

## The naturally occurring $\alpha$ -tocopherol stereoisomer RRR- $\alpha$ -tocopherol is predominant in the human infant brain

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### Abstract

$\alpha$ -Tocopherol is the principal source of vitamin E, an essential nutrient that plays a crucial role in maintaining healthy brain function. Infant formula is routinely supplemented with synthetic  $\alpha$ -tocopherol, a racemic mixture of eight stereoisomers with less bioactivity than the natural stereoisomer RRR- $\alpha$ -tocopherol.  $\alpha$ -Tocopherol stereoisomer profiles have not been previously reported in the human brain. In the present study, we analysed total  $\alpha$ -tocopherol and  $\alpha$ -tocopherol stereoisomers in the frontal cortex (FC), hippocampus (HPC) and visual cortex (VC) of infants ( $n$  36) who died of sudden infant death syndrome or other conditions. RRR- $\alpha$ -tocopherol was the predominant stereoisomer in all brain regions ( $P < 0.0001$ ) and samples, despite a large intra-decedent range in total  $\alpha$ -tocopherol (5–17  $\mu\text{g/g}$ ). Mean RRR- $\alpha$ -tocopherol concentrations in FC, HPC and VC were 10.5, 6.8 and 5.5  $\mu\text{g/g}$ , respectively. In contrast, mean levels of the synthetic stereoisomers were RRS, 1–1.5; RSR, 0.8–1.0; RSS, 0.7–0.9; and  $\Sigma 2S$  0.2–0.3  $\mu\text{g/g}$ . Samples from all but two decedents contained measurable levels of the synthetic stereoisomers, but the intra-decedent variation was large. The ratio of RRR:the sum of the synthetic 2R stereoisomers (RRS+RSR+RSS) averaged 2.5, 2.3 and 2.4 in FC, HPC and VC, respectively, and ranged from 1 to at least 4.7, indicating that infant brain discriminates against synthetic 2R stereoisomers in favour of RRR. These findings reveal that RRR- $\alpha$ -tocopherol is the predominant stereoisomer in infant brain. These data also indicate that the infant brain discriminates against the synthetic 2R stereoisomers, but is unable to do so completely. On the basis of these findings, investigation into the impact of  $\alpha$ -tocopherol stereoisomers on neurodevelopment is warranted.

**Key words:** RRR- $\alpha$ -tocopherol: Natural vitamin E: Brain: Infants

Vitamin E is an essential nutrient consumed through foods including vegetable oils, nuts, dark green leafy vegetables and egg yolk<sup>(1)</sup> and also through supplemented foods. Vitamin E is believed to be particularly important in the nervous system, as deficiency in humans, although rare, leads to neuromuscular abnormalities with ataxia and myopathy, and can be reversed by  $\alpha$ -tocopherol supplementation or consumption<sup>(2–4)</sup>. Vitamin E activity can be derived from four tocopherols ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ) and their corresponding four tocotrienols ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ). However,  $\alpha$ -tocopherol is the most biologically active form of vitamin E<sup>(1)</sup>.  $\alpha$ -Tocopherol is widely accepted to play an important role in the protection of membrane-bound unsaturated fatty acids from oxidation through its potent lipid-soluble, chain-breaking antioxidant activity. In addition, it is thought to support diverse biological processes including regulation of enzymatic activity<sup>(5)</sup>, gene expression<sup>(6)</sup> and cell signalling<sup>(6,7)</sup>.

The three chiral C atoms in the structure of  $\alpha$ -tocopherol result in significant structural complexity. In nature,  $\alpha$ -tocopherol only exists as the RRR stereoisomer (2R, 4R, 8'R- $\alpha$ -tocopherol), commonly referred to as natural vitamin E. In contrast, synthetic

$\alpha$ -tocopherol (*all-rac*- $\alpha$ -tocopherol) is a racemic mixture of the eight possible stereoisomers. On the basis of these differences in stereochemistry, RRR- $\alpha$ -tocopherol has greater biological value than *all-rac*- $\alpha$ -tocopherol. Extensive research has indicated that RRR- $\alpha$ -tocopherol has 1.36–2 times the biological value of *all-rac*- $\alpha$ -tocopherol<sup>(8,9)</sup>. Discrimination among stereoisomers is known to occur in the liver through the action of  $\alpha$ -tocopherol transfer protein ( $\alpha$ -TTP)<sup>(10)</sup>, which preferentially binds 2R stereoisomers, resulting in degradation of the majority of 2S stereoisomers in *all-rac*- $\alpha$ -tocopherol<sup>(11)</sup>.

