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Chapter 2.2

Brain γ-tocopherol levels are associated with presynaptic protein levels in elderly human midfrontal cortex

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Abstract

Introduction: Higher vitamin E intake has been widely related to lower risks on cognitive decline and dementia. Animal models suggest that this relation might be (partially) explained by the protection of vitamin E against presynaptic protein oxidation. In this cross-sectional study, we aimed to examine the associations between brain tocopherols and presynaptic protein levels in elderly humans.

Methods: We examined associations of α- and γ-tocopherol brain levels with presynaptic protein levels in 113 deceased participants (age 88.5±6.0 years, 45 (40%) female) from the prospective Memory and Aging project. Three distinct presynaptic proteins, a SNARE protein composite, a synaptotagmin synaptophysin composite and the protein-protein interaction between synaptosomal-associated protein 25 (SNAP-25) and syntaxin were measured in two cortical brain regions. Linear regression models assessed associations of brain tocopherols with presynaptic protein levels.

Results: Higher brain γ-tocopherol levels were associated with higher levels of the SNARE protein composite, complexin-I, complexin-II, the synaptotagmin synaptophysin composite and septin-5 in the midfrontal cortex (B(SE)= 0.272 to 0.412 (0.084 to 0.091), $p<0.001$ to 0.003). When additionally adjusted for global Alzheimer's disease pathology, cerebral infarcts and Lewy body disease pathology these associations remained largely similar. No associations were found between α-tocopherol and presynaptic protein levels.

Discussion: In this cross-sectional study, we found higher brain γ-tocopherol levels were associated with presynaptic protein levels in the midfrontal cortex. These results are consistent with a proposed role of vitamin E to maintain presynaptic protein levels.

Introduction

Lower intake and blood levels of several vitamin E isoforms are reported to be associated with increased risks of cognitive decline and dementia [1-4]. In a previous study, we found that higher vitamin E brain levels were associated with lower Alzheimer's disease (AD) neuropathology; lower brain γ-tocopherol levels were associated with higher amyloid load and neurofibrillary tangle severity, while higher α-tocopherol in combination with low γ-tocopherol levels were associated with higher amyloid load [5]. These findings support a protective role of vitamin E in AD and specifically an important role for γ-tocopherol. The underlying mechanisms for this relationship, however, remain unclear.

Synapses play a central role in brain function. Previous studies showed that synaptic density and function are highly related to normal neurological function and cognition [6-11]. Synaptic function, often measured as presynaptic protein levels, is therefore considered an indicator of cognitive brain reserve [12]. Consistent with this role for presynaptic proteins as measure of cognitive reserve, we previously reported that presynaptic protein levels are related to a clinical diagnosis of dementia independent of global AD neuropathology and cerebral infarcts [13]. Synapses are thus key for cognitive function, however they are highly susceptible to oxidative stress that induces presynaptic protein loss and synaptic dysfunction [14-16].

A-tocopherol is well known for having antioxidant properties that prevent free radicals from oxidizing polyunsaturated lipids of cell membranes, lipid bodies and lipoproteins [17, 18]. In addition, γ- tocopherol may exert anti-inflammatory effects [19]. Previous animal studies showed that α-tocopherol may counteract oxidative stress processes and help to maintain presynaptic protein levels [20, 21]. We therefore hypothesized that one potential biological mechanism underlying the relation of tocopherols with cognitive health is preservation of cognitive reserve by sustaining higher levels of presynaptic proteins. In this study, we aimed to examine the associations between brain tocopherols and presynaptic proteins in elderly human brains.

Methods

Study Sample

This study sample is comprised of 115 autopsied cases of participants of the Rush Memory and Aging Project (MAP) who were analyzed for both brain tocopherols and presynaptic proteins. The Rush MAP is an ongoing clinical-neuropathological epidemiologic study of persons living in Chicago continuous care retirement communities and subsidized housing that began in 1997 [22]. Volunteers are free

of known dementia at enrollment and agreed to annual clinical evaluations and to brain autopsy at death. Written informed consent was obtained from all study participants and the study was approved by the Institutional Review Board of Rush University Medical Center.

