + Vit A supplemented rats both in hippocampal PC and PE (+21 and +26%, respectively for PC and +21 and +21% for PE respectively; all $p < 0.05$) compared to the middle-aged control group. DHA level in Vit A supplemented group was not different from that of adult control rats for both PC and PE. The EPA and its derivative n-3 docosapentaenoic acid (n-3 DPA) levels in hippocampal PC and PE were also strongly increased in middle-aged EPA/DHA and in middle-aged EPA/DHA + Vit A (EPA: +1200% for PC, $p < 0.0001$ and +730 and 560%, respectively for PE, $p < 0.0001$; n-3 DPA: +140% for PC, $p < 0.0001$ and +412% for PE, $p < 0.0001$) compared to the middle-aged control group. In contrast, AA and n-6 docosapentaenoic acid (n-6 DPA) contents in hippocampal PC and PE were reduced in middle-aged rats supplemented with EPA/DHA or EPA/DHA + Vit A (AA: −19% for PC and −16% for PE, $p < 0.01$; n-6 DPA: −48%, $p < 0.05$ and −39%, $p < 0.0001$, respectively for PC and −73% for PE $p < 0.05$) compared to the middle-aged control group. Thus, EPA/DHA supplementation induced a significant

TABLE 3 | Fatty acid composition (% of total fatty acids) of hippocampal phosphatidylcholine of rats fed the control diet or a supplemented diet (EPA/DHA, Vit A, or EPA/DHA + Vit A).

Fatty acids	Adult	Middle-aged			
	Control	Control	Vit A	EPA/DHA	EPA/DHA + Vit A
16:0	42.10 ± 0.73^{a}	41.68 ± 0.63^{a}	41.70 ± 0.53^{a}	42.45 ± 0.32^{a}	41.42 ± 0.82^{a}
16:1(n-7)	0.38 ± 0.01$^{\mathbf{a}}$	0.43 ± 0.01$^{\underline{\mathbf{b}}}$	0.44 ± 0.02$^{\underline{\mathbf{bd}}}$	0.49 ± 0.02$^{\underline{cd}}$	0.50 ± 0.02$^{\underline{c}}$
18:0	12.78 ± 0.21^{a}	12.97 ± 0.17^{a}	12.83 ± 0.15^{a}	12.74 ± 0.17^{a}	12.98 ± 0.37^{a}
18:1(n-9)	21.29 ± 0.34$^{\mathbf{a}}$	22.79 ± 0.40$^{\underline{bc}}$	22.43 ± 0.31$^{\mathbf{ab}}$	23.76 ± 0.38$^{\underline{c}}$	23.91 ± 0.41$^{\underline{c}}$
18:1(n-7)	5.98 ± 0.07$^{\mathbf{a}}$	5.97 ± 0.11$^{\mathbf{a}}$	5.89 ± 0.10$^{\mathbf{a}}$	5.41 ± 0.04$^{\underline{b}}$	5.47 ± 0.17$^{\underline{b}}$
18:2(n-6)	0.36 ± 0.02$^{\mathbf{a}}$	0.43 ± 0.03$^{\mathbf{a}}$	0.43 ± 0.02$^{\mathbf{a}}$	0.57 ± 0.03$^{\underline{b}}$	0.60 ± 0.04$^{\underline{b}}$
18:3(n-3)	ND	ND	ND	ND	ND
20:4(n-6) (AA)	9.06 ± 0.40$^{\mathbf{a}}$	8.06 ± 0.32$^{\underline{\mathbf{b}}}$	8.21 ± 0.27$^{\mathbf{ab}}$	6.53 ± 0.29$^{\underline{c}}$	6.54 ± 0.46$^{\underline{c}}$
20:5(n-3) (EPA)	0.01 ± 0.01$^{\mathbf{a}}$	0.01 ± 0.01$^{\mathbf{a}}$	0.02 ± 0.01$^{\mathbf{a}}$	0.13 ± 0.01$^{\underline{b}}$	0.13 ± 0.02$^{\underline{b}}$
22:5(n-6) (DPA)	0.36 ± 0.04$^{\mathbf{a}}$	0.32 ± 0.06^{ac}	0.41 ± 0.04$^{\mathbf{a}}$	0.17 ± 0.04$^{\underline{b}}$	0.19 ± 0.04$^{\underline{bc}}$
22:5(n-3) (DPA)	0.06 ± 0.01$^{\mathbf{a}}$	0.08 ± 0.01$^{\mathbf{a}}$	0.07 ± 0.01$^{\mathbf{a}}$	0.19 ± 0.01$^{\underline{b}}$	0.20 ± 0.02$^{\underline{b}}$
22:6(n-3) (DHA)	3.28 ± 0.20^{a}	2.69 ± 0.16$^{\underline{\mathbf{b}}}$	2.84 ± 0.13^{ab}	3.26 ± 0.20^{a}	3.39 ± 0.21^{a}
Total SFAs	56.01 ± 0.55^{a}	55.89 ± 0.53^{a}	55.72 ± 0.49^{a}	56.43 ± 0.37^{a}	55.78 ± 0.36^{a}
Total MUFAs	29.37 ± 0.42$^{\mathbf{a}}$	31.16 ± 0.49$^{\underline{b}}$	30.76 ± 0.42^{ab}	31.47 ± 0.42$^{\underline{b}}$	31.76 ± 0.61$^{\underline{b}}$
Total PUFAs	14.43 ± 0.67$^{\mathbf{a}}$	12.78 ± 0.60^{ab}	13.27 ± 0.46^{ab}	11.90 ± 0.57$^{\underline{b}}$	12.20 ± 0.66$^{\underline{b}}$
Total n-6 PUFAs	10.91 ± 0.47$^{\mathbf{a}}$	9.83 ± 0.41$^{\underline{\mathbf{b}}}$	10.11 ± 0.33$^{\mathbf{ab}}$	8.11 ± 0.35$^{\underline{c}}$	8.27 ± 0.43$^{\underline{c}}$
Total n-3 PUFAs	3.35 ± 0.20$^{\mathbf{ac}}$	2.79 ± 0.16$^{\underline{\mathbf{b}}}$	2.93 ± 0.13$^{\mathbf{ab}}$	3.59 ± 0.22^{c}	3.74 ± 0.24^{c}
n-6/n-3 PUFAs	3.26 ± 0.07$^{\mathbf{a}}$	3.52 ± 0.07$^{\underline{\mathbf{b}}}$	3.45 ± 0.08$^{\mathbf{ab}}$	2.26 ± 0.06$^{\underline{c}}$	2.21 ± 0.08$^{\underline{c}}$

Values are mean ± SEM and were compared using one-way ANOVA and the Fischer PLSD post hoc test. Values not sharing a common superscript within a row are significantly different ($p < 0.05$). Underlined superscript indicates a statistically significant difference from the adult control group and bolded superscript indicates a statistically significant difference from the middle-aged EPA/DHA + Vit A group. ND, not detected; AA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

TABLE 4 | Fatty acid composition (% of total fatty acids) of hippocampal phosphatidylethanolamine of rats fed the control diet or a supplemented diet (EPA/DHA, Vit A, or EPA/DHA + Vit A).

Fatty acids	Adult	Middle-aged			
	Control	Control	Vit A	EPA/DHA	EPA/DHA + Vit A
16:00	5.83 ± 0.21^{a}	5.98 ± 0.15^{a}	5.84 ± 0.06^{a}	6.2 ± 0.17^{a}	6.07 ± 0.12^{a}
16:1(n-7)	0.16 ± 0.01$^{\mathbf{a}}$	0.21 ± 0$^{\underline{\mathbf{b}}}$	0.25 ± 0.03$^{\underline{bc}}$	0.3 ± 0.02$^{\underline{c}}$	0.29 ± 0.03$^{\underline{c}}$
18:00	18.44 ± 0.44^{a}	18.14 ± 0.36^{a}	17.87 ± 0.51^{a}	17.51 ± 0.43^{a}	17.99 ± 0.37^{a}
18:1(n-9)	11.47 ± 0.35$^{\mathbf{a}}$	12.5 ± 0.23$^{\underline{\mathbf{b}}}$	12.78 ± 0.44$^{\underline{bc}}$	13.91 ± 0.47$^{\underline{c}}$	13.63 ± 0.36$^{\underline{c}}$
18:1(n-7)	1.91 ± 0.08$^{\mathbf{a}}$	2.13 ± 0.06$^{\underline{b}}$	2.08 ± 0.09^{ab}	2.13 ± 0.09^{ab}	2.13 ± 0.08$^{\underline{b}}$
18:2(n-6)	0.14 ± 0.01$^{\mathbf{a}}$	0.17 ± 0.01$^{\mathbf{ab}}$	0.2 ± 0.02$^{\underline{\mathbf{b}}}$	0.27 ± 0.01$^{\underline{c}}$	0.24 ± 0.01$^{\underline{c}}$
18:3(n-3)	ND	ND	ND	ND	ND
20:4(n-6) (AA)	13.98 ± 0.25$^{\mathbf{a}}$	13.38 ± 0.12$^{\mathbf{a}}$	13.6 ± 0.21$^{\mathbf{a}}$	11.2 ± 0.27$^{\underline{b}}$	11.24 ± 0.23$^{\underline{b}}$
20:5(n-3) (EPA)	0.02 ± 0.01$^{\mathbf{a}}$	0.03 ± 0.01$^{\mathbf{a}}$	0.04 ± 0.01$^{\mathbf{a}}$	0.25 ± 0.01$^{\underline{\mathbf{b}}}$	0.2 ± 0.02$^{\underline{c}}$
22:5(n-6) (DPA)	1.01 ± 0.13$^{\mathbf{a}}$	0.99 ± 0.06$^{\mathbf{a}}$	0.98 ± 0.02$^{\mathbf{a}}$	0.26 ± 0.02$^{\underline{b}}$	0.26 ± 0.01$^{\underline{b}}$
22:5(n-3) (DPA)	0.13 ± 0$^{\mathbf{a}}$	0.16 ± 0.02$^{\mathbf{a}}$	0.17 ± 0.01$^{\mathbf{a}}$	0.82 ± 0.03$^{\underline{b}}$	0.82 ± 0.02$^{\underline{b}}$
22:6(n-3) (DHA)	17.07 ± 0.58$^{\mathbf{a}}$	15.66 ± 0.41$^{\mathbf{a}}$	15.87 ± 0.29$^{\mathbf{a}}$	19.06 ± 0.71$^{\underline{b}}$	19.01 ± 0.46$^{\underline{b}}$
Total SFAs	39.57 ± 0.58^{a}	38.38 ± 0.44^{ab}	36.98 ± 0.59$^{\underline{b}}$	37.66 ± 0.46$^{\underline{b}}$	38.35 ± 0.47^{ab}
Total MUFAs	21.56 ± 0.69$^{\mathbf{a}}$	24.64 ± 0.36$^{\underline{b}}$	24.62 ± 0.43$^{\underline{b}}$	25.73 ± 0.73$^{\underline{b}}$	24.89 ± 0.67$^{\underline{b}}$
Total PUFAs	38.49 ± 0.97^{a}	36.67 ± 0.6^{a}	37.6 ± 0.55^{a}	36.19 ± 0.98^{a}	36.36 ± 0.75^{a}
Total n-6 PUFAs	21.06 ± 0.38$^{\mathbf{a}}$	20.55 ± 0.26$^{\mathbf{a}}$	21.11 ± 0.31$^{\mathbf{a}}$	15.8 ± 0.31$^{\underline{b}}$	16.04 ± 0.3$^{\underline{b}}$
Total n-3 PUFAs	17.22 ± 0.59$^{\mathbf{a}}$	15.85 ± 0.41$^{\mathbf{a}}$	16.11 ± 0.28$^{\mathbf{a}}$	20.14 ± 0.72$^{\underline{b}}$	20.04 ± 0.47$^{\underline{b}}$
n-6/n-3 PUFAs	1.22 ± 0.02$^{\mathbf{a}}$	1.3 ± 0.03$^{\mathbf{a}}$	1.31 ± 0.02$^{\mathbf{a}}$	0.78 ± 0.02$^{\underline{b}}$	0.8 ± 0.02$^{\underline{b}}$

Values are mean ± SEM and were compared using one-way ANOVA and the Fischer PLSD post hoc test. Values not sharing a common superscript within a row are significantly different ($p < 0.05$). Underlined superscript indicates a statistically significant difference from the adult control group and bolded superscript indicates a statistically significant difference from the middle-aged EPA/DHA + Vit A group. ND, not detected; AA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

decrease in the n-6 PUFA/n-3 PUFA ratio for both PC and PE (−35 and −37%, respectively for PC and −40 and −38%, respectively for PE; $p < 0.0001$) compared to the middle-aged rats.

mRNA Expression in the Hippocampus

The mRNA expression of several PUFA and retinoid nuclear receptors was quantified by real-time PCR in the hippocampus. Results summarized in **Figure 4** show a significant group effect only for RARα, RXRβ and RXRγ mRNAs. Middle-aged rats exhibited a significant decrease in RARα (−17%; $F_{(4,37)} = 3.29$; $p < 0.05$) and RXRβ (−22%; $F_{(4,36)} = 3.44$; $p < 0.05$) mRNA expression in the hippocampus compared to the adult control rats. These mRNA levels were not modified with any dietary supplementations. RXRγ mRNA level was decreased in the middle-aged control group compared to the adult control group, the EPA/DHA group and the Vit A group (−32, −41, and −39% respectively; $F_{(4,35)} = 7.98$; $p < 0.0001$). However, the RXRγ mRNA level in the EPA/DHA + Vit A supplemented group was increased compared to the levels in Vit A and EPA/DHA supplemented groups (+33 and +27% respectively, $p < 0.05$).

The mRNA expression level coding for several proteins involved in both PUFA and RA extra-nuclear signaling pathways were also measured (**Figure 5**). An age-related hypo-expression of the mRNA levels of ERK1 (−19%; $F_{(4,37)} = 3.228$; $p < 0.05$), ERK2 (−21%; $F_{(4,37)} = 2.668$; $p < 0.05$), CAMKII (−21%; $F_{(4,37)} = 6.602$; $p < 0.001$) and AKT (−21%; $F_{(4,37)} = 3.35$; $p < 0.05$) was observed in the hippocampus of middle-aged control compared to adult control rats. Moreover, middle-aged rats supplemented with vitamin A (Vit A and EPA/DHA + Vit A) exhibited mRNA levels for ERK1, ERK2 and CAMKII not different from those of the adult control rats. Interestingly, for AKT, the hippocampal mRNA levels only in the middle-aged rats supplemented with EPA/DHA + Vit A were similar to those in adult control rats (95 vs. 100%; $p = 0.47$). Furthermore, only the mRNA level of ERK1 was significantly higher in the EPA/DHA + Vit A group compared to the middle-aged control group (+21%; $p < 0.01$).

Protein Levels in the Hippocampus

The results of the protein quantification by Western blot are reported in **Figure 6**. As for the mRNA expression, aging was associated with a significant reduced level of ERK1/2 (−30%, $F_{(4,32)} = 4.639$; $p < 0.01$) and AKT (−19%; $F_{(4,32)} = 3.857$; $p < 0.05$). Rats supplemented with EPA/DHA or vitamin A alone displayed similar protein levels of ERK1/2 and AKT compared to middle-aged control rats. However, the EPA/DHA + Vit A supplemented rats exhibited a protein level of AKT not significantly different from the one of adult control rats and an intermediated level of ERK1/2, indicating a additive effect of EPA/DHA and vitamin A supplementations. CAMKII protein level was not significantly different between adult and middle-aged control rats ($p = 0.06$). However, the EPA/DHA + Vit A supplemented rats exhibited a significantly higher level of CAMKII than the one of the middle-aged control rats (+18%; $p < 0.01$) and not different from adult control rats and Vit A supplemented rats. There was no statistical difference between groups in the expression levels of the phosphorylated forms of ERK1/2, CAMKII, and AKT in the hippocampus.

DISCUSSION

Nutritional Status of the Rats

In the present study, middle-aged rats exhibited a significant decrease in serum retinol concentration compared to adult rats, as previously described in aged animals (van der Loo et al., 2004; Féart et al., 2005; Touyarot et al., 2013) and elderly people (Haller et al., 1996). This decrease could be explained by a loss, during aging, in the capacity to mobilize vitamin A from the liver and thereby to regulate serum retinol levels (Azais-Braesco et al., 1995; Borel et al., 1998). Thus, these changes in vitamin A bioavailability during aging could prevent the vitamin A-enriched diet to normalize the serum retinol level in middle-aged rats as previously shown in 13-month-old rats supplemented with 45 IU retinol/g of diet for 4 months (Touyarot et al., 2013).

The analyses of the fatty acid profile in the hippocampal PC revealed a significant decrease in DHA levels of middle-aged control rats compared to the adult control rats as previously described (Dyall et al., 2007; Letondor et al., 2014). However, contrary to the results of Dyall et al. (2007) we did not observe any age-related decrease in DHA levels in the hippocampal PE. This discrepancy could be explained by the very old age of the rats used by Dyall et al. (2007) (28 months in Dyall's study vs. 18 months in the present study), leading to a drastic decrease in DHA levels in most phospholipid classes and in several brain structures. Aging was also associated with a significant increase in total MUFA levels both in hippocampal PC and PE as it was previously observed in the hippocampus and the cerebral cortex of 18-month-old rats (Favrelière et al., 2000). The EPA/DHA-enriched diet in middle-aged rats induced a strong increase in the n-3 LC-PUFA levels, including EPA, DHA and n-3 DPA, in both hippocampal PC and PE. This increase was counterbalanced with lower levels of n-6 LC-PUFAs leading to a decrease in the n-6 PUFA/n-3 PUFA ratio in the hippocampus. It is well known that fish oil diets containing n-3 LC-PUFAs reduce delta-6 and delta-5 desaturase activities leading to a decrease in n-6 LC-PUFAs (Christiansen et al., 1991). These data indicate that the age-related decrease in n-3 LC-PUFA levels in the hippocampal membranes can be reversed by the EPA/DHA-enriched diet as already reported in 21-month-old rats receiving a n-3 PUFA-enriched diet for 3 months (Favrelière et al., 2003).

The originality of the present study was to assess the effect of a 5-months vitamin A supplementation on brain fatty acid profile. Despite previous studies showing a regulation by vitamin A of hepatic desaturase expression involved in fatty acid metabolism (Zolfaghari and Ross, 2003), our results demonstrate that the vitamin A supplementation has no effect on fatty acid contents in the hippocampal PC or PE of middle-aged animals contrary to

the EPA/DHA supplementation. This is in agreement with recent data showing that fat content in the diet has a stronger impact than vitamin A on fatty acid concentrations in the liver and in the plasma (Weiss et al., 2014).