Rodent and pig peripheral tissue  $\alpha$ -tocopherol stereoisomer composition appears to reflect hepatic  $\alpha$ -tocopherol stereoisomer discrimination. The brain and other peripheral tissues of rats fed *all-rac*- $\alpha$ -tocopherol contain dramatically higher levels of 2R than of 2S stereoisomers<sup>(12,13)</sup>. Consistent with enrichment of 2R stereoisomers via hepatic discrimination, mice fed equal quantities of  $d_3$ -RRR- $\alpha$ -tocopherol and  $d_6$ -*all-rac*- $\alpha$ -tocopherol had a brain  $d_3:d_6$  ratio of approximately 1.8<sup>(14)</sup>. Weiser *et al.*<sup>(15)</sup> directly tested individual 2R stereoisomers in brain samples from  $\alpha$ -tocopherol-deprived rats repleted with *all-rac*- $\alpha$ -tocopherol and

**Abbreviations:** FC, frontal cortex; HPC, hippocampus; VC, visual cortex;  $\alpha$ -TTP,  $\alpha$ -tocopherol transfer protein.

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found no discrimination among 2R stereoisomers. Piglets fed sow milk containing a 2:1 d<sub>3</sub>:d<sub>6</sub> ratio had a 2:1 ratio in plasma and 2.6 and 2.4 ratios in the brain after 14 and 21 d of nursing, respectively<sup>(16)</sup>. Although others have reported total  $\alpha$ -tocopherol concentrations in the adult and fetal human brain<sup>(17–19)</sup>, no information is available on the  $\alpha$ -tocopherol stereoisomer composition in the infant human brain.

As human infants are generally born with low levels of plasma  $\alpha$ -tocopherol, their vitamin E status is heavily dependent on the  $\alpha$ -tocopherol content and composition of human milk or infant formula. Infant formula is widely supplemented with *all-rac*- $\alpha$ -tocopheryl-acetate in order to meet the vitamin E requirements of infants. Unlike breast-fed infants, formula-fed infants do not benefit from discrimination of  $\alpha$ -tocopherol stereoisomers in the maternal liver, possibly increasing the likelihood of synthetic stereoisomers reaching the infant brain. Expression of hepatic  $\alpha$ -TTP in neonatal mice is low at birth and increases to adult levels over the first 28 d of life<sup>(20)</sup>. However, it is not known whether the same developmental phenomenon occurs in human infants. Research in knockout mice has revealed that  $\alpha$ -tocopherol is essential for Purkinje cell integrity in the cerebellum through the action of  $\alpha$ -TTP<sup>(21)</sup>. As  $\alpha$ -TTP is known to discriminate between the 2R stereoisomers of  $\alpha$ -tocopherol, we considered it important to determine the  $\alpha$ -tocopherol stereoisomer distribution in infant brain.

## Methods

### Decedents and brain samples

Voluntarily donated, frozen brain samples were obtained from the National Institute of Child Health and Human Development Brain and Tissue Bank for Developmental Disorders at the University of Maryland. Samples from thirty-three decedents were collected between 1995 and 2005 and between 2007 and 2008 from the remaining three decedents. Samples were voluntarily donated by the parents and were identified using a unique numerical identifier, which obscured the identity of the decedent. Identity-obscured autopsy reports were reviewed to confirm that decedents were otherwise healthy and did not suffer from brain abnormalities or other systemic pathologies. A total of seventy-nine samples were obtained from the frontal cortex (FC) (*n* 28), hippocampus (HPC) (*n* 25) and visual cortex (VC) (*n* 26), and it is noteworthy that these regions of the brain are associated with memory (HPC), executive function (FC) and vision (VC). Decedent details are provided in Table 1. Samples were available from all three regions of the brain from fourteen of the thirty-six decedents. Only two regions were analysed for fifteen decedents, and a single region for the remaining seven decedents. Tissues were stored frozen (–70°C) until analysis for  $\alpha$ -tocopherol stereoisomers. Term and preterm decedents were combined as values for total  $\alpha$ -tocopherol and  $\alpha$ -tocopherol stereoisomer profile for the preterm infants fell within the term infant range.