Brain neuropathology

Brains were examined by a board-certified neuropathologist blinded to clinical data. Brain autopsies for the study sample were performed on average, 6.5 hours after death in a standard fashion as previously described [22]. Brains were processed and stored at the Rush Alzheimer's Disease Center (RADC) laboratory. Slabs from one cerebral hemisphere were placed in a -80°C freezer. This tissue was used for the tocopherol and presynaptic protein quantification as described below. The contralateral hemisphere was fixed in 4% paraformaldehyde and stored in 20% glycerol and 2% dimethylsulfoxide. After fixation, tissue was dissected, processed, embedded, cut and stained from multiple standard regions for neuropathologic evaluation of Alzheimer's disease and other dementia related pathologies including infarcts and Lewy bodies [23]. The density of neuritic plaques, diffuse plaques and neurofibrillary tangles was assessed using Bielschowsky silver stain 6 micron sections for visualization and a graticule to count total number of each measure in a 1-mm^2 area of highest density. Counts for each characteristic were completed in the entorhinal cortex, hippocampus, midtemporal, inferior parietal and midfrontal cortex and then converted to standardized scores. The standardized scores were then averaged across the five regions to obtain a summary score for each measure. A measure of global AD pathology was obtained by averaging summary scores for diffuse plaques, neuritic plaques and neurofibrillary tangles [24, 25]. Macroscopic and microscopic infarcts (acute, subacute and chronic measures separately) were converted to a dichotomous variable indicating absence or presence (1 or more) of any chronic infarcts [26]. Lewy bodies were graded on a four-point scale (0-not present, 1-nigral predominant, 2-limbic type 3-neocortical type), as previously described [27].

Brain tocopherol analyses

Frozen brain tissue from two cortical brain regions (inferior temporal cortex and midfrontal cortex) were thawed and analyzed for tocopherol concentrations using high performance liquid chromatography (HPLC) coupled to electrochemical detection as previously described [28, 29]. Extraction losses were corrected for recoveries of the internal standard, δ-tocopherol. For data-analyses, tocopherol levels are expressed as picomoles (pmol) per mg protein. We eliminated from the analyses two cases with extreme values (α-tocopherol > 10.000 pmol/mg; γ-tocopherol> 900 pmol/mg).

Vitamin E intake

Vitamin E dietary and supplement intake was assessed using a semiquantitative food frequency questionnaire (FFQ) validated in a sample of older Chicago residents [30]. Daily intake of vitamin E was obtained by multiplying the vitamin E content of each food item (from the Harvard nutrient database) by reported frequency of intake, and summing over all food items. All nutrients were calorie adjusted by the regression-residual method. Supplement use and dosage was calculated from vitamin E (α-tocopherol) and multivitamin supplements. Dietary and supplement intake were averaged over all valid FFQs available for each participant.

Presynaptic protein measurements

Monoclonal antibodies were used to quantify immunoreactivity for eight presynaptic proteins (synaptophysin, synaptotagmin, septin 5, syntaxin, synaptosomal associated protein 25 (SNAP-25), vesicle-associated membrane protein (VAMP), complexin-I, complexin-II) in two cortical brain regions (inferior temporal cortex and midfrontal cortex). Frozen samples of gray matter were assayed with an enzyme-linked immunosorbent assay (ELISA). The protein concentration needed to acquire equal fixed optical density value across all samples was identified from dilution curve fitting. The total homogenate protein concentration was inversely related to the present quantity of target antigen. Protein-protein interactions between syntaxin/SNAP-25 and SNAP-25/syntaxin were measured using a high-throughput immunoprecipitation strategy implemented with a heterologous capture ELISA [31]. Purified antibody directed against one of the targets was fixed on the ELISA plate, serially diluted brain homogenate samples were incubated on the ELISA plate, subsequently a second antibody was added to detect the protein-binding partner of the initially captured target. Primary antibodies were produced in house. The following quality control actions were performed. Titer tests were performed on tissue culture supernatants to ensure potency of detection antibodies. Purified antibodies for the protein-protein interaction assays were used at an optimized, fixed protein concentration. Duplicate serial dilution curves were carried out for all sample assays on each plate, and linearity was evaluated from the results. Samples were run twice on separate days, with run-to-run correlations required to exceed r=0.8. In each run multiple replicates of a reference sample were included; the within-run coefficient of variation was required to be <10%.

