The Journal of Nutrition
**Genomics, Proteomics, and Metabolomics**

# Brain α-Tocopherol Concentration and Stereoisomer Profile Alter Hippocampal Gene Expression in Weanling Mice

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## ABSTRACT

**Background:** Alpha-tocopherol (αT), the bioactive constituent of vitamin E, is essential for fertility and neurological development. Synthetic αT (8 stereoisomers; *all rac*-αT) is added to infant formula at higher concentrations than natural αT (*RRR*-αT only) to adjust for bio-potency differences, but its effects on brain development are poorly understood.

**Objectives:** The objective was to determine the impact of bio-potency–adjusted dietary *all rac*-αT versus *RRR*-αT, fed to dams, on the hippocampal gene expression in weanling mice.

**Methods:** Male/female pairs of C57BL/6J mice were fed AIN 93-G containing *RRR*-αT (NAT) or *all rac*-αT (SYN) at 37.5 or 75 IU/kg ($n = 10$/group) throughout gestation and lactation. Male pups were euthanized at 21 days. Half the brain was evaluated for the αT concentration and stereoisomer distribution. The hippocampus was dissected from the other half, and RNA was extracted and sequenced. Milk αT was analyzed in separate dams.

**Results:** A total of 797 differentially expressed genes (DEGs) were identified in the hippocampi across the 4 dietary groups, at a false discovery rate of 10%. Comparing the NAT-37.5 group to the NAT-75 group or the SYN-37.5 group to the SYN-75 group, small differences in brain αT concentrations (10%; $P < 0.05$) led to subtle changes (<10%) in gene expression of 600 (NAT) or 487 genes (SYN), which were statistically significant. Marked differences in brain αT stereoisomer profiles ($P < 0.0001$) had a small effect on fewer genes (NAT-37.5 vs. SYN-37.5, 179; NAT-75 vs. SYN-75, 182). Most of the DEGs were involved in transcription regulation and synapse formation. A network analysis constructed around known vitamin E interacting proteins (VIPs) revealed a group of 32 DEGs between NAT-37.5 vs. SYN-37.5, explained by expression of the gene for the VIP, protein kinase C zeta (*Pkcz*).

**Conclusions:** In weanling mouse hippocampi, a network of genes involved in transcription regulation and synapse formation was differentially affected by dam diet αT concentration and source: *all rac*-αT or *RRR*-αT. *J Nutr* 2020;00:1–11.

**Keywords:** vitamin E, α-tocopherol, stereoisomers, gene expression, RNA-seq, network analysis, hippocampus, BDNF

## Introduction

Vitamin E, first discovered in 1922, is an essential vitamin, and must therefore be consumed through the diet (1). Vitamin E consists of 8 structural congeners, 4 tocopherols (α, β, γ, and δ), and 4 tocotrienols (α, β, γ, and δ). However, α-tocopherol (αT) is the most abundant and biologically active form of vitamin E in the diet (2, 3). In mice, studies have established that eliminating αT from the diet or preventing its transfer across cell membranes results in female, but not male, infertility (4), and in other secondary symptoms and dysfunctions in both sexes, such as neurodegeneration, demyelination, and ataxia (5–10). Many common foods, including seeds, vegetable oil, and nuts, are natural sources of αT (11). Also, αT is added to many nutritional products and supplements, such as prenatal vitamins and infant formula (12).

The molecular structure of αT contains 3 chiral carbons yielding 8 different stereoisomers: *RRR*, *RSR*, *RRS*, *RSS*, *SRR*, *SSR*, *SRS*, and *SSS* (13). When the molecule is produced synthetically, all 8 stereoisomers are found in equal proportions


Manuscript received February 27, 2020. Initial review completed April 20, 2020. Revision accepted July 27, 2020.
First published online 0, 2020; doi: https://doi.org/10.1093/jn/nxaa249.

(14). However, in nature, αT is only found as the *RRR* stereoisomer. Nutritional products that contain vitamin E either use natural αT extracted from plant oils or synthetically produced αT (12). It is known that synthetic αT is not as potent as the natural form, based on a number of pieces of evidence from a variety of species (2, 15, 16). In rodents, approximately 40% more αT is needed for fetuses to come to term when the source is synthetic as compared to natural (3, 4, 13). The potency difference is supported by human studies showing that the *RRR* stereoisomer is predominant in the human infant brain and human breast milk (12, 17), and studies showing that infants fed baby formula with twice the concentration of synthetic versus natural vitamin E have similar levels of αT in their plasma (18). This has also been demonstrated in dairy cows, where the *RRR* stereoisomer is predominant in the plasma of calves fed equal concentrations of synthetic and natural αT (19). Part of this difference arises from the activity of αT transfer protein (αTTP) (20, 21), which discriminates among the stereoisomers. The protein preferentially binds to the 2*R* stereoisomers of αT (*RRR*, *RSR*, *RRS*, and *RSS*), causing the 2*R* stereoisomers to comprise the vast majority of αT in plasma (22). For this reason, a 2016 US FDA ruling has changed the *RRR*- to *all rac*-αT ratio of potency to 2:1 for food products (23).

However, the bio-potency of natural versus synthetic αT, based on fetal absorption assays or inferred from liver discrimination studies, may not be the same for all biological processes (24–26). It is possible that some of the synthetic αT stereoisomers interact differently with molecular binding targets compared to *RRR* (2, 15). In addition, it is known that the synthetic stereoisomers competitively reduce the concentration of *RRR* in tissues, thus decreasing the level of the most potent stereoisomer (14).

To the best of our knowledge, there are no studies directly comparing hippocampal transcriptomes of normal mice exposed to different sources of αT. Studies have shown that depletion of αT alters gene expression in the rat hippocampus and cortex (27–29). In addition, a few studies used the αTTP knockout (Ttpa-KO) mouse to produce mice deficient in αT, and observed alterations in gene expression within multiple brain regions, including the hippocampus (5, 30). A recent study fed young Ttpa-KO mice synthetic (*all rac*) αT at twice the concentration as natural (*RRR*) αT, and revealed that the high dose of synthetic αT downregulated genes involved in myelin regulation, relative to a lower dose of natural αT in the cerebellum (31). The cerebellum is important for motor coordination and procedural learning (32). Taken together, these studies suggest that the concentration, and potentially the source, of αT can impact gene expression regulation in the brains of young adult mice. However, it remains unknown to what extent the source of αT impacts gene regulation in the brains of normal, non-transgenic, developing mouse pups. Han et al. (33) showed that equal dietary doses of *all rac*-αT or *RRR*-αT differentially impacted gene expression in lymphocytes isolated from non-transgenic mice (33). However, a study using a hepatic cancer cell line, HepG2 cells, found 215 genes were responsive to the application of either *RRR*-αT or *all rac*-αT, and that both forms of αT induced or repressed the same set of genes, implying they have equivalent transcriptional activity (34, 35).

The goal of the current study was to feed normal, C57BL/6J mouse dams diets containing only *RRR*-αT or *all rac*-αT during pregnancy and throughout lactation, and then to observe the impact on gene expression in the hippocampi of the weanling pups. The hippocampus was explored because it is a region of the brain known to be important for learning and memory (36), and known to continue to develop throughout early postnatal development and young adult life (37, 38). We sought to determine whether the different doses or sources of αT modulated gene expression in the hippocampus and, if so, which specific genes were affected. In testing 2 concentrations of αT, we hypothesized that the lower concentration might reveal differences between sources that would not occur at the higher doses, where higher amounts of the *RRR* stereoisomer were provided. We further reasoned that if similar differences in gene expression between natural and synthetic αT were found at both concentrations, that would suggest the source of αT impacts gene expression because the synthetic stereoisomers compete with bioactive sites and/or interact with molecular partners in a different way than *RRR*.

## Methods

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Illinois, which adheres to policies set forth by the USDA and the Association for Assessment and Accreditation of Laboratory Animal Care.

### Animals

Male and female C57BL/6J mice (Experiment 1, $n = 24$, 12 males and 12 females; Experiment 2, $n = 48$ total, 24 males and 24 females) were obtained from The Jackson Laboratory. Upon arrival, at 7 weeks of age, mice were paired and were immediately placed on 1 of the experimental diets (see below and **Table 1**). The mice were housed in polycarbonate shoebox-style cages (29 x 19 x 13 cm) with 1/4" corncob bedding (Teklad 7097; Harlan Laboratories), and were kept on a constant 12:12 light-dark cycle in a temperature-controlled (21 ± 1 °C) room, with ad libitum access to fresh water and food.