### $\alpha$ -Tocopherol analyses

The concentrations of tocopherols including  $\gamma$ -tocopherol were determined by HPLC after saponification and extraction into heptane. The stereochemical composition of  $\alpha$ -tocopherol was

**Table 1.** Descriptive characteristics of decedents (Numbers and percentages; mean values and standard deviations; thirty-six infants)

Characteristics	<i>n</i>	%
<b>Birth</b>		
Term*	30	
Preterm*	6	
<b>Age (d)</b>		
Mean	131	
SEM	17.6	
Median	100	
Range	5–488	
<b>Sex</b>		
Females	10	38
Males	26	72
<b>Race</b>		
African-American	18	50
Caucasian	17	47
Hispanic	1	3
<b>Cause of death</b>		
Sudden infant death syndrome	17	47
Other	19	53
Asphyxia	4	
Asthma	1	
Bronchopneumonia	4	
Congenital heart defects and heart disease	4	
Complications of prematurity, bronchopulmonary dysplasia	2	
Dehydration	1	
Drowning	1	
Hyperthermia	1	
Idiopathic pulmonary haemorrhage	1	
<b>Postmortem interval (h)</b>		
Mean	18	
SEM	1.1	
Median	19	
Range	2–28	
<b>Postmortem interval v. total <math>\alpha</math>-tocopherol (<i>P</i>)</b>		
FC	0.96	
HPC	0.10	
VC	0.55	
<b>Postmortem interval v. RRR-<math>\alpha</math>-tocopherol (<i>P</i>)</b>		
FC	0.95	
HPC	0.48	
VC	0.96	

FC, frontal cortex; HPC, hippocampus; VC, visual cortex.

\* Term,  $\geq 37$  weeks of gestation at birth, and preterm,  $< 37$  weeks of gestation birth.

determined after methylation of  $\alpha$ -tocopherol stereoisomers into their corresponding methyl ethers and subsequent separation by chiral HPLC<sup>(22)</sup>. This method separates the eight stereoisomers of  $\alpha$ -tocopherol into five peaks: peak 1 contains the four 2S stereoisomers (SSS/SSR/SRS/SRR), peak 2 contains RSS- $\alpha$ -tocopherol, peak 3 contains RRS- $\alpha$ -tocopherol, peak 4 contains RRR- $\alpha$ -tocopherol and peak 5 contains RSR- $\alpha$ -tocopherol. Results are reported as the ratio of the observed stereoisomer among five peaks and the concentration determined by calculation of each stereoisomer concentration from total  $\alpha$ -tocopherol. Recovery of total  $\alpha$ -tocopherol was 96% with a CV (%) of 2.7, whereas recovery of the individual stereoisomers varied from 95.3 to 100.8% with CV (%) varying from 1.2 to 7.9. Data were expressed per wet weight of tissue.

### Statistical analysis

To detect significant main effects and interactions between main factors, data were analysed with one-way

(brain region; stereoisomer) ANOVA using GraphPad Prism version 5.04 for Windows (GraphPad Software, www.graphpad.com). When appropriate, differences between treatment means were evaluated by Tukey's multiple comparison test in GraphPad Prism. Means that differed significantly following a multiple comparison test were denoted using unique letter subscripts ( $P < 0.05$ ). To determine significant correlations between nutrient concentrations and age or other nutrients, data were analysed using Pearson's correlation procedures of GraphPad Prism. All data are expressed as treatment means with their standard errors.