Statistical Analyses

Mann Whitney-*U* tests were used to compare levels of tocopherols in the inferior temporal and midfrontal brain regions. We studied levels of α- and γ-tocopherol, presynaptic proteins and protein-protein interactions in two different brain regions (inferior temporal cortex and midfrontal cortex). Because tocopherol, presynaptic protein data and vitamin E intake were not normally distributed, all

values were log transformed and subsequently standardized to z-scores. Presynaptic protein data were additionally inverted to make higher values correspond to higher protein concentrations. Some presynaptic proteins were highly correlated, serve related functions and were therefore summarized in three composite scores; 1) soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins composite (syntaxin, VAMP, SNAP-25, Spearman correlation coefficients range: 0.74-0.87, p<0.001), 2) Syntaxin/SNAP-25 protein-protein interaction composite (syntaxin/SNAP-25 and SNAP-25/syntaxin, Spearman correlation coefficients range: 0.58-0.59, p<0.001), 3) Synaptophysin synaptotagmin vesicular protein composite (Synaptophysin and synaptotagmin, Spearman correlation coefficients range 0.83-0.86, p<0.001). Composite scores for each brain region separately were calculated averaging z-scores of the individual presynaptic proteins or protein-protein interactions. Spearman's correlations were used to assess correlations between dietary and total (dietary + supplement) intake of vitamin E and brain tocopherol levels. Linear regression models were used to examine the associations of vitamin E intake and brain tocopherol levels in separate models (continuous determinants) with presynaptic proteins and protein-protein interactions (outcome) by region. The basic model was adjusted for age at death, sex, years of education, APOE ε4 genotype carriers and the time interval from death to autopsy (hours). In a second model we additionally adjusted for global AD pathology, cerebral infarcts and Lewy body disease pathology. Models that examined associations of the tocopherols with the syntaxin/SNAP-25 interaction composite were additionally adjusted for the distinct levels of syntaxin and SNAP-25. All analyses were performed in R (3.4.2 (2017-09-28). A probability level of $p<0.05$ was considered statistically significant. Results are reported at a threshold of $p<0.05$. In addition, we report the results that surpass correction for multiple testing using the Bonferroni correction after which p=0.05/48=0.001 was considered statistically significant.

Results

Sample characteristics

Descriptives of the study sample are shown in **Table 1**. Mean±SD age was 88.5±6.0 years and the majority of our sample was female (60%). The mean±SD years of education was 14.9±2.6 and 30 (26%) participants were carriers of at least one APOE ε4 allele. The mean±SD time till autopsy was 6.5±3.2 hours. Levels of α- and γ-tocopherol did not differ between inferior temporal and midfrontal brain regions.

Intake of vitamin E

Total (dietary + supplement) intake of vitamin E was moderately correlated with brain α-tocopherol levels in the inferior temporal cortex (r=0.25, p=0.01) but not in the midfrontal cortex (r=0.17, p=0.07) or with γ-tocopherol brain levels (r=-0.01, p=0.90, inferior temporal cortex, r=-0.06, p=0.52, midfrontal cortex).

Dietary intake alone was not correlated with brain α- or γ-tocopherol levels (r (range)=-0.09 to 0.04).