### Experiment 1: Milk α-tocopherol

We used 12 pairs of mice to determine the extent to which the milk from the dams would contain and reflect the different concentrations and sources of αT in their diet. The pairs were fed a diet based on the standard AIN 93-G, except that the soybean oil used in the formula was stripped of αT, and αT [natural (NAT) or synthetic (SYN)] was then added according to the different treatments, hereafter referred to as NAT-32, SYN-32, and SYN-75. These diets contained either 32 IU/kg *RRR*-αT ($n = 7$), 32 IU/kg *all rac*-αT ($n = 2$), or 75 IU/kg *all rac*-αT ($n = 3$) per kg diet (Table 1). Originally, 24 pairs (8 pairs per treatment) were randomly assigned to these diets, but ultimately only 12 pairs were used, unevenly distributed between the treatments. Uneven sample sizes resulted from differential success in getting pairs

This work was supported by Abbott Nutrition through the Center for Nutrition, Learning, and Memory.
Author disclosures: C-SL, KJS, and MJK are employed by Abbott Nutrition, which supported this work. JSR, CR, JGM, KD, PT, AS, HP, JD, and SC, no conflicts of interest.
Supplemental Methods, Supplemental Tables 1–4, and Supplemental Figure 1 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
Address correspondence to JSR (e-mail: jrhodes@illinois.edu).
Abbreviations used: *all rac*-αT, synthetic α-tocopherol; DEG, differentially expressed genes; FDR, false discovery rate; NAT, AIN 93-G diet containing *RRR*-αT; PKC, protein kinase C; *Pkcz*, protein kinase C zeta; *RRR*-αT, natural α-tocopherol; SYN, AIN 93-G diet containing *all rac*-αT; TRN, transcriptional regulatory network; Ttpa-KO, α-tocopherol transfer protein knockout mouse; VIP, vitamin E interacting protein; α-CEHC, 2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman; αT, α-tocopherol; αTTP, α-tocopherol transfer protein.

**TABLE 1** Composition of the diets

| Experiment | Treatment | Stereoisomers | Diet recipe, g $\alpha$T ingredient/kg diet | Diet, IU $\alpha$T/kg diet |
|---|---|---|---|---|
| Milking study | SYN-32 | *all rac*-$\alpha$T acetate | 0.064 | 32 |
| Milking study | NAT-32 | *RRR*-$\alpha$T acetate | 0.046 | 32 |
| Gene expression | SYN-37.5 | *all rac*-$\alpha$T acetate | 0.075 | 37.5 |
| Gene expression | NAT-37.5 | *RRR*-$\alpha$T acetate | 0.054 | 37.5 |
| Both | SYN-75 | *all rac*-$\alpha$T acetate | 0.150 | 75 |
| Gene expression | NAT-75 | *RRR*-$\alpha$T acetate | 0.107 | 75 |

Diets were prepared by Research Diets, Inc. AIN 93-G was used as the background. Soybean oil stripped of vitamin E was purchased from Dyets, Inc., who use a vacuum distillation process to remove fat soluble vitamins. The concentration of $\alpha$T was measured in samples of each diet using the method described for the brain in the Methods Section, and was found to be within 10% of the reported values. The *all rac*-$\alpha$T acetate was from Fortitech Inc. (500 IU vitamin E/g ingredient) and the *RRR*-$\alpha$T acetate was from Archer-Daniels-Midland Company (700 IU vitamin E/g ingredient). Abbreviations: $\alpha$T, $\alpha$-tocopherol; *all rac*-$\alpha$T, synthetic $\alpha$-tocopherol; NAT, AIN 93-G diet containing *RRR*-$\alpha$T; *RRR*-$\alpha$T, natural $\alpha$-tocopherol; SYN, AIN 93-G diet containing *all rac*-$\alpha$T.

of mice to breed on our time schedule. Instead of 37.5 IU/kg, 32 IU/kg was used; this was our initial estimate of what might be considered a moderately low dose of $\alpha$T, but since this was close to half of 75, we used exactly half of 75, or 37.5, for the gene expression study described below (see Table 1). Each pair of mice was fed the treatment diet from the time they were paired, through conception, pregnancy, lactation, and weaning (21 d).

On day 7 postpartum, the dams were separated from their litters approximately 2 h prior to milking. Pups remained in their original cages and were placed gently on a heating pad to stay warm. The dam was given an intraperitoneal injection of 0.1 ml (2 IU) of oxytocin to induce lactation. An electronic pipet with a modified tip adjusted to an internal diameter of approximately 0.75 mm was used for milking. At 1 min after the intraperitoneal injection, the dam was restrained by hand, back pressure was applied, and the pipette tip was placed gently over the teat. The plunger was then slowly released, creating a vacuum for extracting the milk. This process was repeated on alternating teats until an adequate amount (approximately 200 $\mu$l) was extracted or the milk flow ceased. The collected milk was ejected into a centrifuge tube and kept on dry ice before storing in the −80°C freezer. This procedure was repeated on postpartum days 14 and 21.

## Experiment 2: Gene expression study

We fed 24 pairs of mice a diet based on the standard AIN 93-G, except that the soybean oil used in the formula was stripped of $\alpha$T, and $\alpha$T (NAT or SYN) was then added according to the different treatments (see Table 1), referred to hereafter as NAT-75, NAT-37.5, SYN-75, and SYN-37.5. These diets contained either 75 IU/kg *RRR*-$\alpha$T, 37.5 IU/kg *RRR*-$\alpha$T, 75 IU/kg *all rac*-$\alpha$T, or 37.5 IU/kg *all rac*-$\alpha$T per kg diet. Each pair of mice was randomly assigned to feed on 1 of these diets ($n = 6$ pairs per diet) from the time they were paired, through conception, pregnancy, lactation, and weaning. At the time of weaning (21 d), the mouse pups were weighed and euthanized, and their brains were rapidly removed, weighed, and then divided in half along the midline. Half the brain was used to measure the concentration of $\alpha$T and stereoisomer distribution. The hippocampus in the other half of the brain was carefully dissected and frozen on dry ice, and then stored in the −80°C freezer before RNA-seq (RNA-sequencing) analysis. A total of 126 weanling mice (from 24 dams) were processed this way. Of these 126 mice, 40 were chosen for the gene expression study; mice were chosen such that the mean body mass at weaning was similar between sources within the 37.5 IU/kg and 75 IU/kg doses, in order to control sources of variation in gene expression related to the growth or developmental stage of the pups. These individuals were chosen before observing the $\alpha$T concentrations and stereoisomer distributions in the brain.

## Analysis of $\alpha$T concentrations and stereoisomer distribution

Briefly, ~100 mg of brain tissue was mixed with water, sodium ascorbate, methanol, and potassium hydroxide; flushed with nitrogen; and heated in a 60°C water bath for ~20 min. Samples were immediately cooled; extracted with methanol, hexane, and water; and centrifuged (1500 × *g* at 4°C for 5 min) to separate the hexane and methanol/water layers. For total $\alpha$T analyses, 1 mL of the hexane layer was concentrated to 0.1 mL and analyzed isocratically with normal phase chromatography using a silica column and an isooctane/dichloromethane/isopropanol mobile phase and fluorescence detection. For $\alpha$T stereoisomer analyses, the remaining hexane layer was concentrated to ~100 $\mu$L, and $\alpha$T was isolated chromatographically using a diol column with a hexane/methy-t-butyl-ether mobile phase. The $\alpha$T in the isolated fraction was then methylated using dimethylcarbonate, dimethylsulfoxide, and potassium carbonate, and the 2*S*, *RSS*, *RRS*, *RRR*, and *RSR* isomers were separated using a Chiralcel OD-H, 5 micron, 4.6 x 250 mm column and were detected by fluorescence. The detection limit was approximately 0.2 mg/kg tissue. The preparation procedure for milk samples was similar, except without the cleanup step before methylation.

## RNA sequencing

See **Supplemental Methods** for details on RNA extraction, purification, sequencing, alignment, and normalization procedures.

## DAVID functional annotation

The list of differentially expressed genes (DEGs) which were significant by 1-way ANOVA at less than 20% FDR was submitted to the gene annotation tool in DAVID (Database for Annotation, Visualization and Integrated Discovery). The tool was used to identify those functional categories that were significantly enriched in the DEG list.

## Network analysis

The underlying assumption of the network analysis is that vitamin E influences the activity and levels of vitamin E interacting proteins (VIPs; see **Supplemental Table 1** for description), which subsequently cause global changes in the transcriptome, including the transcript levels of VIPs. Our definition of a VIP here is any protein whose function is affected by the presence of vitamin E. Importantly, our definition does not require that a VIP directly bind to $\alpha$T. Indeed, it is not clear that $\alpha$T binds to many proteins (other than $\alpha$TTP) in a specific manner (39). For example, $\alpha$T could act as an antioxidant and thereby affect the function of proteins involved in oxidative metabolism (40), or it could affect the function of certain proteins (e.g., protein phosphatase), which then affect the function of other proteins (e.g., protein kinase C) in a signaling cascade (41–43). By identifying transcripts that correlate with VIP transcripts, we hypothesized that we could identify key VIP regulators and their corresponding targets. This framework is analogous to "guilt by association" pull-down assays used for identifying protein-protein interactions.