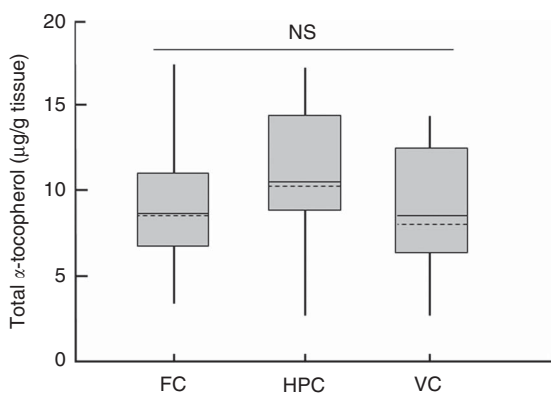
**Results**

*Total  $\alpha$ -tocopherol levels*

Mean total  $\alpha$ -tocopherol concentrations were not different across the three brain regions studied (Fig. 1). All samples analysed contained detectable concentrations of  $\alpha$ -tocopherol, and inter-decedent variation was large with values ranging from  $<5$  to approximately  $17 \mu\text{g } \alpha\text{-tocopherol/g}$  of tissue. Total  $\alpha$ -tocopherol concentration positively correlated with age in each of the three brain regions tested (FC,  $r^2$  0.31,  $P < 0.01$ ; HPC,  $r^2$  0.36,  $P < 0.01$ ; VC,  $r^2$  0.62,  $P < 0.001$ ). Intra-decedent variation in total  $\alpha$ -tocopherol concentrations by brain region is shown for the fourteen decedents who provided samples for each region (online Supplementary Table S1).  $\gamma$ -Tocopherol was undetectable in the majority of samples (FC, twenty; HPC, fourteen; VC, twenty-one). Mean  $\gamma$ -tocopherol for samples with detectable levels was  $0.30$  (SEM  $0.06$ )  $\mu\text{g } \gamma\text{-tocopherol/g}$  tissue ( $n$  8),  $0.36$  (SEM  $0.06$ )  $\mu\text{g } \gamma\text{-tocopherol/g}$  tissue ( $n$  11) and  $0.33$  (SEM  $0.04$ )  $\mu\text{g } \gamma\text{-tocopherol/g}$  tissue ( $n$  5) in FC, HPC and VC, respectively. Average ratios of  $\alpha$ -tocopherol: $\gamma$ -tocopherol were 39, 33 and 25 in FC, HPC and VC, respectively. Neither total  $\alpha$ -tocopherol nor  $RRR$ - $\alpha$ -tocopherol (Table 1) was correlated with postmortem interval.

*$\alpha$ -Tocopherol stereoisomers*

Mean concentrations of  $RRR$ - $\alpha$ -tocopherol were significantly higher than those of each of the synthetic  $\alpha$ -tocopherol



**Fig. 1.** Total  $\alpha$ -tocopherol concentrations in the frontal cortex (FC), hippocampus (HPC) and visual cortex (VC). Data are from thirty-six decedents with twenty-eight FC samples, twenty-five HPC samples and twenty-six VC samples.  $\square$ , 25th and 75th percentiles; —, mean; - - - - -, median; |, minimum and maximum values.

stereoisomers in each of the brain regions tested (Fig. 2(a-c)). Although  $RRR$ - $\alpha$ -tocopherol concentration positively correlated with age in each brain region studied (FC,  $r^2$  0.22,  $P < 0.05$ ; HPC,  $r^2$  0.20,  $P < 0.05$ ; VC,  $r^2$  0.61,  $P < 0.0001$ ), individual synthetic stereoisomers did not. On average, across the brain regions,  $RRR$ - $\alpha$ -tocopherol accounted for 66% ( $P < 0.0001$ , compared with other stereoisomers) of total  $\alpha$ -tocopherol, whereas none of the synthetic stereoisomers exceeded 14%.  $RRR$  accounted for as much as 100% of total  $\alpha$ -tocopherol in two decedents. The minimum percentage of total  $\alpha$ -tocopherol found as  $RRR$  was 58%. More than half of the decedents had at least 30% of total  $\alpha$ -tocopherol as synthetic stereoisomers. Nonetheless,  $RRR$ - $\alpha$ -tocopherol was the most common stereoisomer in all samples and individuals on a per cent basis. In order to provide insight into whether the stereoisomer profile was consistent for different brain regions within individual decedents, the stereoisomer profile is presented by brain region for the fourteen decedents who provided samples for all three regions (online Supplementary Table S1).