Associations between brain tocopherols and presynaptic proteins

Linear regression analysis adjusted for sex, age, education, APOE ε4 genotype and the post-mortem time interval were used to examine the associations of brain tocopherols with presynaptic proteins by region (**Figure 1** and **Table 2**). In the midfrontal cortex, higher γ-tocopherol brain levels were associated with higher levels of the SNARE protein composite, the synaptotagmin synaptophysin composite, complexin-I, complexin-II and septin-5. Only the association with the synaptotagmin synaptophysin composite did not survive Bonferroni correction. When the linear regression models were additionally adjusted for global AD pathology, cerebral infarcts and Lewy body pathology, the effect sizes of associations were not materially changed, but only the association with complexin-II surpassed the more stringent Bonferroni corrected threshold for significance ($p<0.001$) (**Table 2**). Dietary and/or supplement intake of vitamin E was not associated with presynaptic protein levels (data not shown).

Table 1 Descriptives of 113 Memory and Aging Project participants.

General	
Age at death, mean years±SD	88.5 ± 6.0
Female, n (%)	45 (40%)
Education, mean years±SD	14.9 ± 2.6
APOE ε4, n (%) with at least one allele	30 (27%)
Clinical AD diagnosis, n (%)	38 (34%)
Neuropathology	
Post mortem autopsy, mean hours±SD	6.5 ± 3.2
Global AD pathology, mean±SD	0.6 ± 0.5
Presence of cerebral infarctions, n (%)	60 (53%)
Presence of Lewy body pathology, n (%)	19 (17%)
Vitamin E intake (mg/d)	
Dietary vitamin E intake, median (IQR)	5.6 (4.9, 6.6)
Total vitamin E intake, median (IQR)	28.7 (12.8, 146.4)
Brain vitamin E levels (pmol/mg)	
α-tocopherol in the inferior temporal cortex, median (IQR)	172.9 (49.6, 327.0)
α-tocopherol in the midfrontal cortex, median (IQR)	231.8 (93.8, 325.0)
γ-tocopherol in the inferior temporal cortex, median (IQR)	56.7 (34.4, 113.8)
γ-tocopherol in the midfrontal cortex, median (IQR)	57.8 (36.4, 84.4)

Abbreviations: APOE, apolipoprotein E; IQR, interquartile range; SD, standard deviation. Vitamin E intake levels were calorie adjusted, Lewy Body pathology was assessed on a 4 point scale (0-3) as explained in the text, in this table we used a dichotomous variable indicating any presence (score 1-3) of Lewy Body pathology.

Table 2 Association of brain levels of α and γ-tocopherol with presynaptic protein concentrations

Determinant		α-tocopherol				γ-tocopherol			
Outcome	Brain region	Basic model		Basic model + AD pathology + infarcts + Lewy Body pathology		Basic model		Basic model + AD pathology + infarcts + Lewy Body pathology	
		B (SE)	p	B (SE)	p	B (SE)	p	B (SE)	p
SNARE proteins composite[a]	IT	0.098 (0.093)	0.297	0.119 (0.096)	0.215	0.051 (0.095)	0.595	0.065 (0.101)	0.522
SNARE proteins composite[a]	MF	0.038 (0.092)	0.684	0.030 (0.093)	0.749	0.302 (0.086)	<0.001*	0.267 (0.088)	0.003
Complexin-I	IT	0.117 (0.098)	0.235	0.141 (0.101)	0.166	0.023 (0.100)	0.817	0.083 (0.107)	0.439
Complexin-I	MF	0.159 (0.097)	0.105	0.154 (0.100)	0.129	0.327 (0.091)	<0.001*	0.313 (0.095)	0.001
Complexin-II	IT	0.158 (0.098)	0.108	0.154 (0.099)	0.121	0.050 (0.100)	0.617	0.088 (0.104)	0.399
Complexin-II	MF	0.109 (0.094)	0.250	0.103 (0.093)	0.271	0.412 (0.084)	<0.001*	0.369 (0.085)	<0.001*
Syntaxin/SNAP-25 composite[ab]	IT	0.070 (0.091)	0.440	0.082 (0.094)	0.387	-0.004 (0.090)	0.964	-0.027 (0.096)	0.779
Syntaxin/SNAP-25 composite[ab]	MF	0.121 (0.088)	0.172	0.132 (0.090)	0.146	0.118 (0.095)	0.217	0.128 (0.097)	0.187
Synaptotagmin synaptophysin composite [a]	IT	0.100 (0.096)	0.300	0.103 (0.098)	0.292	0.078 (0.097)	0.422	0.075 (0.103)	0.466
Synaptotagmin synaptophysin composite [a]	MF	-0.022 (0.094)	0.816	-0.031 (0.094)	0.741	0.272 (0.088)	0.003	0.233 (0.090)	0.011
Septin-5	IT	0.077 (0.098)	0.434	0.091 (0.099)	0.358	0.038 (0.099)	0.700	0.053 (0.104)	0.612
Septin-5	MF	-0.018 (0.096)	0.850	-0.034 (0.095)	0.723	0.311 (0.089)	<0.001*	0.260 (0.090)	0.005