First, the literature was explored to find all the proteins shown to interact either directly or indirectly with $\alpha$T; that is, changes in $\alpha$T are known to change the expression or function of these proteins in

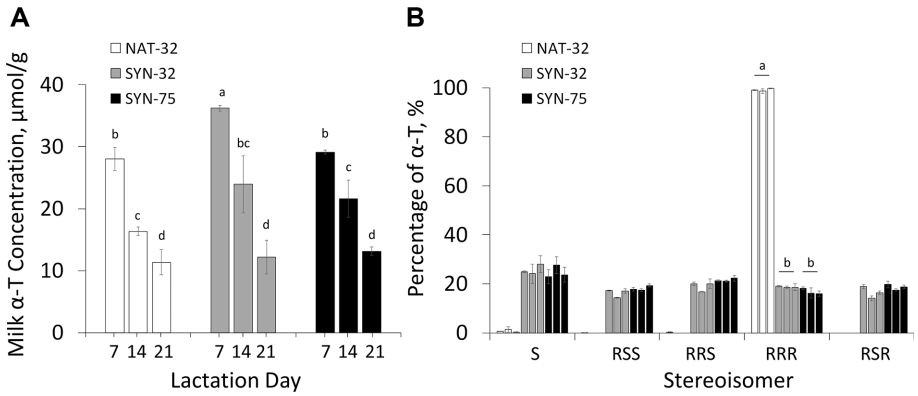


**FIGURE 1** Concentrations (A) and stereoisomer distributions (B) of $\alpha$T in the milk of mouse dams fed diets containing either 32 IU/kg *RRR*-(NAT-32; $n = 7$), 32 IU/kg *all rac*- (SYN-32; $n = 2$), or 75 IU/kg *all rac*-$\alpha$T (SYN-75; $n = 3$), sampled on days 7, 14, and 21 of lactation (Experiment 1). Values are means ± SEMs. The 3 sequential bars within a treatment group represent values for days 7, 14, and 21. The S stereoisomer designation refers to the sum of all four 2*S* stereoisomers. Labeled means (A) or labeled means collapsed across time points (B) without a common letter differ at $P < 0.05$. Abbreviations: $\alpha$T, $\alpha$-tocopherol; *all rac*-$\alpha$T, synthetic $\alpha$-tocopherol; NAT, AIN 93-G diet containing *RRR*-$\alpha$T; *RRR*-$\alpha$T, natural $\alpha$-tocopherol; SYN, AIN 93-G diet containing *all rac*-$\alpha$T.

vitro or in vivo. Our compiled list of such proteins, with references to back up the report of interactions with vitamin E, is shown in Supplemental Table 1. Since some of these proteins have multiple isoforms, this list of 24 became a total of 58 different proteins with known genes. A transcriptional regulatory network (TRN) model was then reconstructed, centered around these VIPs using the ASTRIX (Analyzing Subsets of Transcriptional Regulators Influencing eXpression) algorithm (44, 45). ASTRIX uses gene expression data to identify interactions between regulators and their target genes. The ASTRIX algorithm has been previously used to infer brain TRN models for various organisms, including the honeybee and mouse (46–48). These models showed remarkably high accuracy in predicting gene expression changes in the brain.

Consistent with our previous reports (46–48), the following criteria were used to implicate a VIP in interacting with a given target gene by ASTRIX: *1*) the VIP had to share a significant degree of mutual information, a measure of similarity with the target gene ($P$ value $< 0.001$) (45); 2) the VIP had to explain at least 10% of the variance of the gene, quantified by regression analysis; and *3*) each target gene included in the network had to be predicted with a correlation of at least 0.8 by the ASTRIX model using expression levels of VIPs. A very high correlation threshold was chosen to identify the strongest interactions, as used in prior studies (45). The original implementation of ASTRIX used the least-angle regression algorithm to identify the most parsimonious set of predictors (i.e., least number of regulators) among hundreds of possible features. However, in our case, since there was already a reduced set of regulators (i.e., the 58 VIPs), the least-angle regression analysis was replaced with traditional linear regression.

### Statistical analysis

Data were analyzed using SAS 9.3 (Sas Institute) and R 3.5.1 (R Foundation for Statistical Computing). A $P$ value less than 0.05 was considered statistically significant, except for in gene expression (RNA-seq) data, in which multiple testing correction using the false discovery rate (FDR) method (49) was done separately for all comparisons. The total concentration of $\alpha$T in the milk was analyzed using a repeated-measures 2-way ANOVA with group (3 diets: SYN-32, NAT-32, or SYN-75) entered as a between-subjects factor and time point (3 times: 7, 14, and 21 d) entered as a within-subjects factor. Body mass, brain mass, total $\alpha$T concentration, and percentage of $\alpha$T in the *RRR* stereoisomer were analyzed using a 2-way ANOVA with source (*all rac*-$\alpha$T or *RRR*-$\alpha$T) as 1 factor and dose (37.5 or 75 IU/kg) as the other factor. Data were considered normally distributed if the absolute value of the skewness was less than 1 and kurtosis was less than 2; otherwise, a power transform was used to transform data to within these boundaries. Post hoc pair-wise differences between means were evaluated using Tukey tests when a statistical interaction was observed or when a main effect with multiple levels was significant.

A differential gene expression analysis of the RNA-seq data was performed using the limma-voom method (50). First, 1-way ANOVAs and all pairwise comparisons were computed across the 4 groups. Second, 2-way ANOVAs were computed with concentration (37.5 vs. 75 IU/kg) and source (natural vs. synthetic) entered as the 2 factors, along with the interaction term. The 1-way ANOVAs allowed a straightforward way to filter the results to find those genes whose expression differed for at least 1 of the groups as compared to the others at a set false discovery rate. The 2-way ANOVA provided a way to detect the main effects of source, collapsed across doses, and the main effects of dose, collapsed across sources, as well as interactions; hence, both analyses were included for completeness.

Performance of the TRN model was evaluated first by determining the extent to which expressions of the 58 VIP genes were better at explaining gene expression differences between the groups, as compared to a random sample of 58 genes from the full list of genes measured. Predictions were made 50 times with a different set of 58 random VIPs, and were compared to the actual VIP predictions (i.e., total genes predicted with a significantly high correlation) using a *t*-test.

The TRN model inferred by ASTRIX enabled further prioritization of the list of potential regulators mediating vitamin-E responses. VIP "modules" were defined as the set of genes that are predicted to be regulated by a VIP. The extent to which the VIP modules were enriched for DEGs was evaluated by overlapping the VIP modules with the list of DEGs between each pair of treatment groups. The extent of overlap was assessed using a hypergeometric test.

## Results

### Concentration of $\alpha$T in milk

The concentration of $\alpha$T decreased in dam milk by approximately 40% per week [main effect, $P < 0.0001$; all post hoc pairwise differences between time points (7, 14, 21 d) were significant at $P < 0.001$; **Figure 1**A], and was only slightly altered by the diets ($P = 0.028$). No interaction between time point and diet was detected ($P = 0.35$). Collapsed across weeks, SYN-32 had approximately a 30% higher concentration of $\alpha$T than NAT-32 ($P = 0.025$). No other pair-wise differences between diets were significant (all $P > 0.05$).