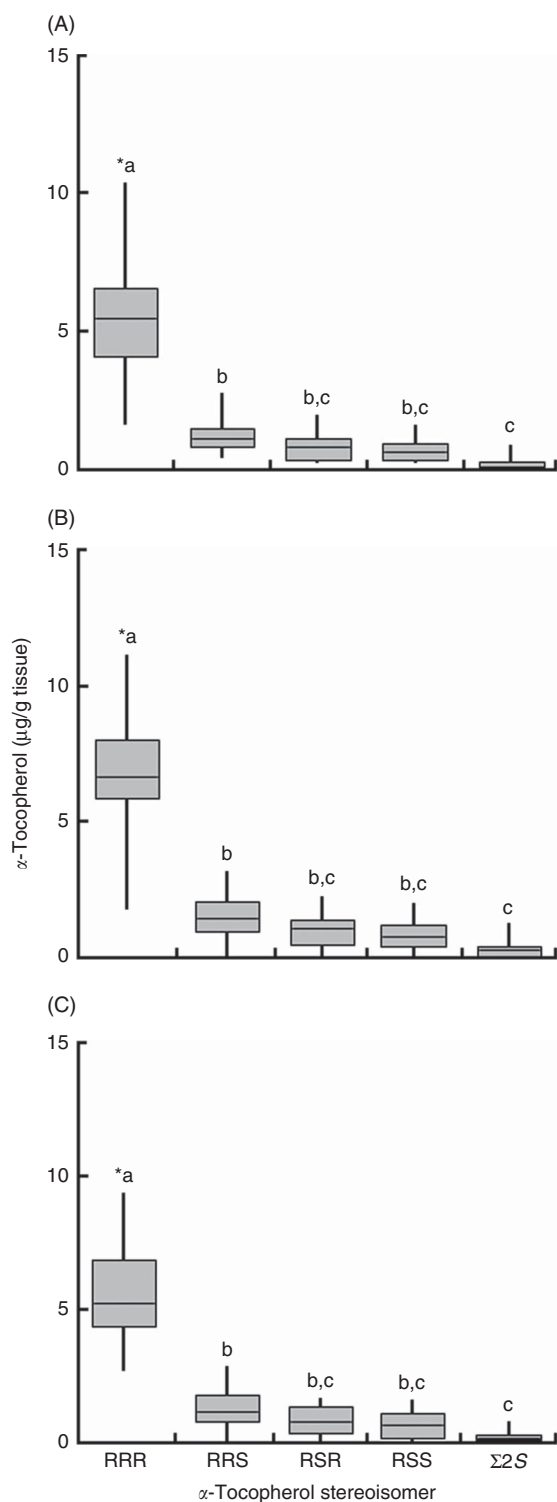
*$\alpha$ -Tocopherol stereoisomer ratios*

The average ratio of the  $RRR$  stereoisomer:the sum of synthetic  $2R$  stereoisomers was  $>2$  (Fig. 3) with a range from a minimum of approximately 1 to a maximum of 6.1, 4.7 and 4.9 for FC, HPC and VC, respectively; two samples, one from HPC and one from VC, from different decedents, did not contribute ratios as the samples contained 100%  $RRR$ .