Abbreviations: IT, inferior temporal, MF, midfrontal, SE, standard error. Basic model adjusted for age at death (years), sex, education (years), APOE ε4 (any ε4 versus none) and post-mortem autopsy time (hours).

[a] SNARE proteins, synaptotagmin+synaptophysin and SNAP-25/syntaxin are calculated composites as described in text.

[b] SNAP-25/syntaxin is additionally adjusted for region specific SNAP-25 and syntaxin concentrations in both models.

* Significant after Bonferroni correction $p<1.0 \times 10^{-3}$

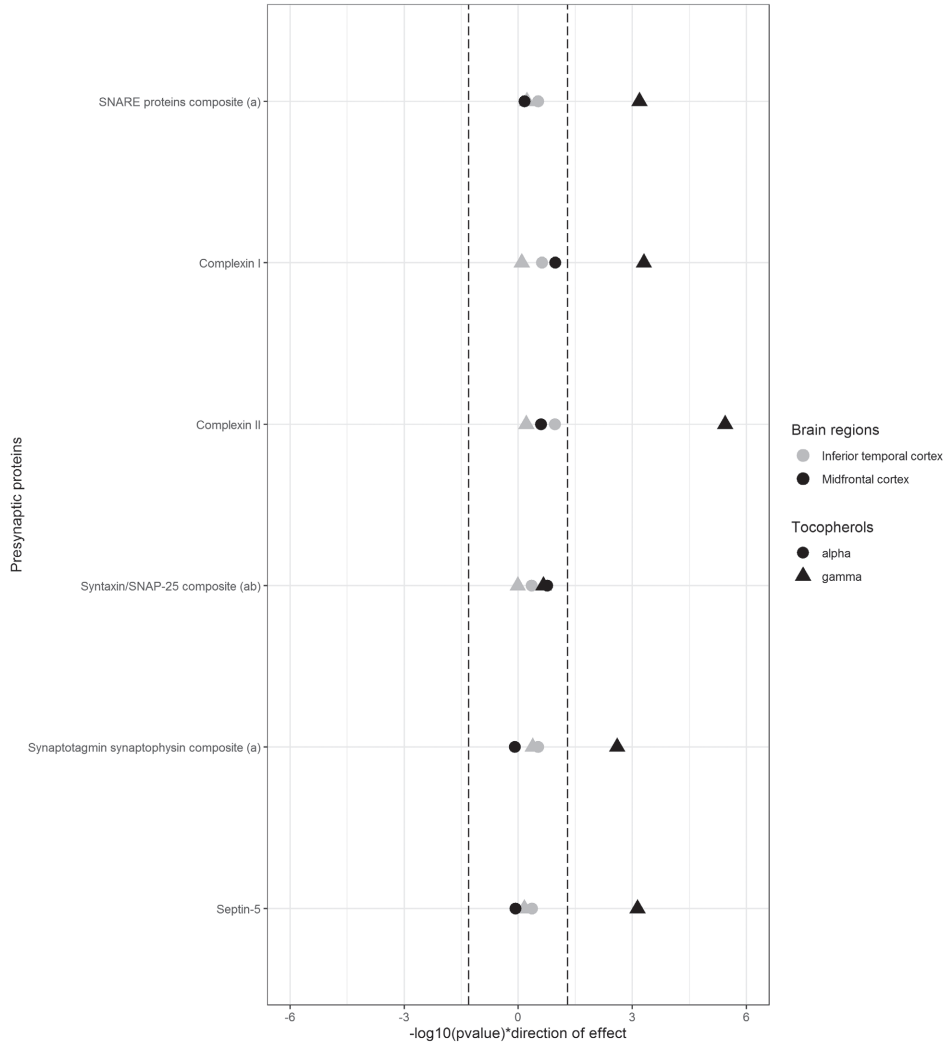

Figure 1 Associations of brain tocopherols with presynaptic proteins by region. Linear regression models corrected for sex. age, education, post-mortem time interval and APOE ε4 genotype. Dashed lines represent p=0.05

(a) SNARE proteins, the synaptotagmin synaptophysin composite and the synatxin/SNAP-25 composite are calculated composites as decribed in the main text. (b) SNAP-25/syntaxin is additionally adjusted for region specific SNAP-25 and syntaxin concentrations.

Discussion

The main finding of this study is that higher brain γ-tocopherol levels relate to higher levels of presynaptic proteins in the midfrontal cortex. To our knowledge, this cross-sectional study is the first study to address the relation between tocopherols and presynaptic proteins in human brain. As such, we can

only speculate on the causality or directionality of effects. Our findings might however, indicate that γ-tocopherol supports synaptic function.

The finding that higher brain tocopherol levels are associated with higher presynaptic protein levels confirms earlier findings from mice studies [20, 21]. Kaneai et al. found that vitamin E deficient mice express lower levels of SNARE proteins [20]. Moreover, oxidative stress reduced levels of presynaptic proteins, but this effect could be partly counteracted with vitamin E supplementation. With these findings, one could hypothesize that tocopherols inhibit the oxidation of presynaptic proteins under oxidative stress. This hypothesis is further supported by two studies describing the potential antioxidant properties of vitamin E in the brain [32, 33].

We found that γ-tocopherol levels were associated with individual presynaptic protein levels in the midfrontal cortex, but not in the inferior temporal cortex. The current knowledge on regional differences in tocopherols and presynaptic protein function is however limited, thus, we can only speculate on the interpretation of these findings. Previous studies have highlighted the midfrontal cortex as highly active area and suitable to study synaptic abnormalities [34-36]. This could perhaps explain why the associations we found between γ-tocopherol and presynaptic protein levels were limited to the midfrontal cortex. Larger studies are however needed to further examine regional differences in tocopherols and presynaptic protein function.

The associations between brain tocopherols and presynaptic protein levels in this study, were independent of AD pathology, cerebral infarcts and Lewy Body disease pathology. In previous work we showed that presynaptic protein levels contributed importantly to cognitive function independent of pathology [13]. Together with the results from this study, this gives support to a model of tocopherols that create an antioxidant environment that protects against oxidation of presynaptic proteins, helps to maintain cognitive reserve, and subsequently might prevent cognitive decline and dementia. In previous work however, we also observed associations between vitamin E and AD brain pathology [5]. Together this might indicate that vitamin E, and in particular γ-tocopherol might have multiple pathways to protect the brain against neurodegenerative processes both via direct effects on pathology and on synapses.