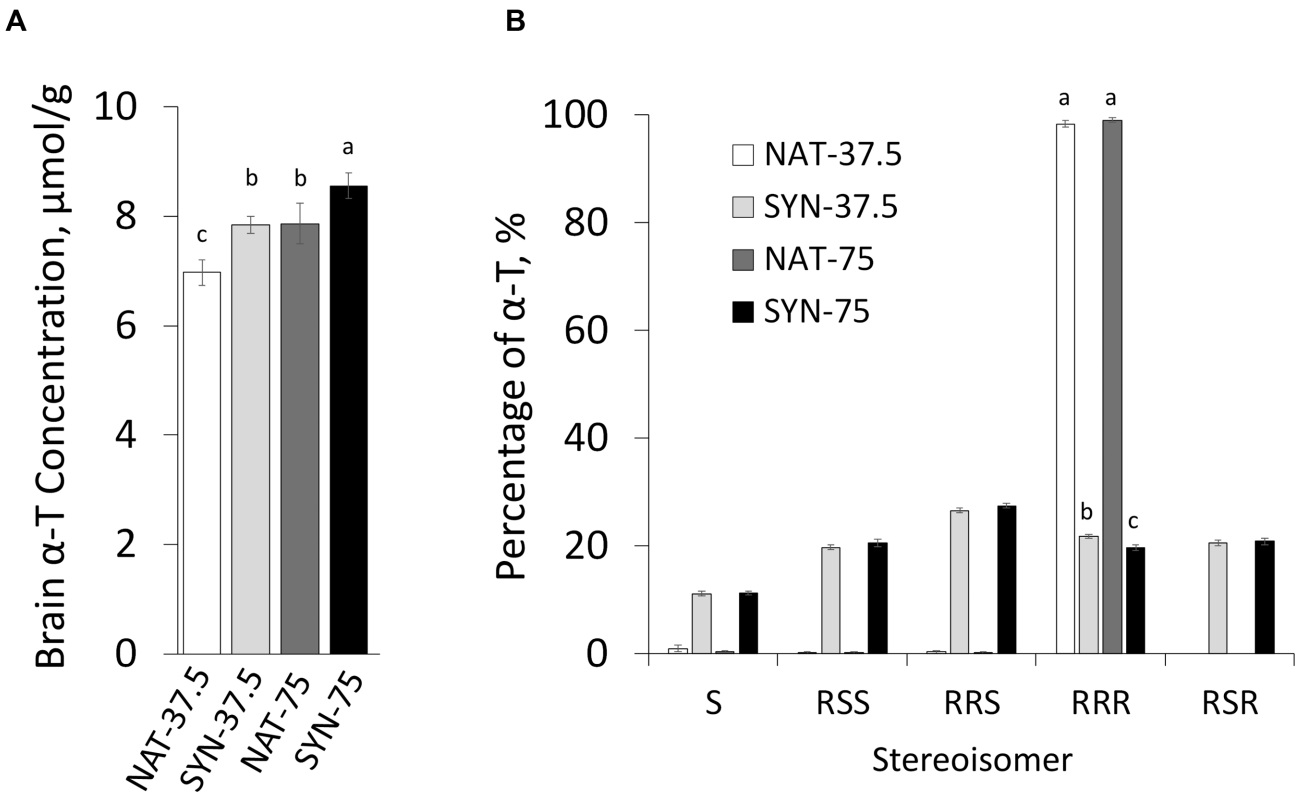


**FIGURE 2** Concentrations (A) and stereoisomer distributions (B) of αT in the brains of mice at weaning age (21 d) from the 37.5 IU/kg *RRR*- (NAT-37.5; $n = 10$), 37.5 *all rac*- (SYN-37.5; $n = 10$), 75 IU/kg *RRR*- (NAT-75; $n = 10$), and 75 IU/kg *all rac*-αT (SYN-75; $n = 10$) groups (Experiment 2). Values are means ± SEMs. Note these 40 weanling mice came from a total of 24 dams. Individuals from within a group each came from a different dam, and weanlings were further matched for body mass between sources within doses (see Methods). The S stereoisomer designation refers to the sum of all four 2*S* stereoisomers. Labeled means without a common letter differ at $P < 0.05$. In (B), a statistical analysis was done only for the percent of *RRR* stereoisomers. Abbreviations: αT, α-tocopherol; *all rac*-αT, synthetic α-tocopherol; NAT, AIN 93-G diet containing *RRR*-αT; *RRR*-αT, natural α-tocopherol; SYN, AIN 93-G diet containing *all rac*-αT.

### Stereoisomer distribution of αT in milk

The stereoisomer distribution of αT in the milk reflected the composition of the diets. Milk from dams fed NAT-32 displayed nearly all αT as *RRR*-αT, whereas milk from dams fed SYN-32 and SYN-75 displayed approximately equal concentrations of the four 2*R* stereoisomers and the sum of the 2*S* stereoisomers (Figure 1B). The presumed lower concentration of individual 2*S* isomers likely reflects incomplete absorption, hepatic catabolism, or transport of 2*S* isomers into tissues, as previously observed for rodents (13, 25, 51). In the NAT-32 group, 99.1% of the αT in the milk consisted of *RRR*-αT (collapsed across the 3 time points of 7, 14, and 21 d), whereas only 18.6% and 16.6% were *RRR*-αT for the SYN-32 and SYN-75 groups, respectively ($P < 0.0001$; Figure 1B). NAT-32 was different from SYN-32 and SYN-75 ($P < 0.0001$), but SYN-32 and SYN-75 were not different from each other ($P = 0.10$).

### Concentration of αT in brain

The 75 IU/kg groups (SYN-75 + NAT-75) had 10.8% more αT in the brain than the 37.5 IU/kg groups (SYN-37.5 + NAT-37.5; $P = 0.004$). Similarly, the brain αT concentrations in the SYN groups (SYN-37.5 + SYN-75) were 10.6% higher than in the NAT groups (NAT-37.5 + NAT-75; $P = 0.005$; Figure 2A). The interaction was not significant ($P = 0.73$).

### Stereoisomer distribution of αT in brain

The stereoisomer distribution of αT in the brain of the pups reflected the diets and milk profiles. Brains of pups in the NAT-37.5 and NAT-75 groups contained nearly 100% αT as *RRR*-αT (98.4% and 99.1%, respectively), whereas only 21.8% and 19.7% of total αT in brains of the SYN-37.5 and SYN-75 was *RRR*-αT, respectively. SYN-37.5 and SYN-75 displayed approximately equal levels of the four 2*R* isomers, and approximately 10% of the total as 2*S* isomers (Figure 2B). These differences were reflected in a significant main effect of the αT source for the percent of *RRR*-αT ($P < 0.0001$; Figure 2B), no effect of dose ($P = 0.14$), but a significant dose-by-source interaction ($P = 0.006$). The interaction was due to slightly lower proportions of *RRR*-αT in the SYN-75 group, as compared to SYN-37.5 group ($P = 0.02$), but no differences between the NAT-75 and NAT-37.5 groups ($P = 0.74$).

The total concentration of *RRR*-αT stereoisomer in the brain was calculated for each individual as the product of the proportion of *RRR*-αT multiplied by the total concentration of αT in the brain. Significant main effects of source ($P < 0.0001$), dose ($P = 0.049$), and dose-by-source interaction were detected ($P = 0.036$). The interaction was due to a 14% higher concentration of *RRR*-αT in the NAT-75 group as compared to the NAT-37.5 group ($P = 0.025$), but no difference between the SYN-75 and SYN-37.5 groups ($P = 1.0$). The mean brain concentrations of

*RRR*-αT (± SEM) were 6.85 μmol/g (± 0.225), 1.72 μmol/g (± 0.042), 7.78 μmol/g (± 0.378), and 1.67 μmol/g (± 0.049) for the NAT-37.5, SYN-37.5, NAT-75, and SYN-75 groups, respectively.

### Body mass and brain mass

Pups were deliberately selected so that the body masses would be similar between the NAT-37.5 and SYN-37.5 groups and between the NAT-75 and SYN-75 groups (see Methods), without knowledge of any other outcome. Therefore, no differences in body mass were detected between the sources of αT ($P = 0.56$). Nonetheless, pups in the NAT-75 and SYN-75 groups were slightly lighter than those in the NAT-37.5 and SYN-37.5 groups ($P = 0.0005$), likely a result of the bias introduced from matching body masses within concentrations rather than from an impact of the diets. The mean body masses (± SEM) were 9.7 g (± 0.212), 9.5 g (± 0.152), 8.8 g (± 0.240), and 8.8 g (± 0.239) for the NAT-37.5, SYN-37.5, NAT-75, and SYN-75 groups, respectively. No differences were detected in the brain masses between any of the groups (all $P > 0.36$). The mean brain masses (± SEM) were 0.42 g (± 0.007), 0.42 g (± 0.006), 0.43 g (± 0.006), and 0.43 g (± 0.006) for the NAT-37.5, SYN-37.5, NAT-75, and SYN-75 groups, respectively. No differences in body mass or brain mass between sources of αT created controlled experimental conditions to test the effects of source of αT on brain gene expression.

### RNA-seq DEGs

A total of 16,755 genes passed our criteria for sufficient expression to be accurately measured (**Supplemental Table 2**). Of these genes, 797 were found to be differentially expressed between the dietary groups, as measured by a 1-way ANOVA with an FDR of less than 20%. Most of these genes were differentially expressed as a result of the concentration of αT in the maternal diet rather than the source (**Figure 3**), and the effect sizes were generally small. Approximately 95% of these differentially expressed genes displayed a fold change less than 1.10 (i.e., less than a 10% change). Of these 797 genes, there were significant post hoc differences in 179 between NAT-37.5 and SYN-37.5 (all $P < 0.05$), 182 between NAT-75 and SYN-75 (all $P$ values $< 0.05$), 600 between NAT-37.5 and NAT-75, and 487 between SYN-37.5 and SYN-75. An analysis of the same data by 2-way ANOVA found that 2522 genes displayed a significant main effect of dose (37.5 vs. 75 IU/kg, collapsed across SYN and NAT), but only 1 gene showed a significant effect of source (SYN versus NAT, collapsed across doses 37.5 and 75 IU/kg), using the same criterion of an FDR less than 20%. No genes showed a significant interaction between dose and source by the 20% FDR criterion.