Cognitive Status and Molecular Mechanisms

Effect of Age

According to numerous data obtained in aged animals (Lister and Barnes, 2009), we demonstrated a clear age-related impairment in hippocampus-dependent spatial reference memory and spatial working memory in 18-month-old rats. These age-related memory impairments were associated with a hippocampal hypoexpression of RARα, RXRβ, and RXRγ mRNAs in middle-aged control animals. These results are consistent with previous data obtained in 23-month-old mice, showing a reduced level of RXRβ/γ mRNA expression in the hippocampus and in the whole brain (Enderlin et al., 1997a), that was associated with a relational memory impairment (Etchamendy et al., 2001). It is well known that the mRNA expression of some RXR and RAR isotypes is regulated by RA in the brain (Enderlin et al., 1997b; Féart et al., 2005) and that RA administration reverses an age-related spatial memory deficit (Etchamendy et al., 2001). In the rodent hippocampus, RA can be synthetized, from circulating vitamin A, in the meninges, by the retinaldehyde dehydrogenase

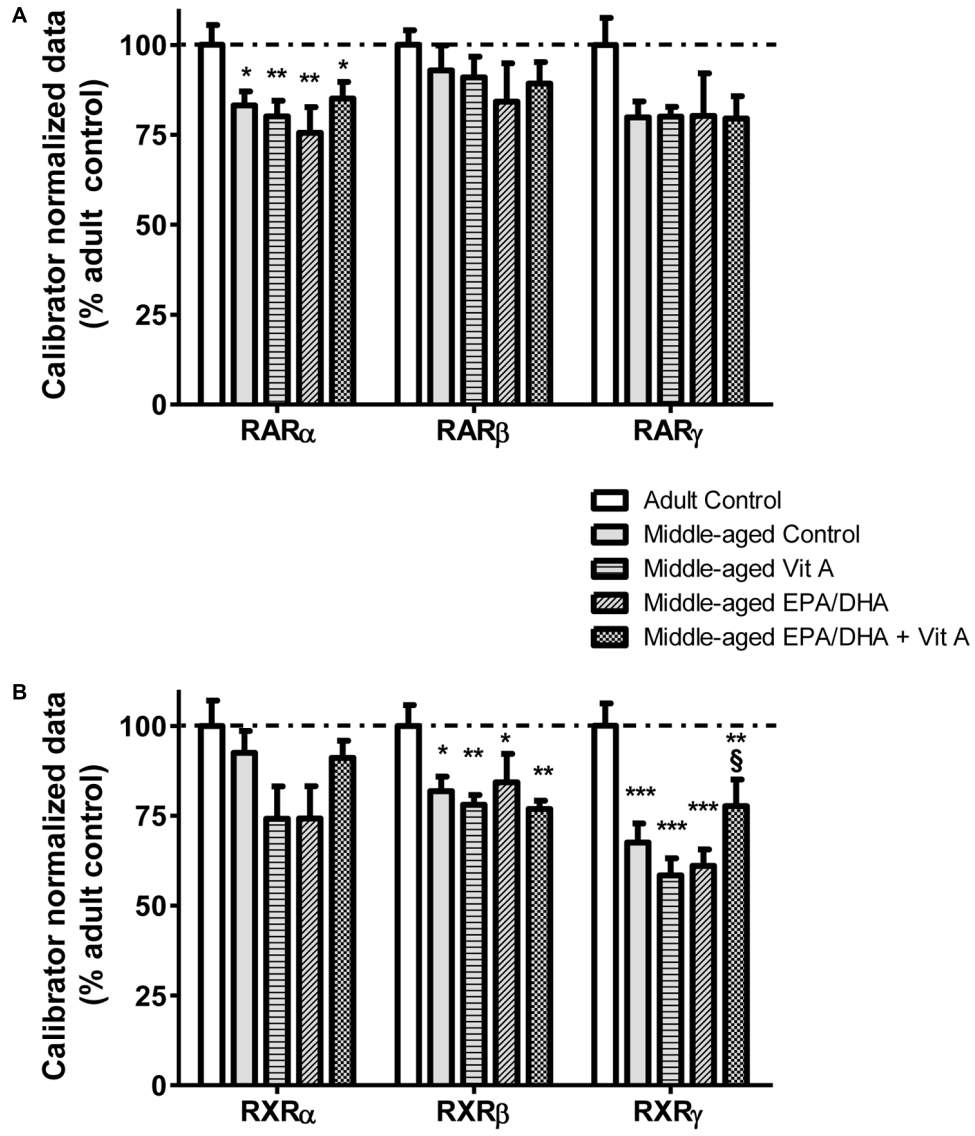

FIGURE 4 | Effect of age and diet (EPA/DHA, Vit A, or EPA/DHA + Vit A) on the mRNA expression of RARα, β, γ (A), and RXRα, β, γ (B). The mRNA levels are expressed as the percentage of Adult control mRNA expression of target/reference ratio normalized by the calibrator. Values are mean ± SEM, $n = 8$–10 rats per group. Data were analyzed by one-way ANOVAs followed by the Fischer PLSD *post hoc* test. Signs indicate values different from Adult control: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ and from middle-aged Vit A and middle-aged EPA/DHA: §$p < 0.05$.

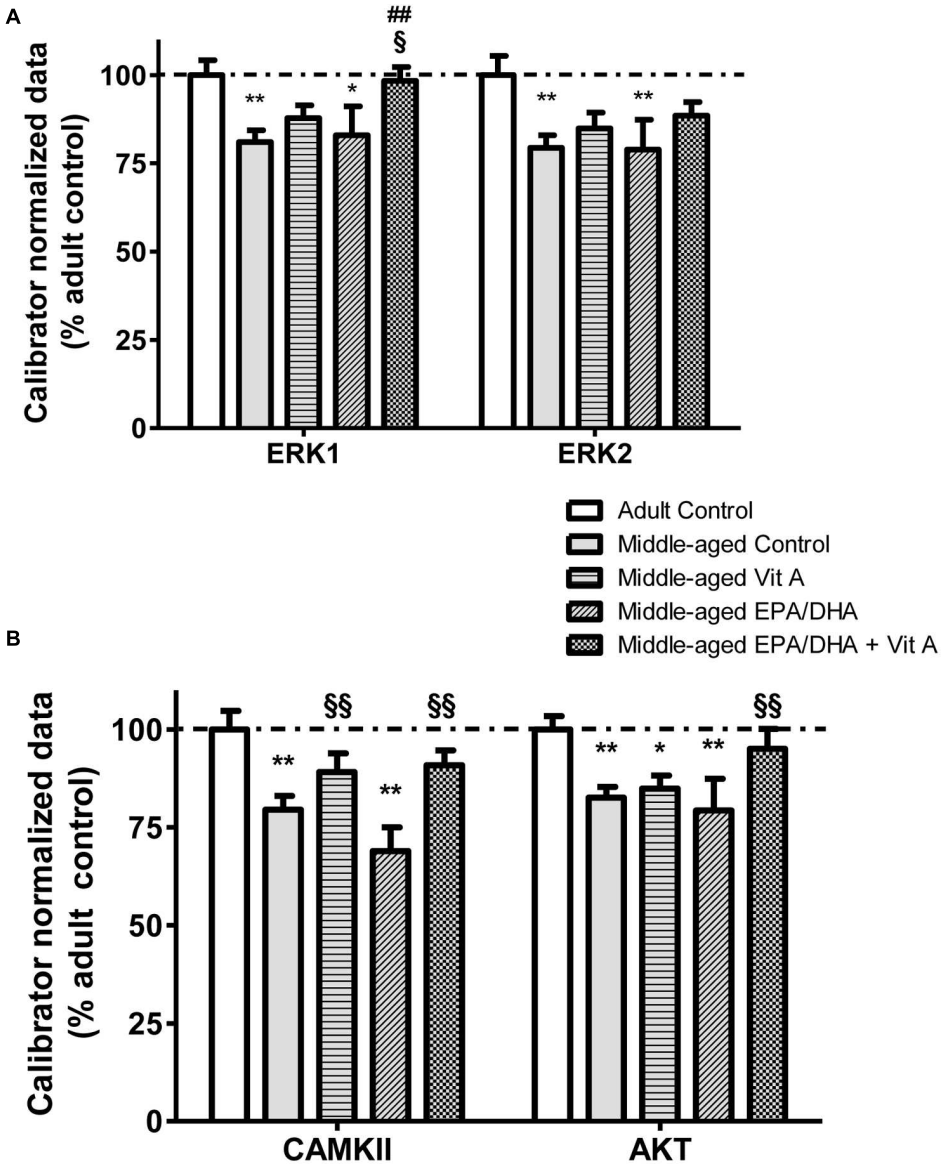

FIGURE 5 | Effect of age and diet (EPA/DHA, Vit A, or EPA/DHA + Vit A) on the mRNA expression of ERK1, ERK2 (A) and CAMKII and AKT (B). The mRNA levels are expressed as the percentage of Adult control mRNA expression of target/reference ratio normalized by the calibrator. Values are mean ± SEM, $n = 8$–10 rats per group. Data were analyzed by one-way ANOVAs followed by the Fischer PLSD *post hoc* test. Signs indicate values different from Adult control: $^{*}p < 0.05$; $^{**}p < 0.01$, from middle-aged control: $\#\#p < 0.01$ and from middle-aged EPA/DHA: $\S p < 0.05$; $\S\S p < 0.01$.

2 (Goodman et al., 2012). Therefore an age-related decrease in serum retinol concentration could lead to a decrease in RA synthesize in the brain, leading to a decrease in RAR and RXR expression in the hippocampus as previously shown in vitamin A deficiency models (Husson et al., 2004; Navigatore-Fonzo et al., 2013).

In addition, disruption of the RXR signaling pathway could be due to a potential decrease in intracellular DHA bioavailability. Indeed, besides the decrease in the DHA level in hippocampal membranes of aged rats, it has been reported a decrease in the independent phospholipase A2 (iPLA2) mRNA expression in the hippocampus of 24-month-old rats compared to 4-month-old rats (Aid and Bosetti, 2007). Knowing that iPLA2 is involved in the release of DHA from membrane phospholipids, this could induce a decrease in the content of DHA in the cell which notably can activate the RXR (de Urquiza et al., 2000). Several studies support the hypothesis that RXRγ plays a key role in learning and memory processes. Indeed, using a gene microarray approach, Blalock et al. (2003) have shown an age-related down-regulation of RXRγ mRNA level in hippocampal CA1 region positively correlated with impaired hippocampus-dependent memory performance. Furthermore, RXRγ knock-out mice display working memory impairments and a pharmacological administration of unesterified DHA improves working memory

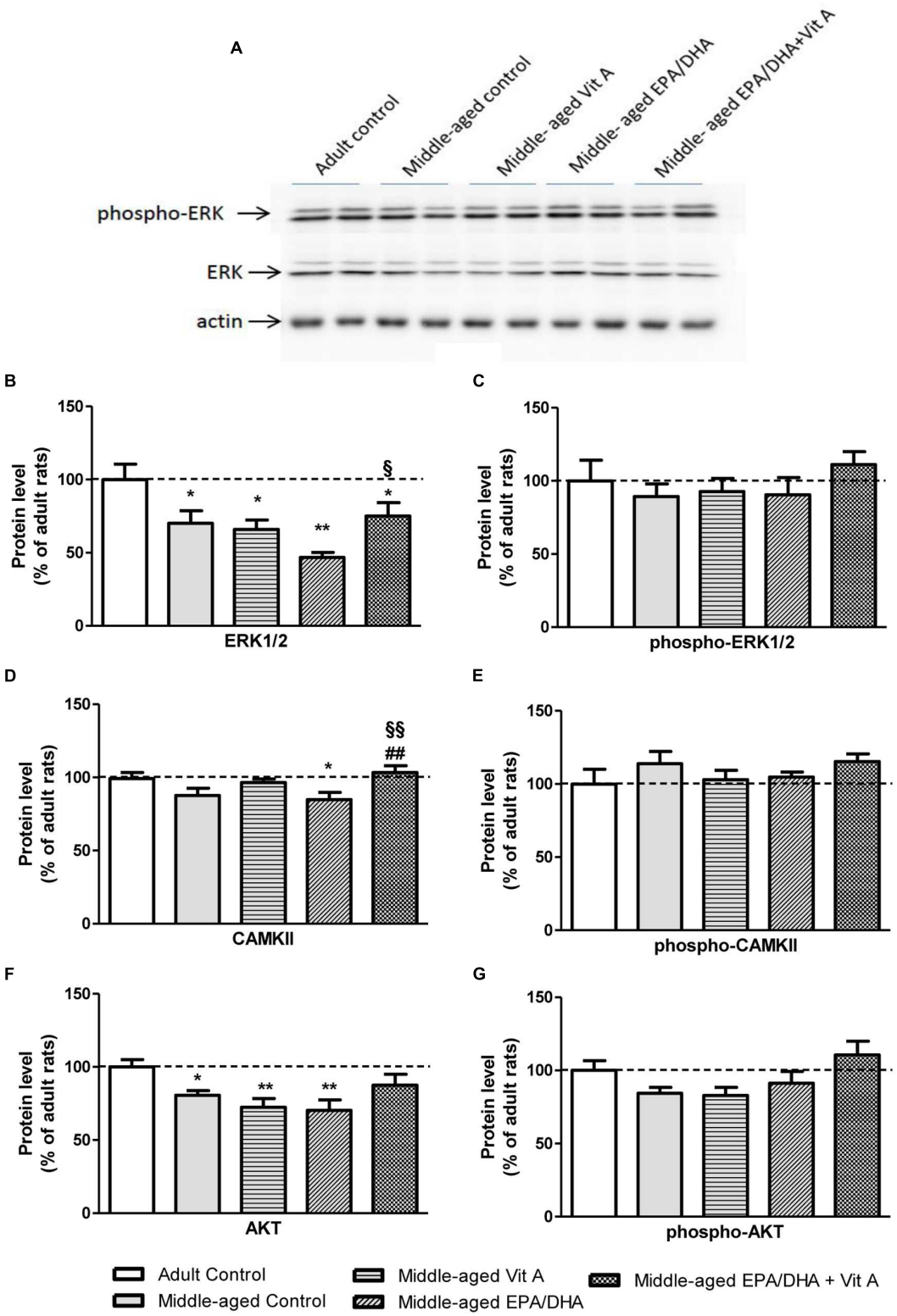

FIGURE 6 | Effect of age and diet (EPA/DHA, Vit A, or EPA/DHA + Vit A) on protein kinase expression in the hippocampus analyzed by Western blot. The upper panel **(A)** illustrates representative immunoblots probed for phospho-Erk1/2, Erk1/2 and actin. Hippocampal Erk1/2 **(B)**, phospho-Erk1/2 **(C)**, CAMKII **(D)**, phospho-CAMKII **(E)**, AKT **(F)**, and phospho-AKT **(G)** protein levels were normalized against actin and expressed as a percentage of adult control expression of the same protein. Values are means ± SEM, $n = 6$–9 rats per group. Data were analyzed by one-way ANOVAs followed by the Fischer PLSD *post hoc* test. Signs indicate values different from Adult control: $*p < 0.05$; $**p < 0.01$; from middle-aged control: $\#\#p < 0.01$ and from middle-aged EPA/DHA: §$p < 0.05$; §§$p < 0.01$.

performance of 5-month-old mice *via* the RXRγ activation (Wietrzych et al., 2005; Wietrzych-Schindler et al., 2011). At the mechanistic level, an *in vitro* study showed that a RXR activation induced by a DHA treatment was associated with a stimulation of neuritogenesis in mice neuroblastoma cells (Calderon and Kim, 2007), demonstrating that RXRs are involved in the modulation of synaptic plasticity. These data support the hypothesis that the age-related memory impairments observed in the present study may be closely linked to the disruption of retinoid and n-3 PUFA signaling pathways, involving notably the RXRγ.

Moreover, the present results showed a decrease in hippocampal CAMKII mRNA expression in middle-aged rats. CAMKII is an ubiquitous kinase in the brain, involved in the regulation of the strengthening synaptic transmission and therefore learning and memory processes (Giese and Mizuno, 2013). Since CAMKII mRNA expression is regulated by RA (Chen and Kelly, 1996), this last result supports the hypothesis of a retinoid signaling pathway disruption in the aged brain that may be responsible, in part, of the memory impairments by altering kinase signaling pathways. Furthermore, the present results demonstrated a decrease in mRNA and protein levels of AKT and ERK1/2 which can be activated by the retinoids and the DHA as a result of a non-genomic effect of these nutrients (Eady et al., 2012; Al Tanoury et al., 2013; Jiang et al., 2013). An age-related disruption of AKT and ERK1/2 signaling has already been observed in rodents. Indeed, Simonyi et al. (2003) reported a decrease in ERK2 mRNA expression in the hippocampus of 12-month-old rats compared to 3-month-old rats. Moreover a reduced AKT signaling pathway activation was observed in the hippocampus of 28-month-old rats compared to 4-month-old rats (Jackson et al., 2009) and in the hippocampus of senescence-accelerated mice (Nie et al., 2009; Armbrecht et al., 2014). These two signaling pathways play major roles in brain functioning since AKT signaling pathway is involved in neuronal survival (Kaplan and Miller, 2000) and ERK1/2 signaling pathway plays a role in hippocampus-dependent learning and memory (Xia and Storm, 2012). Thus the concomitant decrease in ERK1/2 and AKT expression in the hippocampus of middle-aged rats could be responsible, in part, of the age-related spatial memory impairments. Interestingly, the phosphorylation levels of these kinases were not modified in middle-aged rats. It should be noted that kinase activation occurs rapidly and transiently following the induction of synaptic potentiation consecutive to the learning process and constitute a short step before protein synthesis necessary to long term memory formation (Davis and Laroche, 2006). The present result could be due to our experimental settings, notably a 24-h delay between the end of the behavioral procedure and the euthanasia.

Effect of Nutritional Supplementations

Middle-aged rats supplemented with vitamin A displayed memory performance significantly above the chance level, confirming the beneficial effect of a long-term vitamin A supplementation on the aged-related spatial reference memory deficits in rats (Touyarot et al., 2013) or relational memory deficits in mice (Mingaud et al., 2008). This beneficial effect on memory occurred in spite of the decrease in serum retinol concentration measured in middle-aged rats supplemented with vitamin A. This result supports the hypothesis that the newly-absorbed retinol could be used directly by the target tissues such as the brain in order to cover RA needs by an *in situ* synthesis, as it was previously suggested (Ross et al., 2009; Goodman et al., 2012; Touyarot et al., 2013).