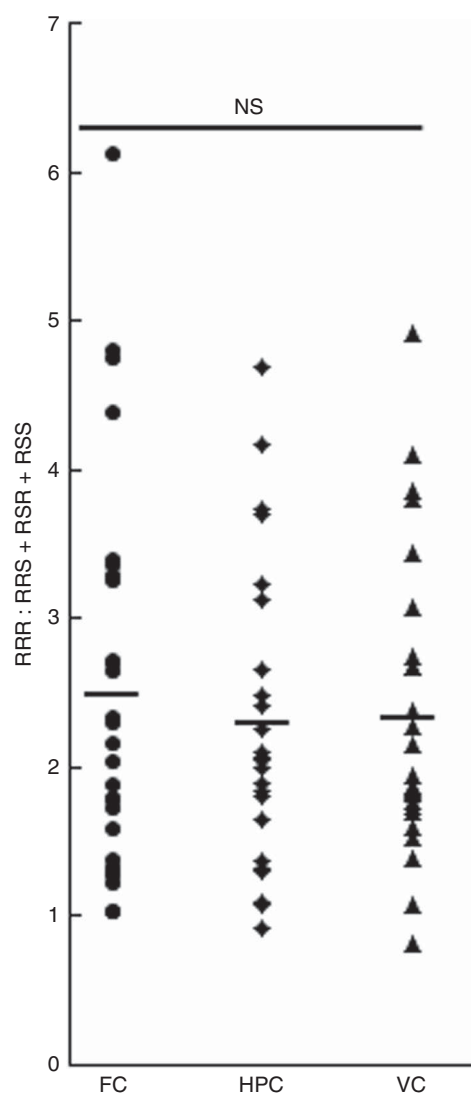
**Discussion**

Although others have previously reported the concentration of total  $\alpha$ -tocopherol in human adult and fetal brain<sup>(17-19)</sup>, we believe we are the first to report the  $\alpha$ -tocopherol stereoisomer profile in the human infant brain. Our data reveal that  $RRR$ - $\alpha$ -tocopherol was the predominant stereoisomer of  $\alpha$ -tocopherol in each of the three regions of the infant brain studied. In fact,  $RRR$ - $\alpha$ -tocopherol was the predominant stereoisomer in each sample tested, despite high inter-decedent variation in  $\alpha$ -tocopherol. We found that the stereoisomer patterns were relatively consistent across the three brain regions in decedents with samples available for each region. The degree of discrimination in favour of  $RRR$ - $\alpha$ -tocopherol is striking as most infant formulae were supplemented with *all-rac*- $\alpha$ -tocopherol. The extent of exposure of the decedents to *all-rac*- $\alpha$ -tocopherol is emphasised by the finding that the brain samples of only one decedent was devoid of synthetic stereoisomers. That both total  $\alpha$ -tocopherol and  $RRR$ - $\alpha$ -tocopherol were positively correlated with age in each brain region might provide further evidence of the importance of  $\alpha$ -tocopherol stereoisomer discrimination.

We believe the  $\alpha$ -tocopherol stereoisomer profile may be of particular importance to infants. First, vitamin E is an essential nutrient that plays an important role in neural function and development. Second, vitamin E requirements of formula-fed infants are routinely met using the less biologically active,



**Fig. 2.**  $\alpha$ -Tocopherol stereoisomer profile in the frontal cortex (FC) (A), hippocampus (HPC) (B) and visual cortex (VC) (C) of the human infant brain. Data are from thirty-six decedents with twenty-eight FC samples, twenty-five HPC samples and twenty-six VC samples. The median values are not shown as they were very similar to the mean values.  $\Sigma 2S$  stereoisomers include SRR, SSR, SRS and SSS. <sup>a,b,c</sup> Stereoisomers with unlike superscript letters were significantly different: <sup>a</sup>  $P < 0.0001$ ; <sup>b,c</sup>  $P < 0.05$ . \* Different from other values for brain region ( $P < 0.0001$ ).  
 □, 25th and 75th percentile values, —, mean; |, maximum and minimum values.



**Fig. 3.** The ratio of RRR- $\alpha$ -tocopherol:sum of RRS- $\alpha$ -tocopherol, RSR- $\alpha$ -tocopherol and RSS- $\alpha$ -tocopherol stereoisomers in infant decedent samples from the frontal cortex (FC), hippocampus (HPC) and visual cortex (VC). Scatter plot of all values by brain region; horizontal bar indicates mean value.

synthetic source of vitamin E – *all-rac*- $\alpha$ -tocopherol. Third, the state of development of the hepatic stereoisomer selection mechanism in infants has not been established.