### DAVID functional annotation

The DAVID analysis indicated significant enrichment of the 797 DEGs from the 1-way ANOVA in the categories of transcription regulation, DNA binding, and synapse regulation (see Supplemental Table 2 and the summary in **Supplemental Figure 1**). Taken together, these results indicate that varying the concentration and source of αT in the diet subtly alters a network of many genes involved in gene transcription and synapse regulation in the hippocampus.

### Network analysis

Among the 16,755 genes in the transcriptome data, the TRN model built by ASTRIX could predict 2233 genes with a correlation of 0.8 or higher using expression levels of the 58 VIPs. The resulting VIP network inferred by ASTRIX contains 11,040 interactions between 58 VIPs and the 2233 target genes. Each target gene, on average, is predicted to interact with 4–5 VIPs. The full TRN is in **Supplemental Table 3**.

The VIPs had significantly higher predictive power than randomly chosen genes. Using a cross-validation analysis, wherein a part of the data was blinded from the algorithm and used information from the remaining nonblinded data, the transcriptome profiles from blinded conditions were predicted. In this case, using VIP transcript levels, ASTRIX was able to consistently predict the expression levels of a significantly higher number of genes in blinded test samples with high correlations ($R > 0.8$) than of a random set of 58 genes; the average predictive power of the random VIPs was 2-fold lower than the actual VIPs (**Figure 4**; *t*-test, $P < 0.0001$). The difference was more significant when we focused on the subset of genes that were differentially expressed between the diets; the average predictive power of the random VIPs was 3-fold lower than the actual VIPs ($P < 0.0001$).

See **Supplemental Table 4** for the total number of genes explained by each VIP (i.e., total number of genes in the VIP module), and the total number of DEGs explained by each VIP for each pair-wise comparison between groups. The table also shows *P* values for whether the VIP module is enriched for DEGs, based on a hypergeometric test. Separate tabs in the table show the names of those DEGs between NAT-37.5 and SYN-37.5 and between NAT-75 and SYN-75 that are explained by the VIPs. For example, the network analysis revealed that the VIP gene protein kinase C zeta (*Pkcz*) predicted 32 DEGs between NAT-75 and SYN-75, including *bdnf*.

## Discussion

The main findings of this study are that the expression of hundreds of hippocampal genes was modestly changed in normal weanling mice due to *1*) slight changes in the brain concentration of αT; and *2*) large modifications in the αT stereoisomer profile. A 2-fold increase in the concentration of αT in the dam diets within the recommended dietary intake range for mice only increased the weanling brain αT by 10%, regardless of the source of αT. In contrast, altering the source of αT (*RRR*-αT or *all rac*-αT) led to dramatic differences in brain αT stereoisomer profiles that reflected the profiles in the dam diet and milk. That the expression of a large number of genes was affected to a modest degree (<10%) suggests that transcriptional regulation was affected on a global scale. The observation that a few hundred genes were differentially expressed between the SYN and NAT groups for both the high and moderately low doses suggests that the synthetic αT stereoisomers may interact with molecular targets in a different way than *RRR*-αT.

Even though the dams were fed diets that varied 2-fold in αT within the recommended reference range, the concentration of αT in the milk was not affected, and the weanling brain concentration changed by only 10%. This suggests the concentrations of αT in milk and the brain are tightly regulated and, when taken together with the gene expression data, indicates that gene expression in the hippocampus is very sensitive to subtle changes in αT concentration. Given the large variation in concentrations of αT observed in the infant brains and milk of humans (12, 17), the impact on the

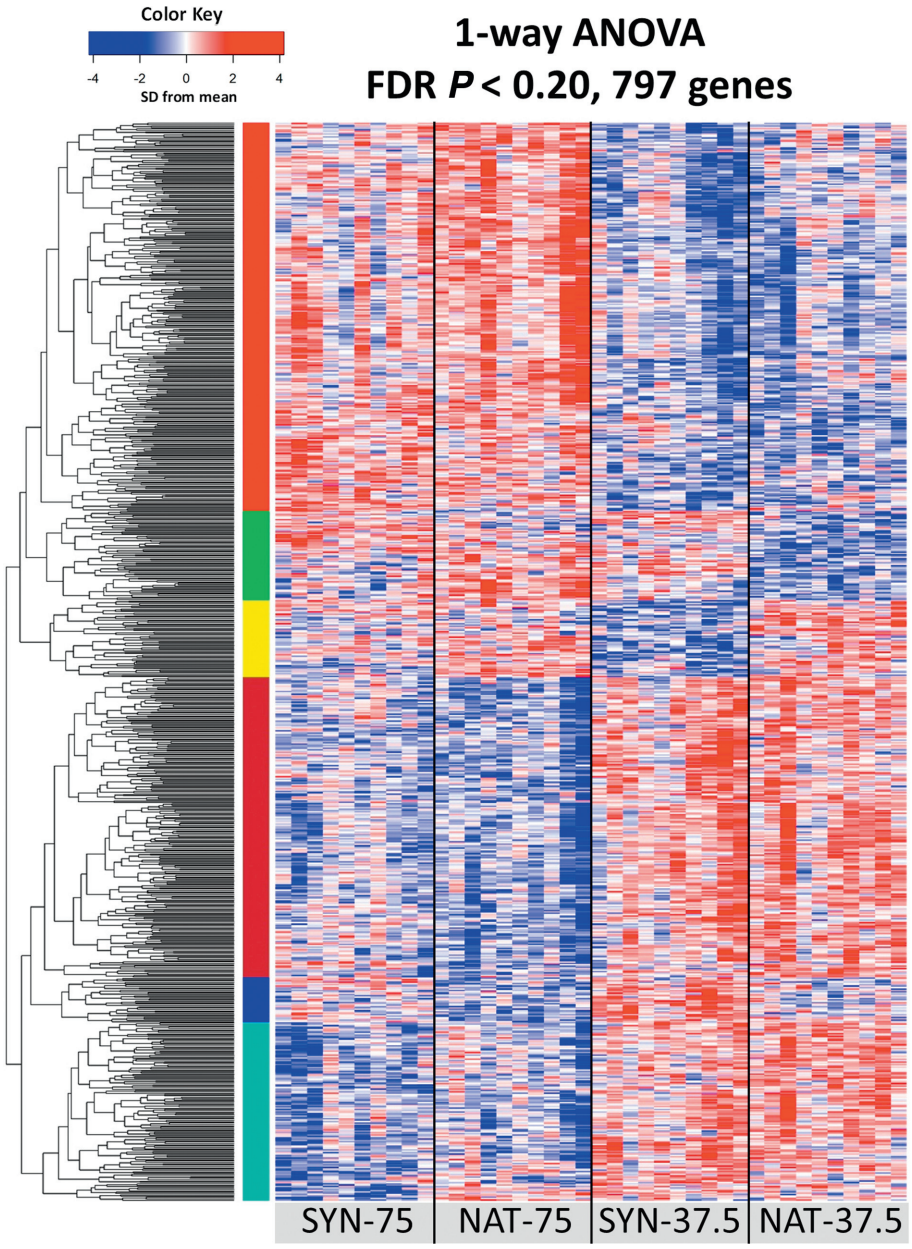


**FIGURE 3** Heat map of differentially expressed genes in the hippocampi of mice at weaning age (21 d) from the 37.5 IU/kg *RRR*- (NAT-37.5; $n = 10$), 37.5 *all rac*- (SYN-37.5; $n = 10$), 75 IU/kg *RRR*- (NAT-75; $n = 10$), and 75 IU/kg *all rac*-α-T (SYN-75; $n = 10$) groups (Experiment 2). Each row represents a single gene out of the 797 that showed a significant 1-way ANOVA result at a false discovery rate of 20%, and each column is a single individual of the 40 in the study. The columns are organized by treatment group, as indicated below the heat map. The rows are organized based on the correlation structure depicted by the dendogram. Standardized relative expression values are shown as shades of red and blue, with darker red indicating the individual displayed higher expression relative to the average, and blue indicating lower expression relative to the average. The colored bars (red, green, yellow, etc.) between the dendrogram and the heat map represent groupings of empirically correlated genes segregating at the second node in the dendrogram, not necessarily biological units. Abbreviations: α-T, α-tocopherol; *all rac*-α-T, synthetic α-tocopherol; FDR, false discovery rate; NAT, AIN 93-G diet containing *RRR*-α-T; *RRR*-α-T, natural α-tocopherol; SYN, AIN 93-G diet containing *all rac*-α-T.

hippocampal transcriptome could be more extreme in humans than that observed here for mice. While the treatments had only slight effects on the concentrations of α-T in the milk and brain, they had marked effects on brain α-T stereoisomer distributions. Mice fed *all rac*-α-T had equal concentrations of the 2*R* stereoisomers and substantial levels of 2*S* stereoisomers, whereas animals fed *RRR*-α-T had nearly 100% α-T in the *RRR* stereoisomer. This result demonstrates that the stereoisomer composition of the diets was, for the most part, faithfully represented in the tissues over the time course of our study, after considering the expected discrimination against the 2*S* isomers.