The main result of the present study highlights for the first time a beneficial additive effect of EPA/DHA and vitamin A supplementation on the reference memory since only the adult and the middle-aged EPA/DHA + Vit A supplemented rats exhibited performance above the chance level in the probe test over 60 s. In order to avoid an extinction effect due to the absence of the platform (Blokland et al., 2004), we also performed analyses only over the first third period of the probe test. In this case, we observed a stronger effect since only the middle-aged EPA/DHA + Vit A supplemented rats exhibited performance closer to those of the adult control rats. On the contrary, the memory performance of either vitamin A or EPA/DHA supplemented rats were still significantly lower than those of adult control rats. Moreover, this combined nutritional supplementation partially alleviated the age-related decrease only in RXRγ mRNA expression in the hippocampus of middle-aged rats. Contrary to our results, Dyall et al. (2010) have shown that a fish oil supplementation for 12 weeks in 25–26 month-old rats reverses the age-related decrease in RARα, RXRα, and RXRβ protein levels in the CA1 and the dentate gyrus of the hippocampus, using an immunohistochemical approach. This discrepancy in fish oil effect on nuclear receptor expression could be explained by the dose or the duration of the fish oil supplementation but also by the age of the rats that are different between the two studies. Moreover, in the present study we measured RAR and RXR expression at the mRNA level in the whole hippocampus contrary to Dyall et al. (2010) who quantified protein levels in discrete hippocampal regions. Therefore, a dilution effect could explain this discrepancy and the use of an *in situ* hybridization approach would permit to compare the two studies. However, this result is consistent with data supporting the major role played by RXRγ in memory processes as discussed above. Moreover, EPA/DHA + Vit A supplemented middle-aged rats exhibited a maintenance of AKT mRNA and protein expression in the hippocampus. AKT signaling pathway is involved in neuronal survival (Kaplan and Miller, 2000) and the beneficial role of DHA on neuronal survival mediated by the AKT signaling pathway has already been reported (Akbar et al., 2005). Vitamin A can also induce the embryonic stem cell renewal *via* an activation of the AKT signaling pathway (Chen and Khillan, 2010). However, to our knowledge, the present study is the first one that demonstrates a combined effect of n-3 LC-PUFAs and vitamin A supplemented diet on AKT in the hippocampus. Furthermore, the two vitamin A supplemented groups exhibited CAMKII and ERK1/2 mRNA and protein levels similar to those measured in the adult control group. According

to the involvement of these kinases in learning and memory, it could be hypothesized that their maintenance during aging could participate to the beneficial effect of vitamin A on the reference memory in middle-aged rats (Giese and Mizuno, 2013). As previously shown for CAMKII, the transcriptional regulation of these kinases could be mediated by retinoids (Chen and Kelly, 1996). Since this is the first study investigating the effects of a combined EPA/DHA and vitamin A supplementation, no experimental data could yet explain this additive effect. However, a study performed in mouse neuroblastoma cells showed that the iPLA2 activity, involved in the release of DHA from membrane phospholipids, in nuclear membrane was induced by a RA treatment (Farooqui et al., 2004). According to this result, it can be hypothesized that vitamin A, the precursor of RA, could potentiate the effects of the EPA/DHA supplementation by increasing intracellular DHA bioavailability leading to a maintenance of RXRγ and kinase signaling pathways necessary for optimal memory processes.

The single EPA/DHA supplementation was not able to prevent the age-related reference memory deficits, as it has already been reported in studies performed with the same diet duration. Indeed, aged rats receiving 140 mg/kg body weight EPA and 109 mg/kg body weight DHA displayed a spatial reference memory enhancement in a eight-arm radial maze (Hashimoto et al., 2015) and aged mice receiving 0.9–23.7 g of DHA/100 g of fatty acids showed an enhancement of maze-learning ability (Lim and Suzuki, 2000). Nevertheless, the present results point out a beneficial effect of n-3 LC-PUFA supplementation on working memory performance assessed over a short retention delay (ITI of 30 s). These results are in accordance with previous data obtained in our laboratory showing that an EPA/DHA supplementation for 4 months in 13-month-old rats prevented the age-related working memory deficits with an ITI of 30 s (Alfos et al., unpublished data).

Interestingly, we demonstrated that the beneficial effect mediated by n-3 LC-PUFA supplementation remained limited since no memory improvement was observed in supplemented middle-aged rats with an ITI of 2 min. There was no effect of the EPA/DHA supplemented diet on the mRNA and the protein levels measured in the hippocampus contrary to the effects observed with the diet enriched with vitamin A, suggesting that the EPA/DHA effect on working memory seems to be mediated by another molecular signaling pathway.

CONCLUSION

Taken together, this study demonstrated impairments in reference memory and working memory, associated with n-3 fatty acid and vitamin A metabolism alterations and a decrease in RXRβ and RXRγ mRNAs and CAMKII, AKT, ERK1/2 expression in the hippocampus of middle-aged rats. Our results highlight for the first time a preventive additive effect of an EPA/DHA- and vitamin A-enriched diet on the age-related decline in reference memory performance. This beneficial effect on memory could be in part mediated both by RXRγ and kinase signaling pathways that were maintained in the hippocampus of middle-aged supplemented rats. Our findings provide new targets within the framework of preventive nutrition to delay brain aging and demonstrate that combinations of dietary nutrients need to be more fully evaluated to determine optimal strategies as recently suggested by Casali et al. (2015) that used a combined therapy with DHA and bexarotene, a RXR agonist, in a mouse model of Alzheimer's disease.

AUTHOR CONTRIBUTIONS

AL, SA, and BB analyzed the data and wrote the manuscript. AL, SA, ER performed the experiments. BB, CV, SL, VP, and SA designed the study and supervised the work.

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Chronic Pyruvate Supplementation Increases Exploratory Activity and Brain Energy Reserves in Young and Middle-Aged Mice

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Numerous studies have reported neuroprotective effects of pyruvate when given in systemic injections. Impaired glucose uptake and metabolism are found in Alzheimer's disease (AD) and in AD mouse models. We tested whether dietary pyruvate supplementation is able to provide added energy supply to brain and thereby attenuate aging- or AD-related cognitive impairment. Mice received ~800 mg/kg/day Na-pyruvate in their chow for 2–6 months. In middle-aged wild-type mice and in 6.5-month-old APP/PS1 mice, pyruvate facilitated spatial learning and increased exploration of a novel odor. However, in passive avoidance task for fear memory, the treatment group was clearly impaired. Independent of age, long-term pyruvate increased explorative behavior, which likely explains the paradoxical impairment in passive avoidance. We also assessed pyruvate effects on body weight, muscle force, and endurance, and found no effects. Metabolic postmortem assays revealed increased energy compounds in nuclear magnetic resonance spectroscopy as well as increased brain glycogen storages in the pyruvate group. Pyruvate supplementation may counteract aging-related behavioral impairment, but its beneficial effect seems related to increased explorative activity rather than direct memory enhancement.

Keywords: Alzheimer's disease, aging, memory, explorative activity, glycogen

INTRODUCTION

One characteristic metabolic change with aging is a blunted glucose response to stress, whether physical, emotional, or cognitive (Gold, 2005). This can be demonstrated as a mismatch between adrenaline (epinephrine) release and subsequent rise in blood glucose levels in aged rats compared to young ones (Gold, 2005). On the one hand, the inability to maintain adequate blood glucose levels upon challenge may lead to insufficient supply of glucose to the brain and cognitive impairment (McNay, 2005). On the other hand, acute glucose infusion has been shown to improve cognitive performance in both rats and humans, especially during a highly demanding

(due to difficulty or duration) task (Gold, 2005). The aging-related susceptibility to episodes with insufficient brain glucose supply is further exacerbated by common age-associated conditions, such as treatment of type 2 diabetes with glucose lowering medications or Alzheimer's disease (AD) associated with reduced levels of glucose transporter 1 and 3 (Liu et al., 2008).

The brain has also a buffer system to counteract sudden decrease in glucose supply. The most important mechanism is glycogen storage in astrocytes (Bélanger et al., 2011), although a recent study suggests that neurons are also capable of storing glycogen (Saez et al., 2014). Recent experimental evidence has established that glycogenolysis in astrocytes is instrumental for some key functions of astrocytes. First, despite efficient uptake of neurotransmitter glutamate by GLT-1 transporter into astrocytes, about 15% of glutamate under this shuttle is used for energy and thus needs to be replenished. This is largely done by pyruvate carboxylase leading to oxaloacetate with further conversion to α-ketoglurate, which then leaves the tricarboxylic acid cycle (TCA) for conversion to glutamate (Hertz et al., 2015). The process is largely abolished by inhibition of glycogenolysis (Hertz et al., 2015). Second, accumulating evidence indicates that the energy for Na^+K^+-ATPase-mediated K^+ uptake derives from astrocytic glycogenolysis (Hertz et al., 2015). Through these two mechanisms, astrocytic glycogen stores may play a pivotal role in controlling excitability of neighboring neurons. This propensity may explain the findings that inhibition of glycogenolysis impairs memory consolidation in neonatal chicks (Gibbs et al., 2007) and rats (Suzuki et al., 2011). Notably, astrocytic glycogen stores are highly dynamic and susceptible to depletion upon physiological challenge. For instance, one-night sleep deprivation in rats leads to ~40% depletion of brain glycogen levels, which recover after 15 h of sleep (Kong et al., 2002). Thus, poor night sleep, a common nuisance at old age and especially AD, may lead to depleted astrocytic glycogen stores and thereby further impair memory.

Neurons are potentially capable of utilizing interstitial lactate as an alternate energy substrate to glucose (Schurr et al., 1988; Amaral, 2013; Ivanov et al., 2014), since even at rest conditions, the interstitial fluid in neocortex and hippocampus contains about 2–3 mM of lactate (Zilberter et al., 2010). However, recent direct *in vivo* measurements in freely moving rats revealed that glucose is preferentially utilized by neurons (Lundgaard et al., 2015), suggesting that lactate may be consumed under conditions of extreme energy demands (Dienel, 2012). Whereas pyruvate is the end product of glycolysis and thus a direct energy substrate for mitochondria, lactate needs to be first converted to pyruvate. The latter reaction depends on the availability of cytoplasmic NAD^+, which may be compromised under metabolic crisis (Zilberter et al., 2015). Pyruvate, and to a lesser extent lactate, incubation prior to glucose deprivation sustained synaptic and metabolic function in hippocampal slices in one study (Shetty et al., 2012). Moreover, pyruvate exposure led to the enhancement of glycogen stores with time, compared to glucose alone. Radiolabeled pyruvate given as a bolus injection could pass the blood–brain barrier (BBB) similarly to glucose and was detected in a large amount in the brain 5 min after the injection (Miller and Oldendorf, 1986; Gonzalez et al., 2005).

Multiple neuroprotective effects of pyruvate after systemic administration have been reported in animal models in the cases of brain injury (Fukushima et al., 2009), ischemia (Kim et al., 2005; Yi et al., 2007), glutamate neurotoxicity (Miao et al., 2011), hemorrhagic shock (Mongan et al., 2003; Su et al., 2013), hydrogen peroxide-induced cell death (Nakamichi et al., 2005), oxygen-glucose deprivation (Ryou et al., 2013), cognitive impairment due to hypoglycemia (Suh et al., 2005), ethanol-induced neurodegeneration (Ullah et al., 2013), and zinc-induced cortical neuronal death (Sheline et al., 2000). In addition, a recent study reported improved spatial memory in the Morris swim task in both 6- and 12-month-old 3xTg AD model mice after repeated i.p. pyruvate injections over months (Isopi et al., 2015).

We have recently reported that 5-week dietary supplementation with pyruvate + β-hydroxybutyrate in the chow reversed impaired tolerance of APPswe/PS1dE9 mice to hypoglycemia, normalized their reduced brain glycogen stores, and attenuated their enhanced neuronal excitability both *in vitro* and *in vivo* (Zilberter et al., 2013). Our subsequent pilot studies showed that a similar effect can be obtained by pyruvate supplementation alone. These findings raised the question whether oral pyruvate supplementation could also provide a means to counteract aging and AD-related memory impairment as alluded to by the study in 3xTg mice (Isopi et al., 2015). However, the previous study used only one behavioral assay (the Morris swim task) and only reported the number of platform crossings and latency in the probe trial without commenting eventual pyruvate effects on non-cognitive factors, such as swimming speed that may affect learning. Since the existing literature on pyruvate mainly focuses on its effects on muscle force and energy metabolism, we wanted to study not only cognitive effects of pyruvate but also its impact on non-cognitive factors, such as spontaneous activity, muscle force, endurance, and anxiety. To this end, we ran three separate experiments with a large behavioral test battery and assessed the direct effects of pyruvate substitution on the indices of brain energy metabolism. The first experiment was run in middle-aged wild-type mice and the second one in adult APPswe/PS1dE9 (APP/PS1) mice that both show only modest memory impairment compared to young adult mice (Minkeviciene et al., 2008). The third experiment addressing alternative non-cognitive mechanisms of pyruvate action was done in adult wild-type mice owing to the limited availability of older wild-type or APP/PS1 mice and to clarify the observed consistency of pyruvate effects independent of age.

MATERIALS AND METHODS

Animals

Experiment 1 comprised 36 male C57Bl/6J mice (breeding at Laboratory Animal Center, University of Eastern Finland, Kuopio, Finland) that started the dietary intervention at 6 months, behavioral testing at 12 months of age, and were euthanized when reaching 13 months. *Experiment 2* comprised 25 male APPswe/PS1dE9 transgenic mice (Jankowsky et al., 2004) that started the dietary intervention at 4.5 months, behavioral testing at 6.5 months of age, and were euthanized at the age of 7 months.

Experiment 3 comprised 20 male C57Bl/6J mice that started the dietary intervention at 3 months, behavioral testing at 5 months of age, and were euthanized at 6.5 months of age. In addition, another group of 19 male C57Bl/6J mice were tested with acute pyruvate vs. saline injection at the age of 5 months. The mice came from a local breeding colony at University of Eastern Finland that was based on breeder mice from Johns Hopkins University (Baltimore, MD, USA). The line was originally generated as C3H × C57Bl/6J hybrid but had been back-crossed to C57Bl/6J strain for 18 generations.

The animals were group-housed until the behavioral tests in controlled environment (temperature 22 ± 1°C, light 0700–1900 hours, and humidity 50–60%), and food and water were freely available. All behavioral tests were conducted during the light phase. The experiments were conducted according to the Council of Europe (Directive 86/609) and Finnish guidelines and approved by the Animal Experiment Board of Finland.

Treatment

Chronic Pyruvate Administration

The test group (PYR) received experimental chow supplemented with 0.6% (w) of Na-pyruvate (Safe Diets, Augy, France). The control group (STD) received the same basic rodent chow (A04, Safe Diets). With the average food intake of 4 g, this corresponds to 800 mg of pyruvate/day, which is at the upper range of effective pyruvate doses in earlier *in vivo* studies (Suh et al., 2005; Fukushima et al., 2009; Isopi et al., 2015).

Acute Pyruvate Administration

The mice received Na-pyruvate (Sigma, St. Louis, MO, USA) 500 mg/kg i.p. or the same molar concentration of NaCl (260 mg/kg i.p.). This single dose affords neuroprotection against cortical concussion injury and increases brain glucose and pyruvate levels as measured by *in vivo* microdialysis (Fukushima et al., 2009). All cage labels about the treatment groups were coded so that the researchers running behavioral tests or assays on postmortem samples were blinded as to the treatment.

Behavioral Testing

Morris Swim Task (Water Maze)

Spatial learning and memory was tested with Morris swim navigation task. The apparatus consisted of a white plastic pool with diameter of 120 cm and of transparent platform (10 cm × 10 cm) submerged 1.0 cm below the water surface. Water temperature was kept at 20 ± 5°C throughout the testing, and 8–10 min recovery period in a warmed cage was allowed between all trials. Before actual navigation task, the mice were pretrained (2 days) to find and climb onto the submerged platform, aided by a guiding alley (1 m × 14 cm × 25 cm) with opaque walls preventing any spatial clues to be seen. In the learning phase (days 1–4), five 60-s trials per day were conducted with the hidden platform. The platform location was kept constant and the starting position varied between four constant locations at the pool rim, with all mice starting from the same position and nose pointing toward the wall in any single trial. If a mouse failed to find the escape platform within 60 s, it was placed on the platform for 10 s by the experimenter. Also, when mice found the platform independently, they were allowed to stay on the platform for 10 s. On day 5, the last trial (fifth) was run without the platform to test the search bias that was followed for 60 s. The experimenter marked the start of each trial using a remote controller and the trial ended automatically when a mouse landed on the platform. Swim paths and other parameters were tracked and recorded by video tracking system EthoVision XT 7 (Noldus, Wageningen, Netherlands).

Wall-swimming tendency (thigmotaxis) was assessed by calculating the time the mouse spent in the outer zone within 10 cm of the wall. The search bias during the probe trial was measured by calculating the time the mice spent in the vicinity of the former platform position. We defined this as a target area, centered on the platform, with a diameter of 30 cm. This target area comprised 6.25% of the total surface, which means that a mouse swimming randomly in the pool would be expected to spend 3.75 s in the target area during the 60-s probe trial.

Odor Recognition Task

The task is based on individual specific odors of mice. Before the test, each mouse to be tested got two small (diameter 20 mm) odorless balls made of birch wood (Step Systems Oy, Lahti, Finland) on the cage bedding. Similarly, a specifically assigned odor donor mouse got several of these balls. These were left overnight to get impregnated with the mouse odor, and in the case of the test mice to let them get adopted to the presence of the balls in their home cage. The wooden balls were removed in the morning. After 2–4 h, the mouse was presented with two balls, one impregnated with its own odor and the other one from the cage of the donor mouse. The test was replicated the next day with one ball of the mouse's own odor and a second ball impregnated with the odor of another unfamiliar mouse. We measured the total time sniffing (the nose pointing to the ball at a distance <2 cm) during a 120-s test session. Only if the total sniffing time exceeded 10 s, we also counted the odor preference as the percent of total time that the mouse was sniffing the ball with unfamiliar odor.

Passive Avoidance

The test was conducted in a shuttle box (*L* 44 cm × *W* 17 cm × *H* 25 cm; Med Associates Inc., St. Albans, VT, USA) that was divided by a partition into two halves. One half was open and well-lit, while the other half was closed by black plastic walls and cover plate. Both compartments had a similar grid floor. The partition had an arch-shaped 6 cm wide opening that could be closed by a slide door. On day 1, the mouse got first 5 min to freely explore both compartments. Then, the mouse was placed in the open compartment and the slide door was closed for 30 s. Once the door was opened, the experimenter took the time for the mouse to enter the dark compartment, closed the opening, and delivered two mild foot shocks (0.30 mA, 2 s, interval 2 s). After the foot shock, the mouse was immediately returned to its home cage. On day 2, the time to enter the dark compartment was measured with a stop watch until a cutoff time of 180 s. No shocks were delivered on day 2.

Spontaneous Explorative Activity

Spontaneous explorative activity was assessed by using an automated activity monitor (TruScan, Coulbourn Instruments, Whitehall, PA, USA) based on infrared photo beam detection. The system consisted of an observation cage with white plastic walls (26 cm × 26 cm × 39 cm) and two frames of photo detectors enabling separate monitoring of horizontal (XY-move time) and vertical activity (rearing). The test cage was cleaned with 70% ethanol before each mouse to avoid odor traces. The following parameters were measured during a 10-min session: ambulatory distance (gross horizontal locomotion) and rearing time. The recording was repeated 48 h later to assess habituation to the environment.