As an essential nutrient, vitamin E must be obtained from food and cross the blood-brain barrier to reach different areas of the brain [37, 38]. In the brain, we found associations between γ-tocopherol levels and presynaptic proteins. We did not observer direct associations between dietary and/or (α-tocopherol) supplement intake and presynaptic protein levels. Moreover, total

(diet+supplement) vitamin E intake was only correlated with α-tocopherol brain levels. This could perhaps be explained by the transport and regulation of vitamin E in humans. While the majority of our dietary vitamin E intake is γ-tocopherol, α-tocopherol is considered the most biologically active form of vitamin E, and comprises the main isoform of vitamin E and multivitamin supplements. Due to the high affinity of vitamin E transporters for α-tocopherol, 90% of the vitamin E in plasma as well as in the brain samples of this study is α-tocopherol [5, 38]. This suggests that our dietary intake of γ-tocopherol only reaches the brain in very low concentrations. Perhaps this explains why the associations between γ-tocopherol and presynaptic proteins in the brain were not seen using the dietary intake measure in this study. Previous studies showed that γ-tocopherol is quickly metabolized into 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman (γ-CEHC) which has similar anti-oxidative and anti-inflammatory properties as γ-tocopherol [39, 40]. It might therefore be of interest to include γ-CEHC measurements as marker for γ-tocopherol function in future investigations. Another explanation could be that the sample size of this study is too small to study the potentially subtle association between dietary intake and presynaptic proteins that could also affected by multiple metabolic factors and recall bias of dietary intake. The additional measurement of γ-tocopherol and γ-CEHC in blood in future studies might help to further assess the complex relationship between dietary intake of γ-tocopherol and presynaptic proteins.

Limitations of this study include the cross-sectional design, and uncertainty concerning the temporal relationship between tocopherols and presynaptic protein levels. The direction of effects cannot be determined. Our data however, support a model where nutritional vitamin E intake contributes to increasing presynaptic protein levels, rather than a brain (disease) effect associated with reduced brain tocopherols or vitamin E intake. First, we found moderate associations of brain α-tocopherol levels with total vitamin E intake, supporting a direct relation between brain tocopherols and nutritional intake. Second, our findings were independent of pathological AD, cerebral infarcts or Lewy Body disease pathology, indicating that disease effects are not (directly) related to our findings. Lastly, our findings are consistent with previous animal studies that showed similar effects in vitamin E supplemented mice. Longitudinal animal studies are however needed to further define the exact cascade of events. Currently, only a few studies measured brain levels of tocopherols; measured concentrations of α-tocopherol and γ-tocopherol levels varied considerably among studies [41, 42]. Inconsistencies in findings could be explained by differences in population or measurement methods. For example, our study sample was younger and included more males, compared to the Georgian Centenarian Study [42]. Although all studies measured tocopherols using HPLC, differences in internal standards or other (pre-)analytical factors could contribute to conflicting

findings. Future research should therefore aim to measure tocopherols in more brain regions and to compare different analytical methods. This study demonstrated associations between γ-tocopherol levels, but not α-tocopherol levels and presynaptic protein levels. Contrary to α-tocopherol, γ-tocopherol has important anti-inflammatory properties, future studies might benefit from including indicators of neuroinflammation [19]. Among the strengths of our present study is that our cohort is very well defined, offered information on the most important potential confounders and detailed neuropathological measures that were obtained after a relatively short post-mortem time interval. In addition, we had information on both dietary and supplement intake of vitamin E as brain tocopherol levels. The sample size of our cohort, with over 100 brain cases, is relatively large in comparison to the limited other autopsy studies in nutrition research. Besides, as a population based cohort, our results might be extrapolated to a larger population. We had many presynaptic protein levels measured in two cortical brain regions, which raises issues of multiple testing. However, we summarized highly correlated presynaptic proteins in composite scores to limit these risks and have additionally used a significance threshold that corrects for multiple testing.

In conclusion, we found associations for higher γ-tocopherol levels with higher levels of presynaptic protein levels in the midfrontal cortex. These results support an important role for γ-tocopherol to maintain presynaptic protein levels and thereby potentially reduce risks of cognitive decline.

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Declaration of interest

None

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