There are at least 2 ways the stereoisomer distribution could have impacted gene expression: either *RRR*-α-T is the active stereoisomer and the animals receiving *all rac*-α-T had less of it in their brains, and/or the synthetic stereoisomers compete for molecular targets and differentially impact the processes that modulate gene expression (52). The latter hypothesis is consistent with our finding that 487 genes were differentially expressed between SYN-37.5 and SYN-75, even though the

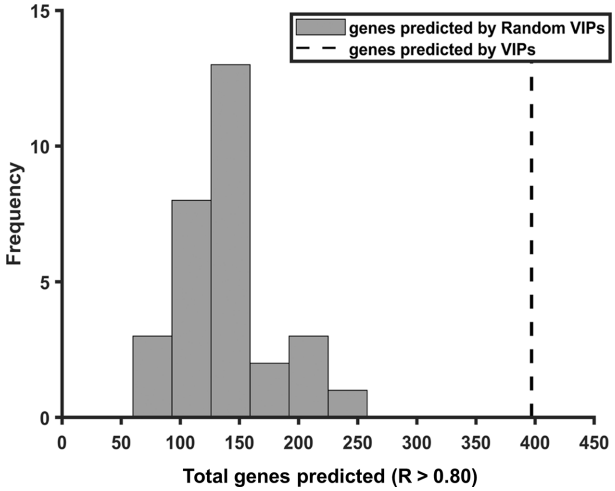


**FIGURE 4** Predictive power of VIPs (black line) vs. a random set of VIPs (50 sets of 58 random VIPs) using 3-fold cross-validation analysis (Experiment 2). Accuracy is measured by the extent of correlation (R) of the predicted expression level vs. measured expression level of transcripts in cross-validation test set. The random VIPs, on average, predicted approximately 140 genes with a correlation of 0.8 or higher in the test set. The actual VIPs predicted 391 genes with $R > 0.8$ (*t*-test; $P < 0.0001$). Abbreviation: VIP, vitamin E interacting protein.

concentration of *RRR*-αT in the brain between these 2 groups was similar. Thus, the synthetic stereoisomers, which were present in the brain at concentrations approximately 10% higher in SYN-75 versus SYN-37.5, most likely accounted for the difference.

The DAVID bioinformatics analysis revealed that genes involved in transcription regulation—that is, zinc fingers, DNA binding molecules, and transcription regulators—were the major categories of genes influenced by the brain αT concentration and αT stereoisomer profile. Previous reports have suggested that αT may act as a cofactor in transcription regulation through interactions with tocopherol-associated protein; this protein was found to translocate to the nucleus after binding αT (53). An alternative interpretation is that the gene expression effects we observed were not due to direct interactions of αT with transcription factors or signaling molecules (41, 52, 54, 55), but rather due to indirect antioxidant effects of αT [or its metabolites, such as 2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman (α-CEHC)], which altered the cellular oxidative environment (40, 56). Although in vitro studies indicate that the αT stereoisomers have similar antioxidative potencies (57–60), the anti-inflammatory activity of αT metabolites is greater than that of αT, and we do not know how the different stereoisomers may have influenced levels of α-CEHC in the brain. Although we did not measure levels of α-CEHC in the mice, other studies have shown that both the enzyme necessary to generate this metabolite and the actual molecule, α-CEHC, are found in the brain tissues of rats (61, 62) and guinea pigs (63). A further possibility is that the αT treatments influenced gene expression by interacting with vitamin K. Vitamin K is present at relatively high concentrations in the brain, where it may play a role in myelin regulation (64). However, we did not observe enrichment in myelin-related genes in this study. We conclude that more work is needed to resolve the issue of how αT influences global hippocampal gene expression, whether through direct interactions with transcription factors and regulators, through interactions with other vitamins, or by altering a signaling pathway involved in transcription regulation indirectly through antioxidant properties of the primary αT molecules or metabolites.

The ASTRIX network analysis successfully predicted hundreds of DEGs, suggesting that αT exerted its influence on gene expression via interactions with known VIPs established in the literature through decades of research. The VIP gene *Pkcz* explained 32 DEGs between the *all rac*-αT and *RRR*-αT groups at the 75 IU/kg diet dose. PKCz belongs to the protein kinase C (PKC) family, which comprises a group of protein kinase enzymes that control the function of other proteins by phosphorylating hydroxyl groups of serine and threonine residues. *Pkcz* has a truncated transcript, known as *Pkmz*, which was the predominant transcript detected in our study (90–96% of reads). PKMz is known to be involved in hippocampal long-term potentiation, a cellular mechanism for learning and memory (65, 66). It has also been specifically implicated in interactions with BDNF (65, 67, 68) via the phosphoinositide 3-kinase pathway (69). *Bdnf* was 1 of the DEGs explained by *Pkcz* in the network analysis. The BDNF protein is widely known for its role in regulating synaptic plasticity and strength, and for promoting the proliferation and survival of neurons in the developing and adult brain (70).

Potential interactions between αT and PKCz are likely indirect, through a signaling cascade and not through direct binding of αT to PKCz. Evidence suggests that although αT can directly bind to the conventional PKC, PKCα, it does not bind to PKCz (71). In addition to the potential for αT to modulate PKCα through direct binding, αT is also known to inhibit PKCα by activating protein phosphatase, which dephosphorylates PKC (41–43). When PKCα is inhibited by αT, *Pkca* gene expression increases in the aortic smooth muscle of rats (42). This is consistent with our observation of a 2% increase in expression of *Pkcz* in the hippocampus in response to elevating the dose from 37.5 to 75 IU/kg, collapsed across SYN and NAT (Supplemental Table 2). Recently, it was hypothesized that BDNF maintains synaptic strength through interactions with PKMz by acting upstream of PKMz, specifically preventing the degradation of PKMz, as opposed to stimulating its transcription (68). If there were more PKMz in the brains of mice fed the higher doses of αT, then there would be less need for BDNF to degrade PKMz to maintain homeostasis, and BDNF expression should go down. Consistent with this logic, *Bdnf* expression was slightly reduced in response to the higher αT dose relative to the lower αT dose, and in the *RRR*-αT groups relative to the *all rac*-αT groups. Taken together, with the known interactions between BDNF and PKMz, our data suggest that *RRR*-αT might more potently inhibit PKMz than the synthetic stereoisomers, resulting in increased expression of *Pkmz* and a subsequent relaxed demand for *Bdnf* in the hippocampus.

An important limitation of our study is that the diets were fed to the sires and the dams, not the developing offspring; therefore, the diets could have impacted offspring gene expression indirectly through their impacts on the dams or sires. For example, studies in dairy cows suggest that αT supplementation is crucial to maintain animal health and milk quality (72). We did not measure the milk quality parameters used in the dairy cow studies, such as levels of somatic cell count/ml in milk. It is possible that the different doses and stereoisomer configurations of the diets affected the quality of the milk, which subsequently affected the gene expression in the hippocampus. Evidence against this idea is that the pups were deliberately matched for body mass, so if effects

were through the dam they did not produce overt effects on the health of the pups that were compared for gene expression.

Another limitation of our study is the inherent difference in biological activity between *RRR*-αT and *all rac*-αT. This difference makes it challenging to match diets supplemented with *RRR*-αT or *all rac*-αT so that differential responses can be attributed to stereoisomer distribution as opposed to αT activity or tissue concentration. We reasoned it would be best to normalize the SYN and NAT diets to vitamin E IU content at each dose of αT. We chose this approach in order to deliver an "equivalent" biologic dose of αT by accounting for the well-documented 1.36-fold difference in vitamin E activity between the 2 sources. Of course, this resulted in the SYN diets containing 1.36 to 1.4 times more αT than the NAT diets (Table 1), and resulted in 10% higher brain αT concentrations for SYN diets than for NAT diets at both doses (Figure 2A). Some have argued that SYN diets should be fortified at twice the concentration of NAT diets (23). Our design allowed us to approximate this correction factor by comparing SYN-75 to NAT 37.5 groups, where the difference in the concentration of αT in the diets was 2.8-fold. SYN-75 pups had significantly higher brain αT concentrations than the NAT-37.5 pups (Figure 2A), indicating that use of a 2-fold equivalence would have likely caused higher concentrations of αT in the brains of SYN-treated mice. That would have further confounded the comparison of αT sources as compared to the IU approach we used. We could have equalized the concentration of *RRR*-αT and *all rac*-αT in SYN and NAT diets; however, that would have caused a 1.36-fold difference in vitamin E activity between the diets. We rejected that option, as the interpretation of gene expression would have been confounded by differences in dietary vitamin E activity, and likely by larger differences in brain αT concentrations. This is especially true given the large number of DEGs observed in response to small, dose-mediated changes in brain αT concentrations. Although we acknowledge the challenge of matching potencies between *RRR*-αT and *all rac*-αT, we believe our choice to normalize the diet αT concentration using vitamin E IU, and directly measure the αT concentration in the brain, was a strength of the analysis and was better than the alternatives described above. Using this strategy, we were able to draw stronger conclusions related to the primary goal of this work, which was to attribute the gene expression differences to stereoisomer distribution rather than to the concentration of αT.