Marble Burying Test

To assess neophobia and general housekeeping activity, the home cage floor was filled with double amount of clean bedding material and nine glass marbles (diameter 1 cm) were inserted in a 3 × 3 array onto the bedding. Visible marbles were then counted after 24 h.

Elevated Plus Maze

The maze, as the name implies, had a shape like a plus sign and consisted of a square platform (5 cm × 5 cm) and four arms (30 cm × 5 cm, two open and two closed), all made of black plastic but covered with white plastic to help video monitoring. The maze platform was 50 cm above the floor in a dimly lit room. Each mouse was placed on the center platform facing an open arm and allowed to explore the maze for 5 min. A video camera hanging from the ceiling recorded the session, which was analyzed offline with EthoVision XT 7 software. The time spent in the open and closed arms, the total distance, and speed in 30 s bins were calculated as well as the percentage of total time spent on the open arms to index anxiety. Only mice making more than five moves between arms during the test were included in the statistical analysis for % time on the open arms.

Open Field

The test was conducted in the dry pool (diameter 120 cm) used for Morris swim task. The mouse was placed in the arena center and its movement was recorded for 10 min. Using EthoVision XT 7 software, we calculated the total distance traversed, speed in 30-s bins, and the % time spend in the arena center (diameter 40 cm).

Pain Threshold

The test was conducted 2 weeks after the passive avoidance test in the same apparatus. The mouse was first given 3 min to fully familiarize with the environment and calm down. Then, electric shocks were delivered every 30 s with gradually increasing intensity (0.05, 0.06, 0.08, 0.10, 0.12, 0.15, 0.2, and 0.3 mA) until the mouse reacted by jumping. Before that, the current intensity eliciting a startle response was also recorded.

Grip Force Test

The grip force was measure using a simple spring scale (Kouluelektroniikka Oy, Rauma, Finland) with a metal grid for the mouse to grip with both front paws. The scale was positioned at a 45° angle at the edge of a table. The mouse was gently pulled by the tail until it could no longer withhold the grip. The test was repeated five times every 30 s and the best reading was recorded.

Treadmill Test

The apparatus was a treadmill designed for mice (LE8710R, Bioseb, France) with adjustable speed and inclination. The mouse was motivated to keep running by a gentle electric shock (0.15 mA during training and 0.2 mA during testing) at the proximal end of the belt. The mice were familiarized with the procedure in two 10-min sessions during 2 days. On the first day, the belt was rolling at a fixed speed of 15 cm/s; on the second day, the speed was increased gradually from 15 to 25 cm/s. On the test day, the speed of the belt was increased from 15 cm/s with a step of 5 cm/s every minute until the speed of 35 cm/s was reached. When the mouse took five electric shocks with 1-s intervals or made only minimum spurts to avoid the shocks, it was judged to be exhausted and the test was terminated.

Metabolic Assays

Histochemical Assay – Periodic Acid Schiff Staining

In Experiment 1, after behavioral testing mice were deeply anesthetized with pentobarbiturate-chloral hydrate cocktail (60 mg/kg i.p. each) and perfused transcardially with 50-ml ice-cold saline (10 ml/min) for 5 min. The brain was removed and split sagittally. The left hemisphere was immersion fixed in 4% paraformaldehyde for 4 h, transferred to a 30% sucrose solution overnight, and stored in a cryoprotectant in −20°C for later histology. The hemisphere was cut on a freezing slide microtome into 20-µm coronal sections. Periodic acid schiff (PAS) staining was done on two adjacent sections at the level of mid-hippocampus (A −2.7 from bregma) in eight STD mice and eight PYR mice. First, the sections were hydrated to water and after that oxidized in 0.5% periodic acid solution for 5 min. After rinsing in distilled water, the sections were soaked in Schiff reagent for 1 min. To get right dark pink color, sections were washed in lukewarm water for 5 min. Dehydration was done with rising alcohol series and coverslipping with DePeX mounting medium. Sections were photographed using the Zeiss Imager M2 microscope (Zeiss, Oberkochen, Germany) and the attached AxioCam ERc 5s camera with a 2.5× objective. Images were transformed to grayscale in Photoshop CS6 software (Adobe Systems Inc., San Jose, CA, USA), which was also used for image analysis. The mean optic densities values (0 for black to 255 for white) were determined from four regions of interest: DG molecular layer, CA1 all layers, white matter (alveus + corpus callosum), and visual cortex.

Enzymatic Analysis of Glycogen Content

At the end of Experiment 3, five young adult mice on STD and five on PYR diet were euthanized with a microwave radiator (5 W, 0.85 s, Muromachi Kikai Co., Ltd., Tokyo, Japan) and the brains were removed, the cortex dissected out, and stored at –70°C. One STD mouse had to be discarded because of failed procedure. The quantitative amounts of glycogen in the tissue were assessed in a coupled enzymatic assay by measuring the production of NADPH in the conversion of glucose-6-phosphate to 6-phosphogluconolactone as described by Brown et al. (2003). To prepare the samples for the assay, the tissues were

homogenized by sonication in ice-cold 70% ethanol. The samples were centrifuged at 20,000 × *g* for 10 min and the supernatant was discarded. The remaining pellet was washed twice with ethanol and once with water and resuspended in an acetate buffer (pH 5). Aliquots of the homogenized pellets were adjusted to pH 2 by the addition of HCl. Amyloglucosidase dissolved in an acetate buffer (pH 5) was added to the acidified homogenates before incubation at 37°C and 50 rpm for 1 h in an alkaline solution (37.1 mM Tris base, 0.007% sodium azide, and 13.8 mM HCl) to ensure pH 7–8, i.e., the pH optimum for hexokinase and glucose-6-phosphate dehydrogenase. The reaction was initiated by the addition of hexokinase and glucose-6-phosphate dehydrogenase to final concentrations of 1.52 and 0.54 U/ml, respectively. The fluorescence was measured before addition of the enzymes and following 40 min of incubation at room temperature, using 360 and 415 nm as excitation and emission wavelengths, respectively. Glucose in the concentration range of 20–200 μM was used as a standard, and the amount of glycogen in the samples is expressed as nanomoles of glucose per milligram of protein. The protein amounts in the samples were determined using the BCA method employing bovine serum albumin as standard.

^{1}H NMR Analysis

After behavioral tests, mice of Experiment 2 (APP/PS1 mice at 7 months of age) were euthanized with cervical dislocation, the brain was rapidly removed, frontal 1/3 was cut off and snap frozen in liquid nitrogen, and stored at –70°C. We chose a frontal cut instead of hippocampal preparation to keep the postmortem delay to the minimum (now 80–100 s). Weighed tissue samples were subjected to perchloric acid (PCA) extraction. The frozen tissue samples were transferred into a precooled mortar and grounded by a pestle with liquid nitrogen to fine powder. The ground tissue powder was transferred into a cold 2-ml Eppendorf tube, and 1.0 ml of cold 0.9M PCA was added. The obtained suspension was homogenized by vortex mixing and sonication (15 min indirect sonication) in a water/ice bath. The supernatant was separated after centrifugation (10 min at 16,000 × *g* + 4°C) and neutralized using 2M potassium hydroxide. Precipitation (potassium perchlorate) was removed by centrifugation (15 min at 16,000 × *g* + 4°C) and the obtained supernatant was freeze-dried. Prior to the NMR measurements, neutralized, freeze-dried PCA extracts were dissolved to deuterium oxide containing 200 μM sodium 3-(trimethylsilyl)propionate-2,2,3,3-d4 (TSP) as a reference substance.

^{1}H NMR spectra of the extracted samples were recorded on a Bruker Avance III HD 600 NMR spectrometer operating at 600.28 MHz and equipped with a Prodigy TCI 5 mm cryogenically cooled probe head. Standard 1D ^{1}H NMR spectra were recorded with 64k data points using 32 transients and applying a standard Bruker zg pulse sequence. The acquisition time was 4.3 s and the relaxation delay 5.7 s. The spectra were measured at 295 K. The free induction decays (FIDs) were multiplied by an exponential window function with a 0.3-Hz line broadening. Identification of the metabolite signals was based on the data in the literature (Govindaraju et al., 2000; de Graaf et al., 2011) or in-house databases and spiking experiments. The quantification of the identified metabolites was done using quantitative quantum mechanical spectral analysis (qQMSA) approach with the program qQMTLS (Tiainen et al., 2014). The concentrations are reported as nanomole per milligram (wet tissue weight).

Statistics

All statistics were calculated using IBM SPSS Statistics 19 software (IBM Corporation, NY, USA). Behavioral tests that were repeated on two or more days were analyzed by ANOVA for repeated measures using the diet as between-subject factor. Otherwise, the comparison of the diet groups was done with Student's *t*-test, with the exception of passive avoidance for young animals where more than half of the animals reached the cutoff time. Here, non-parametric Mann–Whitney test was applied. Data are expressed as group mean ± SEM. Threshold for statistical significance was set at $p < 0.05$.

RESULTS

Experiment 1: Chronic Pyruvate Supplementation in Middle-Aged Wild-Type Mice

Improved Spatial Learning after Chronic Pyruvate

At 12 months of age (and after 6 months on test diet), spatial learning was tested in the Morris swim task. Mice on pyruvate supplementation (PYR) acquired the task faster than mice on standard chow (STD) as evidenced by shorter escape latency across five test days ($F_{1,34} = 7.3$, $p = 0.01$; **Figure 1A**). PYR mice were also faster to quit the inefficient strategy to search for an escape through the pool wall (thigmotaxis) than STD mice ($F_{1,34} = 4.6$, $p = 0.04$; **Figure 1B**). Neither measure showed a diet × day interaction (escape latency, $p = 0.15$; thigmotaxis, $p = 0.36$). The last swim on day 5 was a probe trial without the platform to assess the spatial search bias. PYR mice tended to spend more time in the vicinity of the former platform position that STD mice, but the difference did not reach statistical significance ($t_{34} = 1.6$, $p = 0.11$; **Figure 1C**).

Improved task acquisition, but only a modest augmentation, of memory retention can imply faster learning but also some non-cognitive effect of pyruvate supplementation, such as enhanced endurance in the swim task that is physically strenuous. To control this possibility, we compared swimming speeds between the groups. There was no overall difference in swimming speed across days ($F_{1,34} = 0.5$, $p = 0.48$). We also looked for the possibility of less fatigue on repeated trials in the PYR group, but a separate analysis of swimming speeds on the last trial of the four task acquisition days did not differ between the groups ($p = 0.61$).

Enhanced Odor Recognition but Impaired Fear Conditioning after Chronic Pyruvate

To assess the generalization of augmented learning effect of chronic pyruvate, the same 12-month-old mice were subjected to odor recognition and passive avoidance task. In the odor recognition task, the total sniffing time on day 1 was 45.8 ± 6.1 s (mean ± SEM) for STD mice and 57.1 ± 6.2 s for PYR mice, and on day 2, 42.3 ± 6.1 s for STD mice and 49.1 ± 6.0 s for PYR mice. There was no diet main effect on total sniffing time ($F_{1,34} = 1.4$, $p = 0.24$). However, the odor preference was significantly stronger across the two test sessions in PYR mice compared to STD mice ($F_{1,30} = 4.7$, $p = 0.04$; **Figure 1D**).

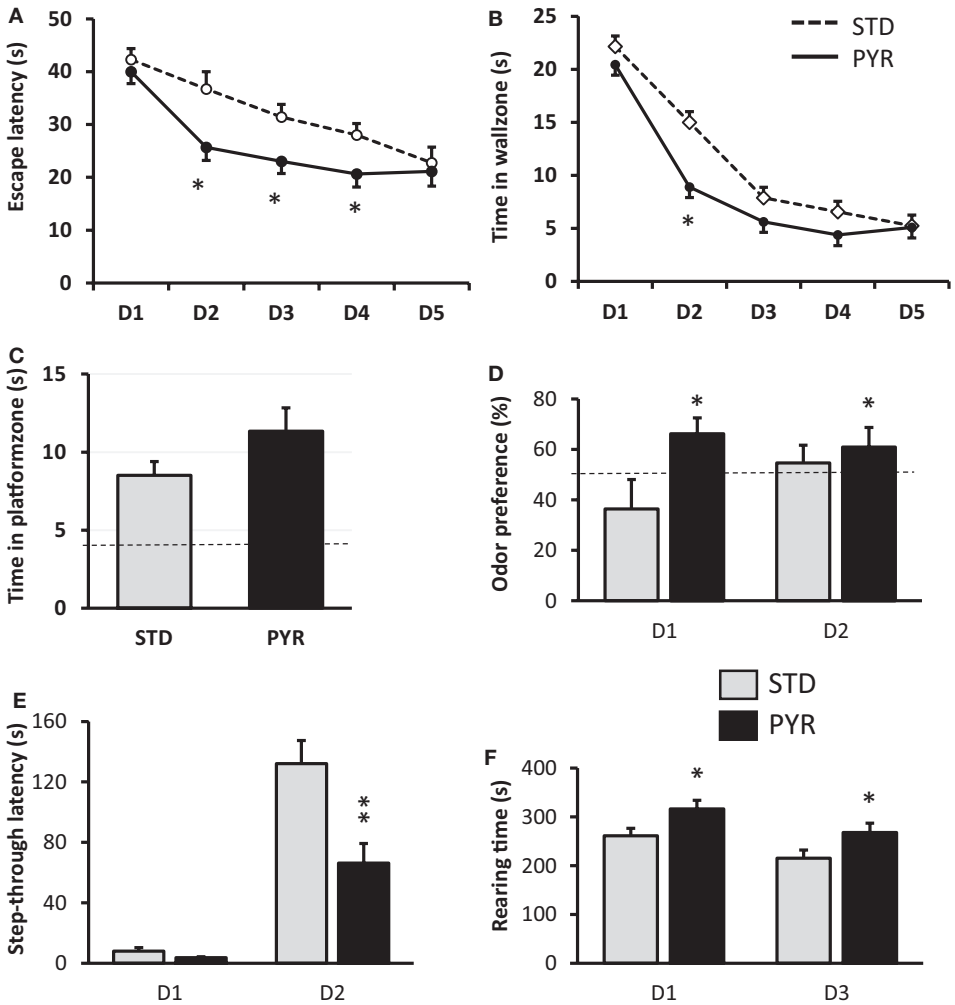

FIGURE 1 | **Behavioral studies in 12-month-old wild-type mice treated with standard chow (STD) or pyruvate-supplemented chow (PYR) for 6 months**. **(A)** Escape latency during 5 days of Morris swim task acquisition. PYR group differs from STD group on this day (*$p < 0.05$, *t*-test). **(B)** Time in the wall zone (10 cm wide). PYR group differs from STD group on this day (*$p < 0.05$, *t*-test). **(C)** Time in the platform zone (15 cm radius from the former platform center) during the probe test on day 5. **(D)** Time preference to explore the odor of an unknown mouse over one's own odor on two test days. The dashed line indicates chance level (50%). PYR group differs from STD group across days (*$p < 0.05$, ANOVA-RM). **(E)** Step-through latency in the passive avoidance task, cutoff 180 s. Day 1 is the training day and day 2 the test day after fear conditioning. PYR group differs from STD group on day 2 (**$p < 0.01$, *t*-test). **(F)** Rearing time in the transparent novel test cage on days 1 and 3, test duration 10 min of each day. PYR group differs from STD group across days (*$p < 0.05$, ANOVA-RM). Group Means ± SEMs are shown.

In the passive avoidance task, the groups did not differ in their latency to enter the dark compartment before conditioning ($t_{34} = 1.8$, $p = 0.09$), although mice in the PYR group tended to enter sooner than STD mice. However, after the fear conditioning, STD mice waited much longer than PYR mice before entering the dark compartment now associated with a foot shock ($t_{34} = 3.3$, $p = 0.002$; **Figure 1E**).

Increased Spatial Exploration but No Effect on Locomotion or Object Neophobia by Chronic Pyruvate

To better understand the ostensibly discrepant result of improved spatial learning and odor recognition but impaired retention of fear conditioning after chronic PYR administration, we ran a battery of control behavioral tasks to assess PYR effects on spontaneous exploration, locomotor activity, and object neophobia.

Although the ambulatory distance was slightly higher in the PYR group, the difference was not significant ($F_{1,34} = 2.0$, $p = 0.17$), and both groups showed robust reduction in the distance traversed during the second exposure to the novel test cage. In contrast, there was a significant difference in the rearing time between the groups ($F_{1,34} = 6.4$, $p = 0.02$), such that PYR mice spent more time rearing on both sessions (**Figure 1F**). Rearing is typically related to exploration of a new environment.

The marble burying test did not reveal significant differences in object neophobia between the diet groups. The STD mice left

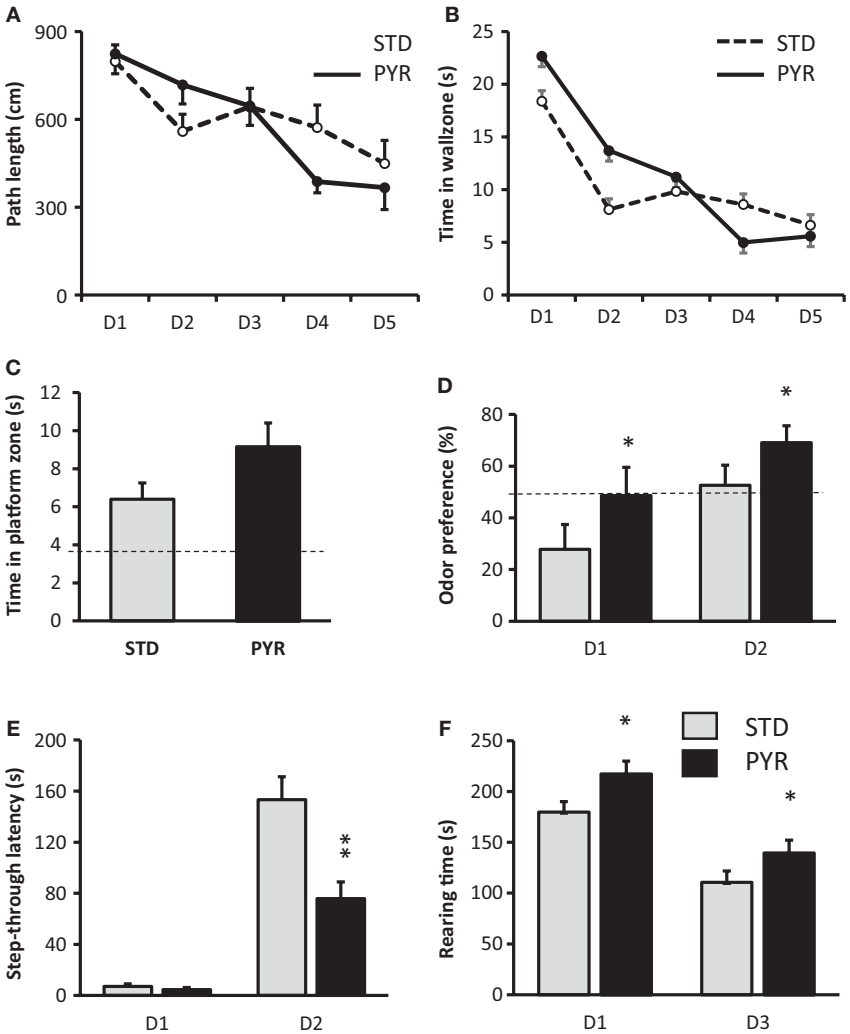

FIGURE 2 | Behavioral studies in 6.5-month-old APPswe/pS1dE9 mice treated with standard chow (STD) or pyruvate-supplemented chow (PYR) for 2 months. **(A)** Escape latency during 5 days of Morris swim task acquisition. **(B)** Time in the wall zone (10 cm wide). **(C)** Time in the platform zone (15 cm radius from the former platform center) during the probe test on day 5. **(D)** Time preference to explore the odor of an unknown mouse over one's own odor on two test days. The dashed line indicates chance level (50%). PYR group differs from STD group across days (*$p < 0.05$, ANOVA-RM). **(E)** Step-through latency in the passive avoidance task, cutoff 180 s. Day 1 is the training day and day 2 the test day after fear conditioning. PYR group differs from STD group on day 2 (**$p < 0.01$, *t*-test). **(F)** Rearing time in the transparent novel test cage on days 1 and 3, test duration 10 min of each day. PYR group differs from STD group across days (*$p < 0.05$, ANOVA-RM). Group Means ± SEMs are shown.

