Brain α-Tocopherol Concentration and Stereisomer 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 SYN-75 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-75, explained by expression of the gene for the VIP, protein kinase C zeta (Pkcζ).

Conclusions: In weanling mouse hippocampus, 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, RKS, RSS, SRR, SSR, SRS, and SSS (13). When the molecule is produced synthetically, all 8 stereoisomers are found in equal proportions...


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 2R stereoisomers of αT (RRR, RSR, RRS, and RSS), causing the 2R 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 synthesis, 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 weaning 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 × 19 × 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 RR-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.
These individuals were chosen before observing the expression related to the growth or developmental stage of the pups.

RNA-sequencing (RNA-seq) analysis. A total of 126 weanling mice (from conception, pregnancy, lactation, and weaning) were fed 24 pairs of mice a diet based on the standard AIN 93-G, except we purchased from Dyets, Inc., who use a vacuum distillation process to remove fat-soluble vitamins. The concentration of αT 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-αT acetate was from Fortitech Inc. (500 IU vitamin E/g ingredient) and the RRR-αT acetate was from Archer-Daniels-Midland Company (700 IU vitamin E/g ingredient). Abbreviations: αT, α-tocopherol; all rac-αT, synthetic α-tocopherol; NAT, AIN 93-G diet containing RRR-αT acetate; SYN, AIN 93-G diet containing all rac-αT.

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 μl) 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 αT, and αT (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-αT acetate or 37.5 IU/kg all rac-αT acetate 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 αT 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 weaning 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 αT concentrations and stereoisomer distributions in the brain.

Table 1. Composition of the diets

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Stereoisomers</th>
<th>Diet recipe, g αT ingredient/kg diet</th>
<th>Diet, IU αT/kg diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milking study</td>
<td>SYN-32</td>
<td>all rac-αT acetate</td>
<td>0.064</td>
<td>32</td>
</tr>
<tr>
<td>Milking study</td>
<td>NAT-32</td>
<td>RRR-αT acetate</td>
<td>0.046</td>
<td>32</td>
</tr>
<tr>
<td>Gene expression</td>
<td>SYN-37.5</td>
<td>all rac-αT acetate</td>
<td>0.075</td>
<td>37.5</td>
</tr>
<tr>
<td>Gene expression</td>
<td>NAT-37.5</td>
<td>RRR-αT acetate</td>
<td>0.054</td>
<td>37.5</td>
</tr>
<tr>
<td>Both</td>
<td>SYN-75</td>
<td>all rac-αT acetate</td>
<td>0.150</td>
<td>75</td>
</tr>
<tr>
<td>Gene expression</td>
<td>NAT-75</td>
<td>RRR-αT acetate</td>
<td>0.107</td>
<td>75</td>
</tr>
</tbody>
</table>

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 αT 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-αT acetate was from Fortitech Inc. (500 IU vitamin E/g ingredient) and the RRR-αT acetate was from Archer-Daniels-Midland Company (700 IU vitamin E/g ingredient). Abbreviations: αT, α-tocopherol; all rac-αT, synthetic α-tocopherol; NAT, AIN 93-G diet containing RRR-αT acetate; SYN, AIN 93-G diet containing all rac-αT.

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Analysis of αT 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 αT 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 αT stereoisomer analyses, the remaining hexane layer was concentrated to ~100 μL, and αT was isolated chromatographically using a diol column with a hexane/methyl-t-butyl-ether mobile phase. The αT in the isolated fraction was then methylated using dimethylcarbonate, dimethylsulfoxide, and potassium carbonate, and the 2S, RRS, 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 αT. Indeed, it is not clear that αT binds to many proteins (other than αTTP) in a specific manner (39). For example, αT 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 phosphatases), 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 αT; that is, changes in αT are known to change the expression or function of these proteins in...
of the gene, quantified by regression analysis; and interactions, as used in prior studies (45). The original implementation least 0.8 by the ASTRIX model using expression levels of VIPs. A

A regression. least-angle regression analysis was replaced with traditional linear there was already a reduced set of regulators (i.e., the 58 VIPs), the among hundreds of possible features. However, in our case, since ASTRIX used the least-angle regression algorithm to identify the information, a measure of similarity with the target gene (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 stereoisomers. Labeled means (A) or labeled means collapsed across time points (B) without a common letter differ at P < 0.05. 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.