In summary, we found that small increases in αT concentrations and major changes in αT stereoisomer distribution in the brain led to subtle changes in the expression of large numbers of genes involved in transcription regulation and synapse formation. We conclude that the synthetic αT stereoisomers likely interacted with molecular targets to produce differential gene expression. This conclusion is based on the finding that a large number of gene expression differences were observed between the SYN-37.5 and SYN-75 groups, concomitant with an increase in synthetic stereoisomers, but not *RRR*-αT. If the synthetic stereoisomers had no impact, we would have expected no gene expression differences between the SYN-37.5 and SYN-75 groups. Synthetic αT is commonly used in prenatal supplements and infant formulas, and although *RRR*-αT is the predominant stereoisomer in human milk, the selection was imperfect, resulting in significant concentrations of synthetic stereoisomers in milk from most women (17, 73). Hence, human infants are being exposed to the synthetic stereoisomers for the first time in evolutionary history. Although subtle, the widespread gene expression differences we observed might have minimal impacts on brain functions and might simply reflect compensatory mechanisms involved in maintaining homeostasis, our findings justify further exploration.


### Acknowledgements

The authors' responsibilities were as follows—JSR, CR, JGM, KD, CL, and MJK: designed the research; CR, JGM, KD, PT, AS, and HP: conducted the research; CR, JGM, KD, JD, SC, and KJS: analyzed the data; JSR and MJK: wrote the paper and had primary responsibility for the final content; and all authors: read and approved the final manuscript.


## References


1. Evans HM, Bishop KS. On the existence of a hitherto unrecognized dietary factor essential for reproduction. Science 1922;56:650–1.
2. Brigelius-Flohe R, Traber MG. Vitamin E: function and metabolism. FASEB J 1999;13:1145–55.
3. Bunyan J, McHale D, Green J, Marcinkiewicz S. Biological potenices of ε-and ζ 1-tocopherol and 5-methyltocol. Br J Nutr 1961;15:253–7.
4. Bryan W, Mason K. Vitamin E deficiency in the mouse. AJP Legacy 1940;131:263–7.
5. Gohil K, Oommen S, Quach HT, Vasu VT, Aung HH, Schock B, Cross CE, Vatassery GT. Mice lacking α-tocopherol transfer protein gene have severe α-tocopherol deficiency in multiple regions of the central nervous system. Brain Res 2008;1201:167–76.
6. Traber MG, Sokol RJ, Ringel SP, Neville HE, Thellman CA, Kayden HJ. Lack of tocopherol in peripheral nerves of vitamin E-deficient patients with peripheral neuropathy. N Engl J Med 1987;317:262–5.
7. Guggenheim MA, Ringel SP, Silverman A, Grabert BE. Progressive neuromuscular disease in children with chronic cholestasis and vitamin E deficiency: diagnosis and treatment with alpha tocopherol. J Pediatr 1982;100:51–8.
8. Satya-Murti S, Howard L, Krohel G, Wolf B. The spectrum of neurologic disorder from vitamin E deficiency. Neurology 1986;36:917.
9. Mariotti C, Gellera C, Rimoldi M, Mineri R, Uziel G, Zorzi G, Pareyson D, Piccolo G, Gambi D, Piacentini S. Ataxia with isolated vitamin E deficiency: neurological phenotype, clinical follow-up and novel mutations in TTPAgene in Italian families. Neurol Sci 2004;25:130–7.
10. Ulatowski L, Parker R, Warrier G, Sultana R, Butterfield D, Manor D. Vitamin E is essential for Purkinje neuron integrity. Neuroscience 2014;260:120–9.
11. Sheppard A, Pennington J, Weihrauch J. Analysis and distribution of vitamin E in vegetable oils and foods. In: Packer L, Fuchs J, editors. Vitamin E in health and disease. New York (NY): Marcel Dekker; 1993: pp. 9–31.
12. Kuchan MJ, Jensen SK, Johnson EJ, Lieblein-Boff JC. The naturally occurring α-tocopherol stereoisomer RRR-α-tocopherol is predominant in the human infant brain. Br J Nutr 2016;116:126–31.
13. Weiser H, Riss G, Kormann AW. Biodiscrimination of the eight α-tocopherol stereoisomers results in preferential accumulation of the four 2 R forms in tissues and plasma of rats. J Nutr 1996;126:2539–49.
14. Jensen SK, Lauridsen C. α-Tocopherol stereoisomers. Vitam Horm 2007;76:281–308.
15. Ranard KM, Erdman Jr JW. Effects of dietary RRR α-tocopherol vs all-racemic α-tocopherol on health outcomes. Nutr Rev 2018;76:141–53.
16. Traber MG. Vitamin E regulatory mechanisms. Annu Rev Nutr 2007;27:347–62.
17. Kuchan MJ, Moulton CJ, Dyer RA, Jensen SK, Schimpf KJ, Innis SM. RRR-α-tocopherol is the predominant stereoisomer of α-tocopherol in human milk. Curr Dev Nutr 2018;2, doi: 10.1093/cdn/nzy055.
18. Stone WL, LeClair I, Ponder T, Baggs G, Reis BB. Infants discriminate between natural and synthetic vitamin E. Am J Clin Nutr 2003;77:899–906.