5.0 ± 0.5 marbles out of nine visible and the PYR mice 4.8 ± 0.5 ($p = 0.74$).

Experiment 2: Chronic Pyruvate Supplementation in 6.5-Month-Old APP/PS1 Mice

Modest Improvement in Spatial Learning after Chronic Pyruvate

At 6.5 months of age (and 2 months on test diet), the diet groups differed significantly in their swimming speed across all test days (STD group faster; $F_{1,23} = 7.1$, $p = 0.01$; data not shown). Therefore, the task acquisition was assessed by the swim path length. The path length did not differ between the groups ($F_{1,23} = 1.0$, $p = 0.76$; **Figure 2A**). No overall difference was found in the time spent in the wall zone, either ($F_{1,23} = 0.5$, $p = 0.48$; **Figure 2B**). However, the diet × day interaction approached significance for the path length ($F_{4,92} = 2.2$, $p = 0.08$) and was significant for the time in wall zone ($F_{4,92} = 2.6$, $p = 0.04$; **Figure 2B**). In the probe test on day 5, PYR mice tended to swim more time in the vicinity of the former platform location ($t_{23} = 1.8$, $p = 0.09$; **Figure 2C**).

Enhanced Odor Recognition but Impaired Fear Conditioning after Chronic Pyruvate

In the odor recognition task, there was no diet main effect on total sniffing time ($F_{1,23} = 0.1$, $p = 0.81$). However, the odor preference was significantly stronger across the two test sessions in PYR mice

compared to STD mice ($F_{1,18} = 6.5$, $p = 0.02$; **Figure 2D**). To be precise, on day 1, APP/PS1 mice on the STD diet actually showed aversion toward the odor of an unknown mouse, while showing no preference on day 2. In contrast, mice on PYR diet showed no preference on day 1 but a clear preference toward the novel odor on day 2 (**Figure 2D**).

In the passive avoidance task, the groups did not differ in their latency to enter the dark compartment before conditioning ($t_{23} = 1.0$, $p = 0.35$). However, after the fear conditioning, STD mice waited much longer than PYR mice before they entered the dark compartment now associated with a foot shock ($t_{23} = 3.5$, $p = 0.002$; **Figure 2E**).

Increased Spatial Exploration, No Effect on Locomotion but Decreased Object Neophobia by Chronic Pyruvate

The ambulatory distance did not differ between the diet groups ($F_{1,23} = 0.3$, $p = 0.60$), and both groups showed robust reduction in the distance traversed during the second exposure to the novel test cage. In contrast, there was a significant difference in the rearing time between the groups ($F_{1,23} = 6.0$, $p = 0.02$), such that PYR mice spent more time rearing on both sessions (**Figure 2F**). Rearing is typically related to the exploration of a new environment.

The pattern of behavioral effects of PYR was thus almost identical in 12-month-old wild-type mice and 6.5-month-old APP/PS1 mice. The only notable differences were the acquisition phase of Morris swim task, where 6.5-month-old mice on PYR did not show improvement, and marble burying test, in which APP/PS1 mice on PYR diet left more marbles visible than STD mice [STD: 2.8 ± 0.4 marbles; PYR: 4.6 ± 0.6 ($p = 0.03$)].

Experiment 3: Pyruvate Supplementation in 5- to 6-Month-Old Wild-Type Mice

To shed light on the robust and reproducible finding that chronic PYR increased spatial learning and odor recognition but clearly impaired fear conditioning, we once again evaluated the possible non-cognitive effects of chronic PYR administration in young adult wild-type mice (the same C57Bl/6J background as in APP/PS1 mice). First, at the age of 5 months (and 2 months on test diet), we repeated the passive avoidance task. The diet groups did not differ in their latency to enter the dark compartment before conditioning ($t_{18} = 0.3$, $p = 0.76$). In contrast to middle-aged wild-type or adult APP/PS1 mice, all but one young adult wild-type mouse in the STD group waited until the cutoff time before entering the dark compartment. However, in the PYR group, half of the mice entered before the cutoff time ($p < 0.05$, Mann–Whitney *U*-test, **Figure 3A**). So, the chronic pyruvate effect was present also in young adult wild-type mice.

To rule out a pyruvate effect on nociception, we measured the minimum current for the foot shock to elicit startle and jump responses. There was no effect of the diet on either the startle response (STD: 0.086 ± 0.003 mA; PYR: 0.083 ± 0.005; $p = 0.60$) or the jump response (STD: 0.21 ± 0.02 mA; PYR: 0.24 ± 0.02; $p = 0.32$).

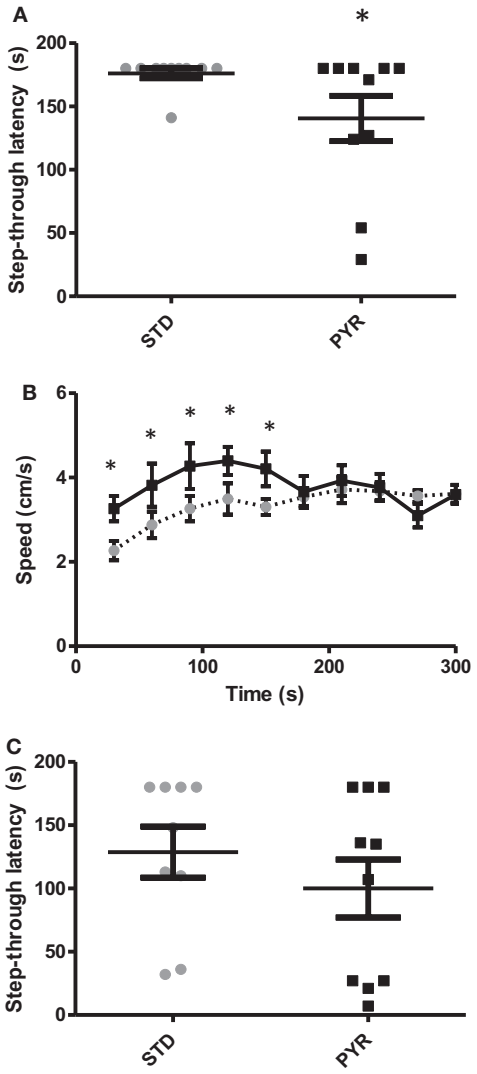

FIGURE 3 | Behavioral studies in young adult wild-type mice. **(A)** Group means and individual values for step-through latency in the passive avoidance test run after chronic (2.5 months) dietary intervention. PYR group differs from STD group (*$p < 0.05$, Mann–Whitney *U*-test). **(B)** Running speed in the elevated plus maze plotted for each 30-s bin. During the first test half (2.5 min), mice on chronic PYR ran faster than STD mice (*$p < 0.05$, ANOVA-RM). **(C)** Group means and individual values for step-through latency in the passive avoidance test in the acute test groups. After the learning phase, half of the mice received Na-pyruvate at 500 mg/kg i.p., while other half received 260 mg/kg NaCl.

To assess the potential role of PYR on anxiety, we ran the standard elevated plus maze test. PYR mice moved a longer distance during the 5-min test than STD mice (STD: 9.9 ± 0.4 m; PYR: 11.9 ± 0.5 m; $t_{17} = 3.3$, $p = 0.004$). A closer analysis on the running speed revealed that PYR mice run at a higher velocity during the first 2.5 min ($F_{1,18} = 6.0$, $p = 0.025$), but then the running speed became even between the treatment groups ($p = 0.97$; **Figure 3B**). However, the % time spent on open arms, which is considered the strongest indicator of anxiety in this

task, did not differ between the diet groups (STD: 21.6 ± 3.5%; PYR: 26.6 ± 4.5%; $t_{18} = 0.9$, $p = 0.40$). We also ran the open field test as an additional measure of anxiety. The relative time spend in the field center during 10 min did not differ between the treatment groups (STD: 4.7 ± 0.7%; PYR: 4.5 ± 0.8%; $t_{18} = 0.2$, $p = 0.86$). However, we could see the same pattern as in elevated plus maze: PYR mice moved faster than STD mice during the first 2.5 min ($F_{1,18} = 4.3$, $p = 0.05$), but then the difference in running speed disappeared ($p = 0.27$, 0.38, and 0.33 for the remaining quartiles).

To assess whether long-term pyruvate administration directly affects muscle function, we measured maximum grip force and endurance on a treadmill. There was no difference in the grip force (STD: 1103 ± 36 N; PYR: 1127 ± 41 N, $p = 0.66$) or the time until exhaustion on the treadmill (STD: 33.6 ± 6.7 min; PYR: 35.7 ± 6.1 min, $p = 0.83$).

Finally, we tested in another batch of young adult wild-type mice whether acute post-trial injection of Na-pyruvate (500 mg/kg i.p.) would also impair retention of fear conditioning in the passive avoidance task. There was no difference between the treatment groups either in the baseline latency ($t_{16} = 0.5$, $p = 0.62$) or latency 24 h after the fear conditioning ($t_{16} = 1.0$, $p = 0.33$; **Figure 3C**). This finding speaks against the possibility that PYR would have impaired memory consolidation.

Metabolic Effects of Chronic Pyruvate Supplementation

Among the middle-aged wild-type mice, the PYR group started with a lower body mass but gained more weight during the 7 months on special diet, resulting in a significant diet × age interaction ($F_{7,28} = 5.0$, $p = 0.001$). At the end of the study, the body weights for STD mice were 38.8 ± 1.0 g and for PYR mice 39.8 ± 0.9 g, $p = 0.48$. Similarly, among the young adult APP/PS1 mice, the PYR mice tended to gain more weight during 3 months on the special diet ($F_{2,22} = 2.8$, $p = 0.08$; end weight STD: 30.2 ± 0.6 g, PYR: 30.8 ± 0.7 g). On the other hand, no extra weight gain was observed in young adult wild-type mice on PYR diet ($F_{1,18} = 0.1$, $p = 0.77$).

To further evaluate the impact of chronic PYR supplementation on brain energy reserves, we euthanized nine young adult wild-type mice of Experiment 3 with microwaves and measured the cortical glycogen content. It was higher in the PYR group than in the STD group ($t_7 = 3.3$, $p = 0.01$; **Figure 4A**). We also stained brain sections from a subset of 16 middle-aged wild-type mice for PAS to visualize brain glycogen. The optic density for PAS was significantly higher in PYR mice than STD mice in the dentate gyrus ($p = 0.01$), CA1 ($p = 0.005$) but not in alveus/corpus callosum ($p = 0.11$) or overlying neocortex ($p = 0.08$) (**Figures 4B–D**).

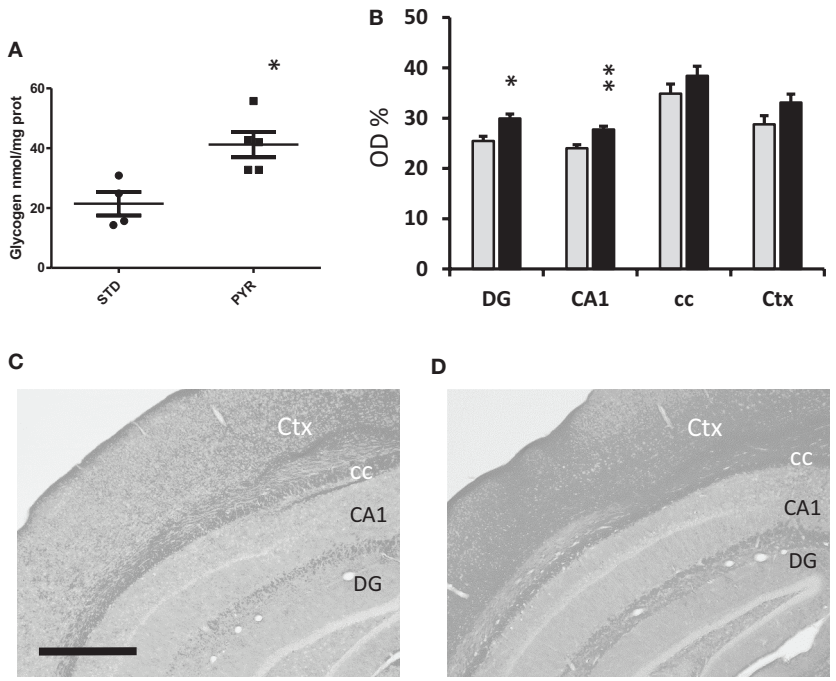

FIGURE 4 | **Metabolic assessment of brain glycogen levels**. **(A)** Group means and individual values for cortical glycogen content in the enzymatic assay in young adult wild-type mice at 6 months of age after been on dietary intervention for 3 months by the time of euthanasia. PYR group has higher brain glycogen levels (*$p < 0.05$, *t*-test). **(B)** PAS staining for brain glycogen in 13-month-old wild-type mice been on the dietary intervention for 7 months. Data are given as relative optic density (OD%) with 0 corresponding to pure white and 100% to pure black. DG = dentate gyrus, molecular layer, CA1 = all layers of this subregion, cc = alveus + corpus callosum, and Ctx = all layers of visual cortex. PYR group has higher PAS staining intensity, *$p < 0.05$, **$p < 0.01$ (*t*-test). **(C,D)** Representative PAS-stained sections through the mid-hippocampus after conversion to grayscale. Scale bar = 500 μm. C = STD group and D = PYR group.

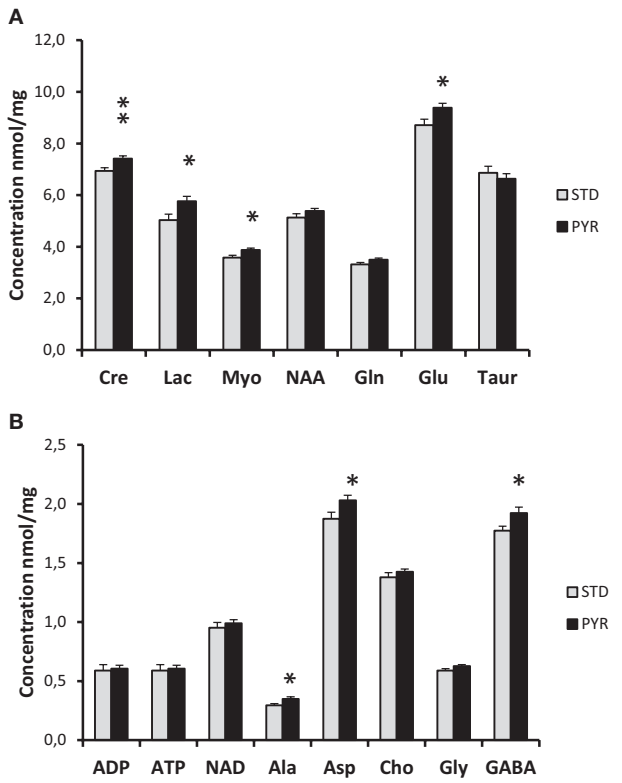

FIGURE 5 | ^{1}H-MRS analysis of snap frozen brains of 7-month-old APPswe/PS1dE9 mice been on the dietary intervention for 3 months by the time of euthanasia. (A) Cre = creatine/creatine phosphate, Lac = lactate, Myo = myoinositol, NAA = *N*-acetyl-aspartate, Gln = glutamine, Glu = glutamate, and Taur = taurine. **(B)** ADP = adenosine diphosphate, ATP = adenosine triphosphate, NAD = nicotinamide adenine dinucleotide, Ala = alanine, Asp = aspartate, Cho = choline, Gly = glysine, and GABA = gamma-amino butyric acid. PYR group differs from STD group at $^{*}p < 0.05$ and $^{**}p < 0.01$ (*t*-test).

The brains of APP/PS1 mice were snap frozen in liquid nitrogen at the end of the study and assessed with ^{1}H-MRS. As summarized in **Figure 5**, levels of several energy metabolites, such as creatinine, glutamate, and lactate, were increased in the PYR group compared to STD group. Collectively, these findings support the idea that long-term dietary supplementation with pyruvate increases brain energy reserves.

DISCUSSION

The present study demonstrated that chronic dietary supplementation with pyruvate (PYR) in mice results in behavioral and metabolic changes in the brain. The pattern of behavioral outcomes was consistent between two independent experiments, one in 12-month-old wild-type mice and another in 6.5-month-old APP/PS1 mice. Long-term PYR supplementation (1) improved learning of the Morris swim task but did not significantly improve spatial memory as assessed by the search bias in the probe task, (2) enhanced preference to explore a novel odor, (3) increased rearing in a novel environment, and (4) robustly impaired performance in the passive avoidance task. The last effect could be observed in 5- to 6-month-old wild-type mice as well after chronic PYR supplementation but not after an acute post-training injection. A common denominator to all these effects is the enhanced exploratory activity, which is consistent with the observed increase in rearing in a new test cage in the PYR groups and increased running speed in a new environment in a set of controls studies. Finally, by using three different methods, we could detect increases in the brain energy metabolism after PYR supplementation confirming that even oral PYR administration can affect the brain energy status provided that the duration of the treatment is long enough.