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 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 αT in milk

The concentration of αT 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 1A], 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 αT than NAT-32 (P = 0.025). No other pair-wise differences between diets were significant (all P > 0.05).

![FIGURE 1](https://academic.oup.com/jn/advance-article/doi/10.1093/jn/nxaa249/5906643) Concentrations (A) and stereoisomer distributions (B) of αT in the milk of mouse dams fed diets containing either 32 IU/kg RRR-

(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.
tissues, as previously observed for rodents (13, 25, 51). In the absorption, hepatic catabolism, or transport of 2 concentrations of individual 2 isomers likely reflect incomplete absorption, hepatic catabolism, or transport of 2 isomers into tissues, as previously observed for rodents (13, 25, 51). In the NAT-32 group, 99.1% of the αT stereoisomer in the brain was calculated for each individual as the product of the proportion of RRR-αT multiplied by the total αT, whereas milk from dams fed SYN-32 and SYN-75 was due to slightly lower proportions of RRR-αT in the SYN-37.5 and SYN-75 groups, respectively (P < 0.0001; Figure 1B). The presumed lower concentration of individual 2S isomers likely reflects incomplete absorption, hepatic catabolism, or transport of 2S isomers into tissues, as previously observed for rodents (13, 25, 51). In the NAT-32 group, 99.1% of the αT in the milk of pups 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 2S 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; 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 2R stereoisomers and the sum of the 2S stereoisomers (Figure 1B). The presumed lower concentration of individual 2S isomers likely reflects incomplete absorption, hepatic catabolism, or transport of 2S 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 2R isomers, and approximately 10% of the total αT was as 2S 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 SYN-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

![Figure 2](https://academic.oup.com/jn/advance-article/doi/10.1093/jn/nxaa249/5906643)
A total of 16,755 genes passed our criteria for sufficient RNA-seq DEGs expression. Mass between sources of differentially expressed as a result of the concentration of αT with an FDR of less than 20%. Most of these genes were and SYN-37.5 (all were significant post hoc differences in 179 between NAT-37.5 and SYN-37.5). Of these 797 genes, there were significant differences in the brain masses between any of the groups (all P > 0.36). The mean brain 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 > 0.05), 182 between NAT-75 and SYN-75. An analysis of the 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 (Pkcζ) 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 IU/kg αT (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). 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: αT, α-tocopherol; all rac-αT, synthetic α-tocopherol; FDR, false discovery rate; NAT, AIN 93-G diet containing RRR-αT; RRR-αT, natural α-tocopherol; SYN, AIN 93-G diet containing all rac-αT.

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 αT in the milk and brain, they had marked effects on brain αT stereoisomer distributions. Mice fed all rac-αT had equal concentrations of the 2R stereoisomers and substantial levels of 2S stereoisomers, whereas animals fed RRR-αT had nearly 100% αT 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 2S isomers.

There are at least 2 ways the stereoisomer distribution could have impacted gene expression: either RRR-αT is the active stereoisomer and the animals receiving all rac-αT 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
Although in vitro studies indicate 6-hydroxychroman (α), protein was found to translocate to the nucleus after binding α, that effects we observed were not due to direct interactions of α and jor categories of genes influenced by the brain involved in transcription regulation—that is, zinc fingers, DNA binding molecules. The difference. The actual VIPs predicted 391 genes with R > 0.8 (t-test; P < 0.0001). Abbreviation: VIP, vitamin E interacting protein.

collection 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 antioxidant 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 Pkkez explained 32 DEGs between the all rac-αT and RRR-αT groups at the 75 IU/kg diet dose. PKCβ 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. Pkkez 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, 66, 68) via the phosphoinositide 3-kinase pathway (69). Bdnf was 1 of the DEGs explained by Pkkez 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, PKCa, it does not bind to PKCz (71). In addition to the potential for αT to modulate PKCa through direct binding, αT is also known to inhibit PKCa by activating protein phosphatase, which dephosphorylates PKC (41–43). When PKCa is inhibited by αT, Pkea gene expression increases in the aortic smooth muscle of rats (42). This is consistent with our observation of a 2% increase in expression of Pkea 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 alter the gene expression directly.
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.

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