19. Meglia GE, Jensen SK, Lauridsen C, Waller KP. α-Tocopherol concentration and stereoisomer composition in plasma and milk from dairy cows fed natural or synthetic vitamin E around calving. J Dairy Res 2006;73:227–34.
20. Ferslew KE, Acuff RV, Daigneault EA, Woolley TW, Stanton Jr PE. Pharmacokinetics and bioavailability of the RRR and all racemic stereoisomers of alpha-tocopherol in humans after single oral administration. J Clin Pharmacol 1993;33:84–8.
21. Traber MG, Burton G, Ingold K, Kayden H. RRR-and SRR-alpha-tocopherols are secreted without discrimination in human chylomicrons, but RRR-alpha-tocopherol is preferentially secreted in very low density lipoproteins. J Lipid Res 1990;31:675–85.
22. Acuff RV, Thedford SS, Hidiroglou NN, Papas AM, Odom Jr TA. Relative bioavailability of RRR- and all-rac-α-tocopheryl acetate in humans: studies using deuterated compounds. Am J Clin Nutr 1994;60:397–402.
23. Food and Drug Administration, HHS. Food labeling: revision of the nutrition and supplement facts labels. Fed Reg 2016;81:33741.
24. Blatt DH, Pryor WA, Mata JE, Rodriguez-Proteau R. Re-evaluation of the relative potency of synthetic and natural α-tocopherol: experimental and clinical observations. J Nutr Biochem 2004;15:380–95.
25. Ingold K, Burton G, Foster D, Hughes L, Lindsay D, Webb A. Biokinetics of and discrimination between dietary RRR- and SRR-α-tocopherols in the male rat. Lipids 1987;22:163–72.
26. Hoppe P, Krennrich G. Bioavailability and potency of natural-source and all-racemic α-tocopherol in the human: a dispute. Eur J Nutr 2000;39:183–93.
27. Gaedicke S, Zhang X, Huebbe P, Boesch-Saadatmandi C, Lou Y, Wiswedel I, Gardemann A, Frank J, Rimbach G. Dietary vitamin E, brain redox status and expression of Alzheimer's disease-relevant genes in rats. Br J Nutr 2009;102:398–406.
28. Rota C, Rimbach G, Minihane A-M, Stoecklin E, Barella L. Dietary vitamin E modulates differential gene expression in the rat hippocampus: potential implications for its neuroprotective properties. Nutr Neurosci 2005;8:21–9.
29. Hyland S, Muller D, Hayton S, Stoecklin E, Barella L. Cortical gene expression in the vitamin E-deficient rat: possible mechanisms for the electrophysiological abnormalities of visual and neural function. Ann Nutr Metab 2006;50:433–41.
30. Gohil K, Godzdanker R, O'Roark E, Schock BC, Kaini RR, Packer L, Cross CE, Traber MG. α-Tocopherol transfer protein deficiency in mice causes multi-organ deregulation of gene networks and behavioral deficits with age. Ann NY Acad Sci 2004;1031:109–26.
31. Ranard KM, Kuchan MJ, Bruno RS, Juraska JM, Erdman JW. Synthetic α-tocopherol, compared with natural α-tocopherol, downregulates myelin genes in cerebella of adolescent Ttpa-null mice. J Nutr 2019;150:1031–40.
32. Goddyn H, Leo S, Meert T, D'Hooge R. Differences in behavioural test battery performance between mice with hippocampal and cerebellar lesions. Behav Brain Res 2006;173:138–47.
33. Han SN, Pang E, Zingg J-M, Meydani SN, Meydani M, Azzi A. Differential effects of natural and synthetic vitamin E on gene transcription in murine T lymphocytes. Arch Biochem Biophys 2010;495:49–55.
34. Muller PY, Netscher T, Frank J, Stoecklin E, Rimbach G, Barella L. Comparative quantification of pharmacodynamic parameters of chiral compounds (RRR- vs. all-rac-α tocopherol) by global gene expression profiling. J Plant Physiol 2005;162:811–7.
35. Hundhausen C, Frank J, Rimbach G, Stoecklin E, Muller PY, Barella L. Effect of vitamin E on cytochrome P450 mRNA levels in cultured hepatocytes (HepG2) and in rat liver. Cancer Genom Proteom 2006;3:183–90.
36. Andersen P, Morris R, Amaral D, Bliss T, O'Keefe J. The hippocampus book. Oxford: Oxford University Press; 2006.
37. Kohman RA, Rhodes JS. Neurogenesis, inflammation and behavior. Brain Behav Immun 2013;27:22–32.
38. Kempermann G, Song H, Gage FH. Neurogenesis in the adult hippocampus. Cold Spring Harb perspect biol 2015;7: a018812.
39. Zulkiflee N, Awang S, Woo X, Kamilan M, Mariappan M, Tan J. In silico docking of vitamin E isomers on transport proteins. Curr Comput-Aid Drug 2019.
40. Traber MG, Atkinson J. Vitamin E, antioxidant and nothing more. Free Radic Biol Med 2007;43:4–15.
41. Azzi A, Gysin R, Kempna P, Munteanu A, Negis Y, Villacorta L, Visarius T, Zingg J. Vitamin E mediates cell signaling and regulation of gene expression. Ann NY Acad Sci 2004;1031:86–95.
42. Azzi A, Aratri E, Boscoboinik D, Clément S, Özer NK, Ricciarelli R, Spycher S. Molecular basis of alpha-tocopherol control of smooth muscle cell proliferation. Biofactors 1998;7:3–14.
43. Mahoney CW, Azzi A. Vitamin E inhibits protein kinase C activity. Biochem Biophys Res Commun 1988;154:694–7.
44. Chandrasekaran S. Predicting phenotype from genotype through reconstruction and integrative modeling of metabolic and regulatory networks. In A systems theoretic approach to systems and synthetic biology I: models and system characterizations. Dordrecht: Springer; 2014: pp. 307–25.
45. Chandrasekaran S, Ament SA, Eddy JA, Rodriguez-Zas SL, Schatz BR, Price ND, Robinson GE. Behavior-specific changes in transcriptional modules lead to distinct and predictable neurogenomic states. Proc Natl Acad Sci 2011;108:18020–5.
46. Bukhari SA, Saul MC, Seward CH, Zhang H, Bensky M, James N, Zhao SD, Chandrasekaran S, Stubbs L, Bell AM. Temporal dynamics of neurogenomic plasticity in response to social interactions in male threespined sticklebacks. PLoS Genet 2017;13:e1006840.
47. Shpigler H, Saul M, Murdoch E, Cash-Ahmed A, Seward C, Sloofman L, Chandrasekaran S, Sinha S, Stubbs L, Robinson G. Behavioral, transcriptomic and epigenetic responses to social challenge in honey bees. Genes Brain Behav 2017;16:579–91.
48. Saul MC, Seward CH, Troy JM, Zhang H, Sloofman LG, Lu X, Weisner PA, Caetano-Anolles D, Sun H, Zhao SD. Transcriptional regulatory dynamics drive coordinated metabolic and neural response to social challenge in mice. Genome Res 2017;27:959–72.
49. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Series B Stat Methodol 1995;57:289–300.
50. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 2015;43:e47.
51. Nitta C, Hayashi K, Ueda T, Igarashi O. Distribution of α-tocopherol stereoisomers in rats. Biosci Biotechnol Biochem 1993;57:1406–7.
52. Azzi A, Stocker A. Vitamin E: non-antioxidant roles. Prog Lipid Res 2000;39:231–55.
53. Yamauchi J, Iwamoto T, Kida S, Masushige S, Yamada K, Esashi T. Tocopherol-associated protein is a ligand-dependent transcriptional activator. Biochem Biophys Res Commun 2001;285:295–9.
54. Zingg J-M. Vitamin E: a role in signal transduction. Annu Rev Nutr 2015;35:135–73.
55. Rimbach G, Moehring J, Huebbe P, Lodge JK. Gene-regulatory activity of α-tocopherol. Molecules 2010;15:1746–61.
56. Traber MG, Elsner A, Brigelius-Flohé R. Synthetic as compared with natural vitamin E is preferentially excreted as α-CEHC in human urine: studies using deuterated α-tocopheryl acetates. FEBS Lett 1998;437:145–8.
57. Burton G, Ingold K. Autoxidation of biological molecules. 1. Antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants in vitro. J Am Chem Soc 1981;103:6472–7.
58. Mukai K, Kageyama Y, Ishida T, Fukuda K. Synthesis and kinetic study of antioxidant activity of new tocopherol (vitamin E) compounds. J Org Chem 1989;54:552–6.
59. Pryor WA, Cornicelli JA, Devall LJ, Tait B, Trivedi B, Witiak D, Wu M. A rapid screening test to determine the antioxidant potencies of natural and synthetic antioxidants. J Org Chem 1993;58:3521–32.
60. Pryor WA, Strickland T, Church DF. Comparison of the efficiencies of several natural and synthetic antioxidants in aqueous SDS (sodium dodecyl sulfate) micelle solutions. J Am Chem Soc 1988;110:2224–9.
61. Wang Y, Zhao J, Kalsotra A, Turman CM, Grill RJ, Dash PK, Strobel HW. CYP4Fs expression in rat brain correlates with changes in LTB4 levels after traumatic brain injury. J Neurotrauma 2008;25:1187–94.
62. Farley SM, Leonard SW, Stevens JF, Traber MG. Deuterium-labeled phylloquinone fed to α-tocopherol-injected rats demonstrates sensitivity

of low phylloquinone-containing tissues to menaquinone-4 depletion. Mol Nutr Food Res 2014;58:1610–9.
63. Hagl S, Kocher A, Schiborr C, Eckert SH, Ciobanu I, Birringer M, El-Askary H, Helal A, Khayyal MT, Frank J. Rice bran extract protects from mitochondrial dysfunction in guinea pig brains. Pharmacol Res 2013;76:17–27.
64. Crivello NA, Casseus SL, Peterson JW, Smith DE, Booth SL. Age- and brain region-specific effects of dietary vitamin K on myelin sulfatides. J Nutr Biochem 2010;21:1083–8.
65. Sacktor TC. How does PKMζ maintain long-term memory? Nat Rev Neurosci 2011;12:9–15.
66. Hernandez AI, Blace N, Crary JF, Serrano PA, Leitges M, Libien JM, Weinstein G, Tcherapanov A, Sacktor TC. Protein kinase mζ synthesis from a brain mRNA encoding an independent protein kinase cζ catalytic domain implications for the molecular mechanism of memory. J Biol Chem 2003;278:40305–16.
67. Callender JA, Newton AC. Conventional protein kinase C in the brain: 40 years later. Neuronal Signal 2017;1:NS20160005.
68. Mei F, Nagappan G, Ke Y, Sacktor TC, Lu B. BDNF facilitates L-LTP maintenance in the absence of protein synthesis through PKMζ. PLoS One 2011;6:e21568.
69. Nakanishi H, Brewer KA, Exton J. Activation of the zeta isozyme of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem 1993;268:13–6.
70. Poo M-m. Neurotrophins as synaptic modulators. Nat Rev Neurosci 2001;2:24–32.
71. McCary CA, Yoon Y, Panagabko C, Cho W, Atkinson J, Cook-Mills JM. Vitamin E isoforms directly bind PKCα and differentially regulate activation of PKCα. Biochem J 2012;441:189–98.
72. Politis I. Reevaluation of vitamin E supplementation of dairy cows: bioavailability, animal health and milk quality. Animal 2012;6:1427–34.
73. Gaur S, Kuchan MJ, Lai C-S, Jensen SK, Sherry CL. Supplementation with RRR- or all-rac-α-tocopherol differentially affects the α-tocopherol stereoisomer profile in the milk and plasma of lactating women. J Nutr 2017;147:1301–7.