Improved Spatial Memory and Odor Recognition

The first behavioral finding, decreased escape latency in the Morris swim task by middle-aged wild-type mice on PYR supplementation, and a trend toward a better search bias in the probe task are in line with the recent findings in 3xTg mice showing improved spatial search bias by PYR treatment both at 6 and 12 months of age (Isopi et al., 2015). Unfortunately, Isopi et al. did not show the results of the acquisition phase in their report, making the direct comparison between the studies difficult. On the other hand, we did not find significant improvement in spatial search bias in 6.5-month-old APP/PS1 mice. Unfortunately, due to time and budgetary restrictions, we could not let our APP/PS1 mice age beyond 6.5 months of age when the spatial learning impairment is still modest. Thus, we cannot exclude the possibility that PYR supplementation would have been beneficial in 12-month-old APP/PS1 mice with more robust memory impairment. Why the effect was particularly pronounced during the acquisition phase and not during the probe test? Morris swim task is a very complex task and changes in many aspect of the behavior may lead to faster task acquisition. At least we could rule out the simple explanation that PYR worked against fatigue and slower swimming speed that would automatically lead to longer escape latencies. There was no difference in swimming speed among middle-aged wild-type mice overall, and importantly, not during the last trial of the day when the mice should have showed fatigue. It is worth noticing that we let the animals recover and warm up for 10 min between the trials to avoid hypothermia that easily develops in mice during swimming (Iivonen et al., 2003). Improved learning could also be seen in terms of decreased thigmotaxis, tendency for searching escape through the pool wall. This can be interpreted as improved cognitive flexibility (abandoning of an inefficient strategy) or decreased fear to move from the wall to the pool center. Together, these facts suggest that the treatment effects are more likely mediated by altered brain function rather than altered muscle function.

The odor recognition task has proven to be sensitive to age-dependent cognitive impairment in APP/PS1 mice (Koivisto et al., 2014). The task is based on the innate tendency of mice to explore a novel odor more than a familiar one. Not only should the mouse be familiar with its own odor but also to the presence of the wooden balls in its home cage during the familiarization phase. We cannot exclude the possibility that

reduced exploration of a novel behaviorally meaningful odor stems from impaired olfaction, but in other tests, APP/PS1 mice have shown no impairment in habituation to the odor ball upon repeated exposures or learning to associate the odor of a natural extract to the presence of odorless food reinforcement. However, the test is not a pure recognition memory, either, since the outcome is also influenced by the interest to explore novelty vs. fear for an unknown smell. Interestingly, PYR supplementation led to significant improvement in the task in both middle-aged wild-type mice and adult APP/PS1 mice similar to our recent finding in middle-aged APP/PS1 mice after dietary supplementation with the omega-3 fatty acid DHA (Koivisto et al., 2014). However, the young adult APP/PS1 mice also showed a clear fear-related aversion toward the unknown conspecific on the first test day but no more on the second day. The most likely explanation for the pattern (which to a smaller extend could be seen in middle-aged wild-type mice as well) is that we see a combined effect of two factors: repetition-dependent fear for the unknown odors and PYR-induced increased tendency for exploration.

Paradoxical Impairment in Passive Avoidance

The most consistent finding across age groups was the impairment in passive avoidance task. At first, it seems counterintuitive to see "memory improvement" in two behavioral tasks by PYR supplementation, but then a robust "memory impairment" in a third classic memory task. Further, an impaired memory consolidation would be unexpected, since both glucose (Gold, 2005) and lactate (Suzuki et al., 2011) given as post-training injections have been reported to enhance memory retention in this task. Consistent with this, we did not observe impairment after post-training injection of PYR at the dose shown effective in several previous studies (Lee et al., 2001; Suh et al., 2005; Isopi et al., 2015). However, passive avoidance is known to be a "quick and dirty" task with several behavioral confounds. We could rule out the possibility that PYR supplementation affects pain threshold. Decreased level of anxiety could easily mask the effect of fear conditioning and encourage the animal to enter the dark compartment. However, we did not find much evidence for PYR-induced anxiolysis. Although the marble burying task revealed a response that could be interpreted as anxiolytic, this was only seen in APP/PS1 mice and not in the middle-aged wild-type mice with PYR treatment. Further, the classic anxiety tests parameters, relative time spent on the open arms in the elevated plus maze or time in the center of the open field, were not influenced by PYR supplementation. The best explanation we can offer for the time being is that chronic PYR increased exploration of novelty, leading to the situation where the drive for exploration weighed more than the fear for the foot shock. In fact, increased exploration provides the best possible explanation to all present behavioral findings (less thigmotaxis and faster learning in the Morris swim task, increased exploration of a novel odor, increased rearing in a novel test cage, and increased running speed in the plus maze or open field during the first 2.5 min).

Direct Pyruvate Effect or Secondary after Conversion to Lactate in the Liver?

The chow containing 6 g/kg pyruvate led to an average daily ingestion of 800 mg/kg/day after adjusting for food intake and spillage. Extrapolating to humans, these doses correspond to ~10 g daily pyruvate intake. A recent study showed that a single pyruvate dose of 0.1 g/kg resulted in a shift in fuel utilization toward accelerated carbohydrate oxidation lasting for several hours (Olek et al., 2015). In rodents, the neuroprotective effects of intraperitoneal pyruvate have been reported for doses ranging from 200 to 1000 mg/kg [for review, see Zilberter et al. (2015)]. The dose of pyruvate used in this study proved to be efficient; however, it is yet unclear what amount of pyruvate actually enters the brain at chronic oral administration. One can suggest that pyruvate may first be converted to lactate in the liver before it enters the brain. It has been shown, however, that glucose but not lactate is a preferred fuel for neurons *in vitro* (Patel et al., 2014), *ex vivo* (Ivanov et al., 2014), and *in vivo* in freely moving mice (Lundgaard et al., 2015). Meanwhile, the concentration of lactate in the extracellular cerebral fluid is close to that of glucose, and in the cortex is about 2–3 mM (Zilberter et al., 2010). Why then lactate is not utilized efficiently in spite of its relatively high level? We speculate that in the absence of extreme energy demands, the balance (presumably the redox state) is shifted toward glycolysis and subsequent release of excessive lactate from neurons (Dienel, 2012). In contrast, pyruvate is a "direct" energy substrate for mitochondria, while lactate needs to be converted first to pyruvate in the reaction dependent on the availability of cytoplasmic NAD^+. In addition, besides being a mitochondrial fuel, pyruvate possesses a whole array of neuroprotective properties (Zilberter et al., 2015) lacking for lactate. A recent study on rats with Aβ microinfusion (i.c.v.) reported beneficial effects of pyruvate (500 mg/kg) after 10 daily intraperitoneal injections on spatial learning, LTP, ROS production, and neuronal survival. Corresponding effects were not obtained by systemic lactate injections, speaking for a direct pyruvate effect on neurons (Wang et al., 2015).

One may wonder why we did not find increased brain levels of pyruvate in the MRS assay. This is probably due to rapid pyruvate transformation to lactate. The kinetics of BBB transport of pyruvate is estimated to be 30- to 100-fold slower than the rate of pyruvate to lactate conversion. A recent MRI study employing hyperpolarized ^{13}C-pyruvate could demonstrate the BBB penetration of a small bolus injection pyruvate and its conversion to lactate within 2 min in the brain (Hurd et al., 2010). A massive i.p. injection of 1000 mg/kg in an *in vivo* microdialysis study resulted in increased pyruvate levels in the plasma and interstitial fluid for 60 min, while the increased lactate levels were maintained for the entire 75-min follow-up time (Fukushima et al., 2009). Indeed, we could also detect increased levels of lactate in the brain, as well as those of glutamate and creatine/creatine phosphate. Considering that the mice were euthanized between 8:00 and 11:00 a.m. at least a couple of hours after significant ingestion of the chow during the dark period, it would have been impossible to even detect increased pyruvate levels in the circulation according to the microdialysis data. Thus, we must leave open the possibility

that a fraction of pyruvate, when administered orally, may first be converted to lactate in the liver before it enters the brain.

In any event, we could confirm our previous finding that pyruvate as dietary supplement is able to increase brain glycogen levels (Zilberter et al., 2013). At this point, we cannot say whether the observed behavioral changes were directly linked to increased glycogen stores or whether increased brain glycogen only acts as a surrogate marker for improved brain energy status. Anyway, several neuronal processes have been shown to depend on astrocytic glycogen as their primary energy source. First, neurotransmitter glutamate is largely recycled, but ~15% is used for energy and needs to be replenished. This is mainly done by pyruvate carboxylase, which largely depends on glycogenolysis (Hertz et al., 2015). Second, the energy for Na^+, K^+-ATPase-mediated K^+ uptake into astrocytes after prolonged neuronal activity derives from astrocytic glycogenolysis (Hertz et al., 2015). Third, inhibitor of glycogen phosphorylase inhibits memory consolidation in neonatal chicks (Gibbs et al., 2007) and rats (Newman et al., 2011; Suzuki et al., 2011).

Indications for Pyruvate Supplementation

Besides emergency medicine, pyruvate has been extensively studied in sports medicine for its possible effects to improve muscle mass and endurance. Some studies have reported enhanced exercise endurance capacity by oral pyruvate combined with dihydroxyacetone (Stanko et al., 1990a,b) and enhanced fat loss when combined with low-energy diet (Stanko et al., 1992). However, in one study, the combination of pyruvate with creatine increased anaerobic performance while pyruvate supplementation alone was ineffective (Stone et al., 1999). Similarly, oral pyruvate alone for a week (Morrison et al., 2000) or in combination with creatine (Van Schuylenbergh et al., 2003) was ineffective to improve aerobic performance in cyclists, and 30-day puruvate supplementation failed to increase aerobic exercise performance in untrained subjects (Koh-Banerjee et al., 2005). Our findings in young adult mice are in line with these results, in that long-term pyruvate did not increase aerobic endurance or maximal anaerobic muscle force. At present, pyruvate supplements (creatine pyruvate) are actively marketed to body builders. The present study suggests a new indication for pyruvate by showing that dietary supplementation of pyruvate may offer a simple and effective way to correct for insufficient glucose supply and depleted glycogen stores in the aging brain. In addition to energy supplementation, pyruvate is a potent antioxidant, has anti-inflammatory, and anti-epileptic properties (Zilberter et al., 2015). It is well tolerated: up to millimolar plasma concentrations after i.v. infusion are reported not to have apparent adverse effects in humans (Dijkstra et al., 1984).

CONCLUSION

Long-term dietary supplementation of pyruvate led to clear behavioral changes. Improved spatial learning, increased exploration of a novel odor, and a novel environment were seen in middle-aged wild-type mice as well as in adult APP/PS1 mice. The most consistent finding in all subgroups of mice was a shorter latency to enter the dark compartment in the passive avoidance task, which can be attributed to the increased drive for exploration. We found no evidence that pyruvate would act by changing muscle force or endurance. Increases in brain energy metabolites and glycogen stores after long-term pyruvate supplementation also speak for an effect on the brain metabolism. Dietary pyruvate supplementation may prove beneficial against aging-related cognitive impairment and inactivity.

AUTHOR CONTRIBUTIONS

HK: performed behavioral studies in middle-aged and APP/PS1 mice, analyzed the data, and collected all brain samples. HL: planned, performed, and supervised behavioral studies in young adult wild-type mice with chronic treatment, analyzed part of the data, and participated in writing the manuscript. MP and HH: performed behavioral studies in young adult wild-type mice with chronic treatment and analyzed the data. GB: set up PAS staining methods, performed histology on middle-aged mice, and analyzed the data. MS and HW: set up the analysis method for brain glycogen and ran the assay, analyzed the results, and participated in manuscript writing. MT and PS: ran all NMR spectroscopy assays, analyzed the results, and participated in writing the manuscript. YZ: planned the study with HT and major contribution in writing the manuscript. HT: PI of the whole study, supervision and coordination of all subprojects, and writing the manuscript.

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Neural Signaling of Food Healthiness Associated with Emotion Processing

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The ability to differentiate healthy from unhealthy foods is important in order to promote good health. Food, however, may have an emotional connotation, which could be inversely related to healthiness. The neurobiological background of differentiating healthy and unhealthy food and its relations to emotion processing are not yet well understood. We addressed the neural activations, particularly considering the single subject level, when one evaluates a food item to be of a higher, compared to a lower grade of healthiness with a particular view on emotion processing brain regions. Thirty-seven healthy subjects underwent functional magnetic resonance imaging while evaluating the healthiness of food presented as photographs with a subsequent rating on a visual analog scale. We compared individual evaluations of high and low healthiness of food items and also considered gender differences. We found increased activation when food was evaluated to be healthy in the left dorsolateral prefrontal cortex and precuneus in whole brain analyses. In ROI analyses, perceived and rated higher healthiness was associated with lower amygdala activity and higher ventral striatal and orbitofrontal cortex activity. Females exerted a higher activation in midbrain areas when rating food items as being healthy. Our results underline the close relationship between food and emotion processing, which makes sense considering evolutionary aspects. Actively evaluating and deciding whether food is healthy is accompanied by neural signaling associated with reward and self-relevance, which could promote salutary nutrition behavior. The involved brain regions may be amenable to mechanisms of emotion regulation in the context of psychotherapeutic regulation of food intake.

Keywords: functional neuroimaging, food, healthiness, amygdala, midbrain, gender

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INTRODUCTION

The ability to perceive food as being advantageous or disadvantageous to one's own health, and guiding one's nutritional behavior accordingly, promotes good health. This is particularly important considering that the burden and the economic impact of nutrition related medical conditions and unhealthy lifestyles is high (Striegel-Moore and Bulik, 2007; Hoek and King, 2008). Nutrition, or food intake, may trigger, or may cause pleasant and unpleasant feelings, and can such be considered to be associated with the activation of brain regions that process emotions (Rolls, 2005, 2008; Siep et al., 2009; Ziauddeen et al., 2012; Meye and Adan, 2014; Morton et al., 2014).

A feature of tasty, but unhealthy nutrition consists of appetitive emotions occurring with impaired impulse control and self-guiding related to respective food stimuli, despite knowledge of a possible disadvantageous health value (Glanz et al., 1998). For example, eating chocolate is accompanied by a positive emotion, whereby resisting eating an offered chocolate may result in unpleasantness and may require impulse control. This unpleasantness signal makes sense from an evolutionary point of view, as the ingestion of high caloric food is advantageous for an organism's survival. However, many people, for instance those with obesity, diabetes or other nutrition related conditions, have to control eating certain foods that they desire. Evaluating and choosing healthy food on an individual and situation based level in this way is especially important.

In everyday life, many people do not actively reflect on whether the food they eat is healthy, but more so on whether it is tasty (Glanz et al., 1998). However, consciously reflecting about the healthiness of a food item can influence eating behavior. Identifying the brain regions involved in the evaluation of food healthiness might help to understand which cognitive strategies are utilized to promote salutary nutrition. We investigated brain activity associated with single subject's conscious food healthiness evaluation and rating and focused on brain regions known to be involved in emotion processing and regulation. We were interested in which brain areas signal differentially when evaluating a food item to be of higher healthiness compared to lower healthiness.

Earlier studies on the neural processing of the visual presentation of food stimuli in healthy subjects showed heightened activation in insular and orbitofrontal cortex (OFC) regions, when hungry (Porubská et al., 2006) and not hungry (Killgore et al., 2003; Simmons et al., 2005). These results are consistent with reports of gustatory representation in these areas (Rolls, 2001; Kringelbach, 2004). Furthermore, the OFC has been shown to integrate different sensory modalities such as gustation and olfaction (Rolls, 2001). Fuhrer et al. (2008) analyzed brain activity during the presentation of food items compared to other stimuli. They found stronger activation of the medial prefrontal, insular, anterior cingulate, and striatal regions when participants were presented with food stimuli. Siep et al. (2009) reported activity in reward-associated brain regions, the amygdala and OFC, when assessing high versus low caloric nutrition. Rolls (2008) discussed a central involvement of the orbitofrontal and anterior cingulate cortex in the multimodal representation of food particularly associated with reward. These suggestions were also supported by Frank et al. (2010). Medial and lateral prefrontal cortex areas are also involved in value based decision making (Deco et al., 2013; Dixon and Christoff, 2014), which was also required in our task. Following these findings, regions of interest to be considered in our study were the medial and dorsolateral prefrontal cortex regions, anterior/subgenual cingulate gyrus, OFC, anterior insula, amygdala, ventral striatum, medial thalamus, and midbrain.

In previous studies that have investigated neural activation associated with food healthiness, food healthiness processing in general was related to cognitive domains such as attention (e.g., Hare et al., 2011; Grabenhorst et al., 2013), but signaling of healthiness versus non-healthiness in distinct brain areas was not addressed and the individual estimation of healthiness evaluation was not considered (e.g., Frank et al., 2010; Killgore and Yurgelun-Todd, 2010). The novelty of our study relies on (i) the direct comparison of brain activation associated with high and low health value in healthy subjects, and (ii) on the investigation of the single subject level of food healthiness evaluation. Therefore, our analysis was not based on a general a priori categorization of the food items into healthy and unhealthy categories, but on the single subject rating of each food item concerning perceived health value. Given that the subjective valence of different foods can vary, we individually determined the grade of healthiness related to each presented food item and considered the individual results for the analysis. As it was previously shown that males and females may differ regarding their estimation of healthiness of nutrition and other food related aspects (Killgore and Yurgelun-Todd, 2010; Geliebter et al., 2013), we further aimed to identify gender differences at the level of neural activation concerning food evaluation. We expected the differential evaluation of food healthiness to be associated with the activation of brain areas related to emotion processing, especially in more primordial brain regions such as the midbrain, amygdala and ventral striatum regarding an emotional connotation, and in the insula and OFC regarding viscero-sensitive interoception. A cognitive approach, however, would involve higher cortical regions such as medial prefrontal and dorsolateral prefrontal cortex regions.

MATERIALS AND METHODS

Ethical Statement

The study was approved by the Kantonale Ethikkommission Zuerich, Switzerland (as stated in the submission questionnaire).

Subjects

Forty-one healthy subjects (age 20–46 years, mean 24.8, SD 4.6; all right handed; 22 males, BMI mean 22.9, SD 2.4, range 19.9–28.6 with $n = 1 > 26$; 19 females, BMI mean 21.3, SD 2.1, range 18.0–24.6; none with dietary needs, 39 with academic background, mostly students, 2 medical assistance professionals) were recruited to participate in this study and gave written informed consent. The study was approved by the local ethics committee. Further, the subjects were neither hungry throughout scanning, nor had they had a major meal within an hour prior scanning. Four subjects were excluded afterward because of sudden movement artifacts (exceeding more than 3 mm in at least one direction) or other technical reasons, so that the data of 37 subjects (age 20–46, mean 24.9, all right handed, 19 males, 18 females; **Table 1**) were analyzed. The subjects were healthy (assessed with clinical interview based on ICD-10 and DSM-IV) and did not take any psychotropic medication or have any psychiatric, neurological, or other relevant medical history that would affect the results of this study. We also assessed self-ratings of depression (SDS, German version; Zung, 2005) and state-trait anxiety inventory (STAI) to control for affective or anxiety symptoms (**Table 1**).

TABLE 1 | Demographic and psychometric data of the subjects.

	Total mean (*SD*, range)	Males mean (*SD*, range)	Females mean (*SD*, range)	Males vs. females *p*
Subjects (*n*)	37	19	18	
Age (years)	24.8 (4.6, 20–46)	24.4 (4.0, 20–36)	25.3 (5.5, 20–46)	0.58
SDS	45.6 (3.8, 36–53)	45.2 (1.9, 42–48)	46.1 (4.2, 36–53)	0.48
STAI				
– State	43.6 (2.8, 38–49)	43.4 (2.9, 38–49)	43.8 (2.7, 39–48)	0.71
– Trait	43.7 (4.3, 37–56)	43.0 (4.2, 37–52)	44.4 (4.2, 39–56)	0.32

SDS, Self-ratings of depression; STAI, state-trait anxiety inventory.

Experimental Design

During fMRI scanning, the subjects evaluated the healthiness of different food items presented in photographs. The photographs showed the food items on a white background, in such a way that only the food item was visible (examples in **Figure 1**). The food items were divided into two halves representing more or less healthy or unhealthy food, respectively. The food photographs were presented for 5940 ms (equivalent to three repetition times, TR, for the fMRI volumes). In this "evaluation" period, the subjects were instructed to look at the photo and to estimate the healthiness of the respective food item. Subsequently, a visual analog scale ranging from 1 to 5 was presented for 3960 ms (two volumes) on which the subjects indicated the individually estimated nutrition value between very healthy (5) to very unhealthy (1) by moving a cursor using a trackball with the right hand (**Figure 1**), the "rating period". Altogether, 40 food stimuli were presented in a randomized order. The following baseline period (13700 ms, 7 TR) was of sufficient duration to allow the blood oxygen level-dependent signal to wear off before the next trial. The task was programmed with Presentation™ (Neurobehavioral Systems, USA) and presented via digital video goggles (Resonance Technologies, Northridge, CA, USA). Photographs were sized to fill approximately two thirds of the screen diameter, so that the food item could have been identified immediately with minimal eye movements required. After scanning, the subjects were asked to rate the healthiness of the food items again and also the grade of subjective tastiness, from very tasty (5) to not tasty at all (1), on visual analog scales.

We specifically assessed the difference in healthiness evaluation within the sample of food pictures on a single subject level. As such, we explicitly compared the items as being of high or low healthiness as they were rated by the individual. In order to better discriminate healthy ratings from unhealthy ones, we separated both groups by a group of stimuli with intermediate healthiness (group definition below).

Data Acquisition

Imaging was performed with a 3.0 T GE Signa™ HD Scanner (GE Medical Systems, Milwaukee, WI, USA). Echo planar imaging was performed for fMRI (repetition time TR/echo time TE 1980 ms/32 ms, 22 sequential axial slices, whole brain, slice thickness 3.5 mm, 1 mm gap, resulting voxel size 3.125 mm × 3.125 mm × 4.5 mm, matrix 64 × 64 pixels, field of view 200 mm, flip angle 70°). 528 volumes were obtained per subject, 12 per trial. Four initial volumes were discarded to allow for equilibration effects, seven volumes were added for a final baseline. High-resolution 3-D T1 weighted anatomical volumes were acquired (TR/TE 9.9/2.9 ms; matrix size 256 × 256; 1 mm × 1 mm × 1 mm resolution) for co-registration with the functional data.

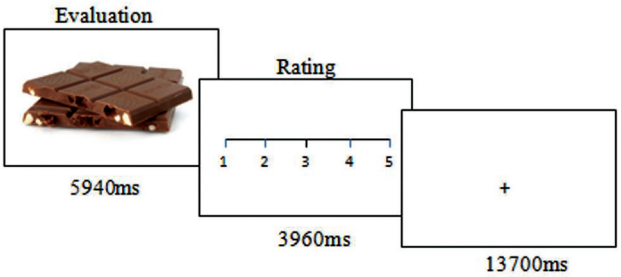

FIGURE 1 | Experimental task. The trials started with displaying a food photograph for nearly 6 s while the participants evaluated the healthiness of the food item. This was followed by a rating period of nearly 4 s. A baseline condition of nearly 16 s was implemented between trials.

Data Analysis

FMRI data were analyzed using BrainVoyager™ QX 2.0 (Brain Innovation, Maastricht, The Netherlands). Pre-processing of the functional scans included motion correction, slice scan time correction, high frequency temporal filtering, and removal of linear trends. Functional images were superimposed on the 2D anatomical images and incorporated into volume time courses. The individual volume time courses were transformed into Talairach space resulting in a voxel size of 3 mm × 3 mm × 3 mm and then spatially smoothed with an 8 mm Gaussian kernel for subsequent group analysis. From each included subject ($n = 37$), the individual food healthiness evaluation periods of each single food item presentation were considered. We pre-defined three categories of healthiness ratings: high, medium, and low. The categories were mathematically divided considering the highest and lowest 1.5 score periods on the scale for the analysis of high and low healthiness, respectively. Thus, low healthiness was defined between 1.00 and 2.50, medium healthiness between 2.51 and 3.49, and high healthiness between 3.50 and 5.00, based on the distribution of the evaluation ratings. Individual

experimental design matrices for each subject for the fMRI-analysis were built comprising the individually rated items meeting the three conditions (low, medium, high healthiness) and the respective three conditions with presentation of the rating scale as predictors, resulting in six predictors for the design matrix. The periods were modeled as epochs using a two-gamma hemodynamic response function provided by BrainVoyager^{TM} and were adapted to the applied period duration.

The fMRI data analysis, based on the general linear model (GLM), comprised the following steps: First, fixed effects analyses were calculated separately for each subject for the contrasts comparing the individual conditions of evaluation and rating of 'high healthiness' versus 'low healthiness', resulting in summary images. The summary images were subjected to second level group analyses. Thus, those trials in which the food photographs were rated as 'high' and 'low' healthiness were considered for contrast analysis. The 'medium' rated items were also modeled as a condition in the analysis protocol but not considered for the final fMRI analysis and served therefore as a "buffer" between 'low' and 'high' for better discrimination. We further differentiated between the evaluation and the rating period. The evaluation period was the primary period of interest with the pure mental act of reflecting about and estimating the healthiness of the presented food item without a motor command or other distracting activity. The rating period was the period of giving feedback concerning the healthiness estimation. Both, evaluation and rating period, were functionally and timely coupled, nevertheless, we decided on a separated analysis. To analyze the evaluation and rating periods, three-dimensional statistical parametric maps were calculated for the groups using a random effects analysis.

Because of the approach with individual data of each subject for each food item, the data were not suitable for a continuous or regression analysis, which would have been suitable for a mean value derived from all subjects for the single food items.

The main analysis therefore focused on the contrasts "evaluation high healthiness > evaluation low healthiness" (e-hi > e-lo) and "rating high healthiness > rating low healthiness" (r-hi > r-lo). The voxel-wise threshold for reporting results in the random effects analysis was set at $p < 0.005$. To correct for multiple comparisons, a Monte Carlo simulation was used (Goebel et al., 2006) for estimating cluster-level false-positive rates on these maps, yielding after 10.000 iterations a minimum cluster size threshold of 10 voxels of 3 mm × 3 mm × 3 mm (270 mm^3), corresponding to a corrected cluster level $p < 0.02$.

We also assessed the brain activity associated with food healthiness evaluation and rating in predefined anatomical cubic ROIs, which are known to be related to emotion processing. For the larger cortical regions of the insula, subgenual (sg)ACC, OFC, DLPFC, and DMPFC, ROIs were constructed using 4 × 4 × 4 functional voxels (edge length 12 mm × 12 mm × 12 mm, volume 1728 mm^3 each). The ROIs were placed according to the Talairach Client (Lancaster et al., 2000) and prior studies: DMPFC $x = 6/-6$, $y = 6$, $z = 50$, covering Brodmann Area (BA) 6 and 8 in the superior frontal gyrus; and DLPFC $x = 43/-43$, $y = 18$, $z = 30$, covering mainly BA 9 in the middle frontal gyrus (Northoff et al., 2006; Herwig et al., 2010, 2011); anterior insula $x = 33/-33$, $y = 16$, $z = -1$ (Craig, 2009; Paulus and Stein, 2010); ventral striatum (10/–10, 6, –6; McClure et al., 2004; Heimer and Van Hoesen, 2006), amygdala (Costafreda et al., 2008, edge length 6 mm, 22/–22, –6, –12), medial thalamus (0, –12, 4), midbrain (0, –23, –12), OFC (0, 52, –1), ACC (0, 38, 1), sgACC (0, 17, –9).

Finally, in order to assess the influence of gender on the food evaluation and food rating periods, we introduced this variable as a covariate in a further analysis using the same statistical approach and thresholds.

RESULTS

Behavioral Data

Thirty-seven subjects were included in the analysis (demographic data including normal anxiety and depressiveness ratings in **Table 1**). The subjects attributed high healthiness to 16.2 items (SD 3.0), medium healthiness to 5.7 items (SD 3.1), and low healthiness to 18.0 items (SD 2.1), on average. This resulted in $n = 601$ trials with high healthiness, $n = 212$ trials with medium healthiness, and $n = 667$ trials with low healthiness, overall. Correlating healthiness with tastiness in the groups of pictures that were rated as healthy and in the group rated as unhealthy did not reveal any significant results: healthy/taste $r = -0.06$ (mean healthy food pictures 4.4, taste health group 4.1), unhealthy/taste $r = -0.11$ (mean health rating in the unhealthy food pictures 1.8, mean taste in that group 3.6). However, when correlating the grades of healthiness and tastiness in the whole group, we found a positive correlation ($r = 0.52$), meaning that healthier food items were also rated as tastier.

fMRI Results

We performed whole brain analyses on the contrasts "evaluation high healthiness > evaluation low healthiness" (e-hi > e-lo) and "rating high healthiness > rating low healthiness" (r-hi > r-lo).

Regarding the evaluation period, we found higher activation in a left superior/medial prefrontal cortex region covering BA 6, 8, 9, [premotor cortex (PMC) and DLPFC, **Figure 2A**] and in precuneus and lateral parietal cortex regions associated with the evaluation of food pictures subjectively rated as high in healthiness. Higher activation in primary and associative visual cortex was associated with the low healthiness food pictures (**Table 2**; **Figure 2**). The ROI analysis was used in order to assess activation in emotion processing related brain areas, and revealed higher activity in the right amygdala associated with evaluation of unhealthy food stimuli compared to healthy stimuli ($p = 0.048$, $t = -2.05$). Both evaluations (healthy and unhealthy) activated the amygdala compared to baseline (right and left $p < 0.00001$, $t > 7$; **Figure 3**).

Regarding the rating period, right ventral striatal activity was stronger when rating food as being healthy compared to unhealthy ($p = 0.008$, $t = 2.82$). DLPFC BA 46 was bilaterally more active with the rating of unhealthy food compared to healthy (right $p = 0.020$, $t = -2.44$; left $p = 0.041$, $t = -2.12$).

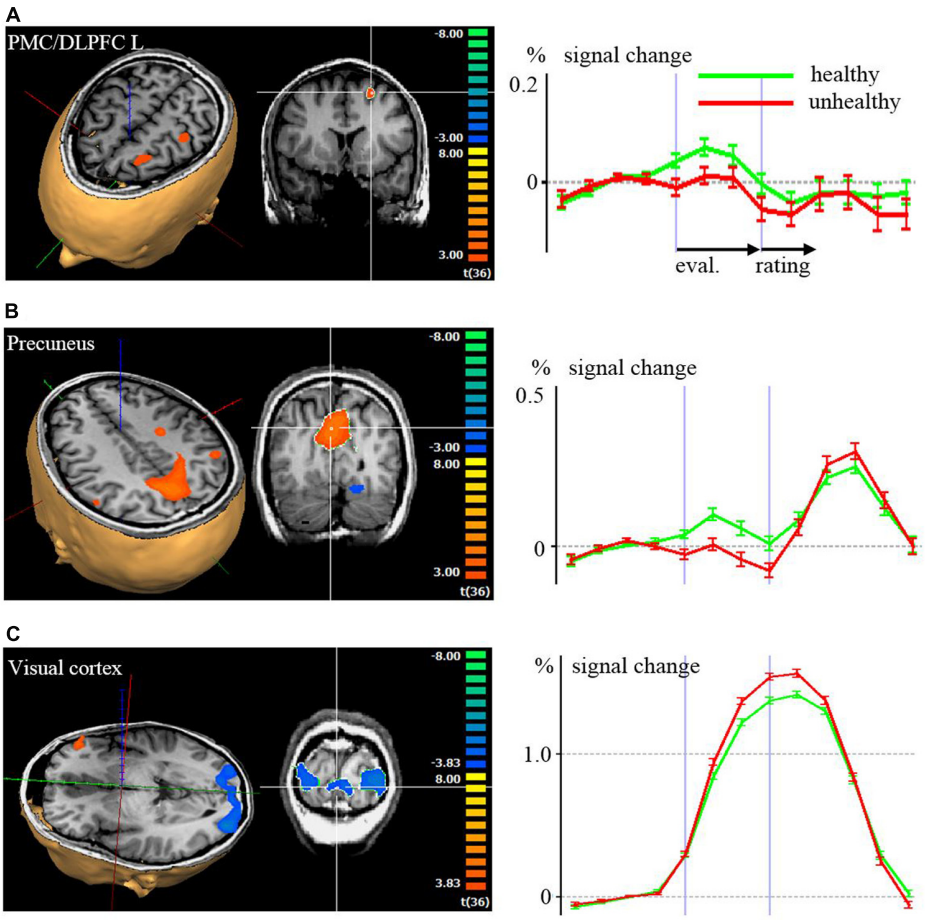

FIGURE 2 | Brain activation with color coded maps and time courses with signal changes according to a random effects analysis ($p < 0.001$) of the evaluation (eval.) and rating period, comparing high healthiness against low healthiness: (A) left dorsolateral prefrontal cortex (DLPFC), (B) precuneus, (C) visual cortex. In the 3D visualizations, red, blue, and green axes indicate the coordinate system. In the 2D coronal slice, the region with statistical significant activation of which the time course is derived is marked with the white crosshair. In the time course diagrams on the left side, the first vertical violet bars after the y-axis correspond to the activations during the first volume of the evaluation period. The second vertical violet bars correspond to the activations during the first volume of the rating period.

TABLE 2 | Contrast evaluation period of healthy vs. non-healthy food photographs in the whole brain analysis.

	BA	Peak *x*	Peak *y*	Peak *z*	*t*	*P*	mm^3
PMC/DLPFC L	6, 8, 9	–18	8	49	3.73	0.0007	766
IFG R	11, 44, 45	42	44	–8	4.96	0.0000	6832
Posterior insula R	13	36	–37	22	3.63	0.0009	521
Superior temporal ctx	22	36	–40	1	3.46	0.0014	649
Precuneus	7	–3	–70	34	4.75	0.0000	16393
Occipital ctx	18	–30	–88	–2	–5.41	0.0000	31725
Postcentral gyrus L	3	–33	–28	52	3.55	0.0011	472
Temporo-occipital ctx L	22, 39	–39	–52	16	4.84	0.0000	8614
Temporo-parietal ctx R	39	45	–58	28	4.50	0.0001	5269

PMC, premotor cortex; DLPFC, dorsolateral prefrontal cortex; IFG, inferior frontal gyrus ctx cortex; BA, Brodmann Area; R, right; L, left; SD, standard deviation.

Food healthiness evaluation and rating were both associated with significant neural activation in the other ROIs such as the MPFC, OFC, ACC, insula (apart for right anterior insula and evaluation), medial thalamus and midbrain, but without differences in regard to subjective healthiness (**Figure 4**; **Table 3**).

When assessing differences between males and females in the ROI analysis, we found higher activity in the right ventral

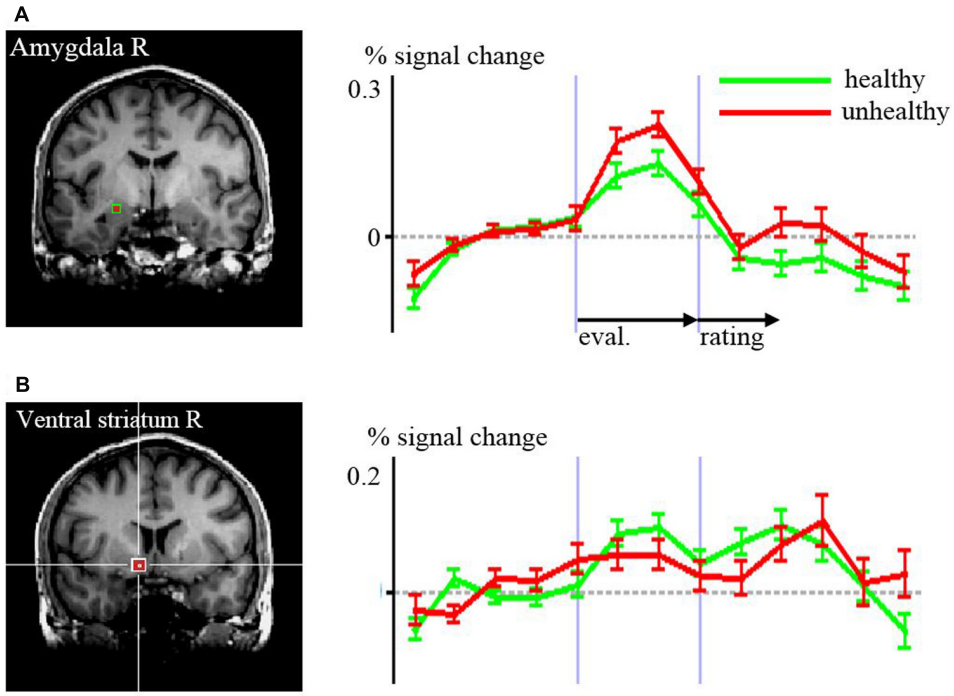

FIGURE 3 | Time courses of signal changes according to the region of interest analyses of healthy versus unhealthy food in (A) amygdala, (B) ventral striatum. The regions are indicated by the red squares in the structural MR images Differences in the time courses were significant for the evaluation (eval.) period in the amygdala ($p = 0.048$) and for the rating period in the ventral striatum ($p = 0.005$).

striatum among males compared to females in the perception and evaluation of unhealthy stimuli ($m > f$; $p = 0.008$, $t = 2.813$). This contrast was at the borderline to significance in the medial thalamus ($p = 0.058$, $t = 1.959$). Activation in the midbrain region was higher among females associated with the rating period of healthy stimuli compared to unhealthy stimuli ($p = 0.028$, $t = 2.293$; **Figure 5**). We found no differences between males and females in the amygdala, insula, and prefrontal regions.

DISCUSSION

Our main interest was on neural signaling associated with evaluating higher versus lower healthiness of food stimuli on a single subject level. We found an association of higher healthiness evaluation with higher activity of the PMC/DLPFC BA 6/8/9 and in the precuneus in the whole brain analysis and in the ROI analysis of the rating period in the ventral striatum and the OFC. Contrary to our hypothesis, we found an association of lower food healthiness with higher activation in the amygdala,. This was also found bilaterally in more anterior and lateral regions of the DLPFC (BA 46) as well as in primary and secondary visual areas. Nearly all other areas known to be involved in a network of brain areas associated with emotion processing, such as the MPFC, OFC, ACC, insula, medial thalamus, and midbrain, were activated generally when associated with the act of food healthiness evaluation and/or respective rating, but not specifically for healthy or unhealthy, and of course within the frame of our study without exclusion of other cognitive components that may be attributed to the activation. However, this at least implicates a relationship between the estimation of nutritional healthiness and emotional signaling in distinct emotion processing areas. The only gender difference was reflected by a higher activation in the midbrain of females associated with healthy stimuli.

Food Health Evaluation and Emotion Processing

The general association between nutrition and emotion has been proven by multiple studies (Rolls, 2005, 2008; Siep et al., 2009; and many others). Denton et al. (2009) have shown hunger and satiation as belonging to "primordial emotions" deeply rooted in central-nervous information processing and developed early in evolution. From an evolutionary point of view, one can even suggest that the complex system of emotions may at least in part originate from the bodily signaling associated with the ingestion of food and its evaluation in early species throughout evolution (Denton et al., 2009). Getting nutritious food is necessary for survival and associated with feelings of reward. Conversely, being deprived of food is unpleasant and potentially life threatening. As such, one may argue that neural emotion processing might have evolved at least in part from nutrition related neural processing. Considering the

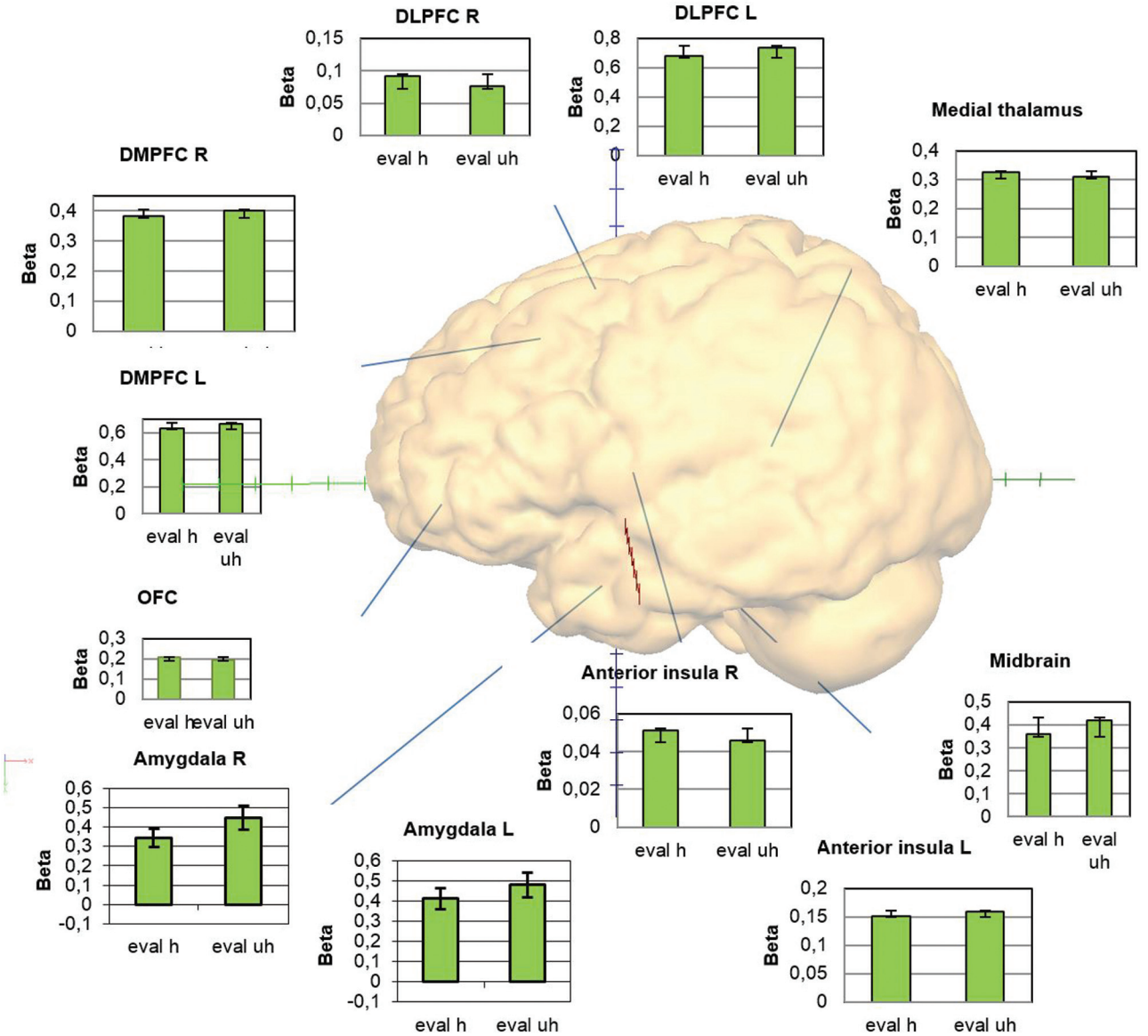

FIGURE 4 | Overview of the activations within the regions of interest providing graphs with the beta weights for the conditions evaluation healthy and unhealthy.

long term consequences of healthiness and favoring them over the short term benefits of tasty but unhealthy food, however, is often difficult and requires awareness for health aspects and self-control (Liberman and Trope, 2008; Hare et al., 2011).

According to our findings, regions associated with a differential healthiness signal comprise the DLPFC, MPFC, precuneus, amygdala, and ventral striatum. The PMC/DLPFC region showed stronger activation in more posterior and superior regions (BA 6/8/9) associated with the rating of higher healthiness, and in more rostral regions (BA 46) associated with the rating of lower healthiness. Hare et al. (2011) reported the lateral prefrontal cortex in BA 8/9 and 46/47 showed increased activity during a condition where subjects were asked to generally consider the healthiness of a food item. Further, BA 9 of the DLPFC appeared to modulate ventromedial PFC to promote health information (Hare et al., 2009, 2011). In a study assessing brain activation during the regulation of the desire for food intake by using reappraisal strategies, the DLPFC, as well as medial and inferior frontal PFC areas, were activated. This suggests that these areas have an impact in controlling food intake (Hollmann et al., 2012). The identified regions of the DLPFC, which is generally known to be involved in cognitive and executive control (e.g., Disner et al., 2011; Fuster, 2000), are suggested to be involved centrally in guiding nutrition relevant evaluation and

TABLE 3 | Results of the comparisons of the conditions healthy and unhealthy in different regions of interest providing statistical *p*- and *t*-values for each, the evaluation (Eval) and the rating (Rat) periods.

Anatomic region Tal. *X/Y/Z*	Evaluation healthy > unhealthy *p/t*	Evaluation healthy *p/t*	Evaluation unhealthy *p/t*	Rating healthy > unhealthy *p/t*	Rating healthy *p/t*	Rating unhealthy *p/t*
Amygdala R						
22/–6/–12	0.048/–2.05	<0.0001/7.26	<0.0001/7.16	0.54/–0.62	0.53/–0.64	0.93/–0.09
Amygdala L						
–22, –6, –12	0.14/–1.50	<0.00001/6.92	>0.00001/6.01	0.85/0.19	0.63/0.49	0.71/0.37
Ventral striatum R						
10/6/–6	0.083/–0.25	0.001/3.64	0.005/3.03	0.005/3.00	0.001/3.71	0.08/–1.81
Ventral striatum L						
–10/6/–6	0.48/–0.75	0.0002/4.12	0.0003/4.03	0.30/–1.05	<0.00001/5.74	<0.00001/6.12
DMPFC R						
6/6/50	0.73/–0.342	<0.00001/5.005	<0.00001/5.27	0.94/0.071	<0.00001/22.23	<0.00001/17.14
DMPFC L						
–6/6/50	0.51/–0.66	<0.00001/8.77	<0.00001/8.59	0.56/–0.59	<0.00001/19.11	<0.00001/17.16
DLPFC R						
43 / 18 / 30	0.77/0.30	0.20/1.28	0.34/0.96	0.020/–2.44	<0.00001/9.18	<0.00001/9.62
DLPFC L						
–43/18/30	0.34/–0.98	<0.00001/9.60	<0.00001/10.04	0.036/–2.17	<0.00001/8.58	<0.00001/9.48
Anterior cingulated						
0/38/1	0.079/–1.81	<0.00001/9.26	<0.00001/9.32	0.38/–0.89	<0.00001/13.69	<0.00001/12.20
Subgenual cingulated						
0/17/9	0.29/–1.08	0.00015/4.28	0.00002/4.91	0.59/0.54	0.036/–2.18	0.027/–2.31
Anterior insula R –33/16/–1	0.94/0.07	0.37/0.90	0.39/0.86	0.37/0.90	<0.00001/10.48	<0.00001/9.19
Anterior insula L 33/16/–1	0.90/–0.13	0.039/2.14	0.030/2.26	0.73/0.35	<0.00001/9.98	<0.00001/9.16
Medial thalamus						
0/–12/4	0.74/0.33	<0.00001/5.23	<0.00001/4.40	0.79/0.27	<0.00001/14.57	<0.00001/11.28
Midbrain						
0/23/–12	0.19/–1.32	<0.00001/8.06	<0.00001/7.95	0.71/–0.37	<0.00001/11.17	<0.00001/11.9
Orbitofrontal cortex						
3/49/–12	0.80/0.25	0.00015/4.23	0.0036/3.12	0.0056/2.97	< 0.00001/–6.00	<0.00001/–8.03

The x, y, z coordinates correspond to the centers of the named regions.

behavior. The anterior and subgenual cingulate cortices, involved in conflict detection (Carter and van Veen, 2007), were not activated specifically with evaluation of healthy or unhealthy food.

Important concomitant signals for evaluations of food may arise from the amygdala and ventral striatum. However, contrary to what we hypothesized, the amygdala in our study exerted a stronger signal associated with lower healthiness and the ventral striatum signaled higher healthiness. Both regions are known to be tightly coupled with emotion processing. The ventral striatum is part of the reward system (Salamone and Correa, 2012) and the amygdalae are central processors of emotional signals (Costafreda et al., 2008; Pessoa and Adolphs, 2010). In the context of food processing, Grabenhorst et al. (2013) found that nutritional information biased food evaluations in the amygdala, potentially reflecting an active amygdala participation in food choice. Siep et al. (2009) also reported the amygdala as being active when attending and evaluating food. However, when simply watching food, the amygdala was reported as inactive (Siep et al., 2009; Frank et al., 2010). This underlines the role of the amygdala in evaluation and, possibly, choice or "what if" processes. It may provide bottom–up signaling of the grade of healthiness and, as our findings imply, "warn" by a higher activation when an unhealthy food item is detected. Furthermore, amygdala signaling may be associated with the retrieval of autobiographic and episodic content concerning food, biasing approach or avoidance behavior. On the other hand, the amygdala is a central recipient of cognitive control processes (e.g., Ochsner and Gross, 2005; Herwig et al., 2007) and thus is susceptible to deliberate regulation of food choice and consumption. In that context, "warning" signals can also be intentionally ignored or suppressed when deciding to eat unhealthy food.

The ventral striatum was consistently activated during evaluation and rating bilaterally, and showed right-sided higher activity during the rating of higher healthiness. This may be interpreted as a reward signaling, since the ventral striatum is centrally involved in the brain reward system (Hollmann et al., 2012; Kringelbach et al., 2012). Earlier studies reported,

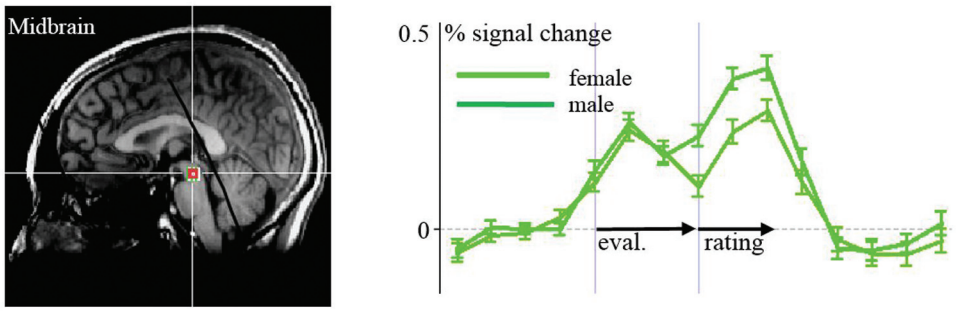

FIGURE 5 | ROI-Analyses of females vs. males: activation in the midbrain to healthy food items is stronger in females than in males.

however, that salutary food might be regarded as less tasty than unhealthier food items (Glanz et al., 1998), because healthy food might cause less reward signaling than unhealthy food. We found a positive correlation between health and taste rating, which might result from a bias due to actively making oneself aware of the health value, which promotes a reward signal in the case of healthy foods. It has been shown that food and drug cues activate the same reward related brain regions (Tang et al., 2012). The cognitive act of intentionally reflecting on the healthiness of food prior to the concrete selection of nutrition, which is often not regularly done, might lead to preferring healthy food, as biased by a reward signal in the context of health evaluation. This might be a simple cognitive strategy for healthier nutrition and better impulse control compared to every-day nutrition without actively reflecting on health value.

The OFC is assumed to be involved in the representation of emotional value linked to reward and decision-making, thereby guiding behavior (Kringelbach, 2005; Schoenbaum et al., 2011). In our case, the OFC was more strongly activated when rating healthy food than when rating unhealthy food. This supports an association between health evaluation and reward in our context. We expected insular regions to be differentially activated by healthiness, but despite a bilaterally prominent general activation during the rating period, no specific healthiness signal was detected. Nevertheless, the strong activation reflects its involvement in associated interoceptive awareness processes (Critchley et al., 2004; Paulus and Stein, 2010). Finally, the unhealthy food items activated the areas within the primary and associative visual cortex more strongly. Whether this may be due to neural processing related to the unhealthiness or to basic visual aspects remains open.

A potential clinical application could be the utilization of cognitive regulation strategies such as reappraisal in order to control food intake when needed (Siep et al., 2012; Yokum and Stice, 2013). A recent reappraisal study for instance supported applying the strategy of reflecting about the long-term benefits of not eating (Yokum and Stice, 2013). Incorporating health aspects in such strategies may advance the application within psychotherapeutic control of eating behavior.

Nutrition and Self-Related Brain Activation

Another interesting finding was the prominent activation of the precuneus, particularly its cognitive self-representation related domain, associated with health evaluation. In an earlier study, participants had estimated the risk of certain hazards presented as verbal terms. It was found that the precuneus was activated when evaluating a higher risk (Herwig et al., 2011). The precuneus is regarded evolutionarily as a newer brain region, particularly present in primates (Cavanna and Trimble, 2006). The precuneus was also reported to be involved in self-imagery, representation of the mental self and autobiographical memory (Cavanna and Trimble, 2006). Furthermore, the precuneus was found to be involved in the evaluation of risks and benefits when establishing good reputations (Watanabe et al., 2014). Another study reported evidence on the role of the precuneus in the integration of both visuospatial information and self in the context of navigation within personal space (Freton et al., 2014). Cavanna and Trimble (2006) summarized the function of the precuneus as a richly connected multimodal associative area that belongs to a neural network, subserving awareness and producing a conscious self-percept. Regarding the activation of the precuneus in our current study, self-relevance and a link to self-representation appears to be a relevant common denominator, with a higher signaling associated with healthier food particularly in the anatomic subdivision of the cognitive/associative central area of the precuneus (Margulies et al., 2009). This area has connections to the prefrontal cortex, BA 10, 46, 8, and also to the dorsal thalamus including the lateral pulvinar, pretectal area, and superior colliculi, thus being connected with very early visual processing that is also related to emotion processing (Tamietto and de Gelder, 2010).

Gender Differences

Several studies on food processing in the central nervous system have reported gender differences. Killgore and Yurgelun-Todd (2010) showed that women, when compared to men, had significantly greater activation to high-caloric foods within dorsolateral, ventrolateral, and ventromedial prefrontal cortex, middle/posterior cingulate, and insular brain regions. They concluded that when viewing high-calorie food images, women

appear to be more responsive than men within cortical regions involved in behavioral control and self-referential cognition. Frank et al. (2010) revealed that satiation seems to influence the processing of food pictures differently in men and women in areas such as the MPFC and fusiform gyrus. On the other hand, Grabenhorst et al. (2013) did not find gender differences concerning taste preference and health-based decision variables. Geliebter et al. (2013) found obese men and women exert different brain activation toward high versus low calorie food in fed and hunger states, comprising prefrontal and subcortical areas such as the caudate. The only difference we found was stronger midbrain activation toward evaluating more healthy food items in women compared to men. One might consider a very deeply rooted healthiness signal in midbrain regions in women reflecting a primordial emotion, even though this remains speculation.

Reflecting on limitations, we have to consider the experimental condition in which the task was restricted to evaluating healthiness without the implementation of another cognitive control condition in order to assess specificity for this aspect. However, we attempted to differentiate between high and low healthiness, with the active cognitive requirement to reflect on healthiness, so that both conditions served as a control for each other with the general basis of health estimation.

CONCLUSION

Differential signaling of perceived food healthiness is associated with activity in the DLPFC, MPFC, precuneus, amygdala, and ventral striatum. The overlap between food processing and emotion processing is obvious. Certainly, this overlap can be explained from an evolutionary perspective and one may even suggest that emotion processing might have its roots, at least in part, in food processing. Regarding possible implications for interventions toward nutrition behavior, one might propose an intentional active mental evaluation of the health value of food intended to be consumed. This might be combined with emotion regulation strategies aimed to reflect the accompanied appetitive emotions, such as incorporating a reality check and reappraisal toward unhealthy food. Actively reflecting on the health value of food may also enhance impulse control. The healthiness associated activation of areas involved in basic emotion processing, such as the amygdala, supports applying emotion regulation strategies in psychotherapeutic attempts to support healthy nutrition.

AUTHOR CONTRIBUTIONS

Substantial contributions to the conception or design of the work: UH, AB, MS, MD; acquisition, analysis, or interpretation of data for the work; UH, AB, MS, MD, CK, SO, AH, SS; drafting the work (UH) or revising it critically for important intellectual content: UH, AB, MS, MD, CK, SO, AH, SS; final approval of the version to be published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: UH, AB, MS, MD, CK, SO, AH, SS.

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