Total  $\alpha$ -tocopherol concentrations reported here are lower than, but in line with, those previously reported for autopsied adult human brain<sup>(17–19)</sup> and very similar to those for rat<sup>(15)</sup> and piglet<sup>(16)</sup> brain. Although we cannot rule out the potential that tissue degradation before sample preservation could introduce artifact, no relationship was found between total  $\alpha$ -tocopherol concentration and time until tissue freezing. We believe that the slow turnover rate of  $\alpha$ -tocopherol in the brain<sup>(16,23,24)</sup> may have reduced the potential for preservation artifact.

We found that all individuals tested had very low levels of 2S stereoisomers and corresponding high levels of the 2R stereoisomers of  $\alpha$ -tocopherol. This observation is largely consistent with previous animal research, revealing that rodent

and piglet brains preferentially contain the *2R* stereoisomers of *all-rac-α-tocopherol*<sup>(13,15,16)</sup>. Discrimination against *2S* stereoisomers is believed to occur in the liver. Hepatic  $\alpha$ -TTP-mediated discrimination in the infant and mother likely explains the low concentrations of  $\gamma$ -tocopherol we found in the infant brain samples. Despite high levels of  $\gamma$ -tocopherol in the American diet, breast milk generally has a 5:1 ratio of  $\alpha$ -tocopherol: $\gamma$ -tocopherol. In addition, some infant formulae contain relatively high levels of  $\gamma$ -tocopherol, indicating that the infant liver also discriminates against  $\gamma$ -tocopherol in favour of  $\alpha$ -tocopherol<sup>(25)</sup>.

Previous research in animals does not provide definitive insight into whether there is discrimination between the *2R* stereoisomers in the brain. The single study published to date that directly quantified *2R*  $\alpha$ -tocopherol stereoisomers in the brain revealed no selection among *2R* stereoisomers in  $\alpha$ -tocopherol-deficient rats repleted with *all-rac-α-tocopherol*<sup>(15)</sup>. Our data, however, are consistent with preferential discrimination for *RRR-α-tocopherol* in the human infant brain. Extensive research in human adults and corroborative evidence in infants indicate that plasma from individuals consuming *all-rac-α-tocopherol* has roughly equal concentrations of the *2R* stereoisomers<sup>(8,9,26)</sup>. Equal concentrations of *2R* stereoisomers result in a ratio of *RRR-α-tocopherol*:the sum of *RRS-α-tocopherol*, *RSR-α-tocopherol* and *RSS-α-tocopherol* of approximately 0.33<sup>(8,26,27)</sup>. Therefore, if there was no stereoisomer discrimination in the infant brain, the minimum *RRR*:(*RRS* + *RSR* + *RSS*) would be approximately 0.33<sup>(15)</sup>. It is highly likely that some of the decedents studied received predominantly *all-rac-α-tocopherol*; however, the minimum ratio we observed was 0.7 with median brain values of 1.8 or higher in each brain region studied. These observations are consistent with significant selection among the *2R* stereoisomers in favour of *RRR-α-tocopherol*.

Potential over-representation of breast-fed infants in the study cohort does not explain our observations. Consistent with observations in sow milk<sup>(16)</sup>, unpublished observations from our group (M Kuchan) suggest that breast milk from North American women contains a significant amount of synthetic *2R*  $\alpha$ -tocopherol stereoisomers. This observation is based on 300 samples from more than 200 women and likely reflects the use of synthetic  $\alpha$ -tocopherol in maternal supplements. In addition, the results of this study indicate that both total and *RRR-α-tocopherol* increased with age during the 1st year, despite the rapid increase in brain volume known to occur over this period. We believe this diminishes the likelihood of possible confounding effects of maternal liver–placenta–infant liver tandem selection before birth. Enhanced discrimination between stereoisomers in the infant liver compared with that known to occur in the adult liver is inconsistent with published results in the human infant<sup>(25)</sup>. For these reasons, we believe that these data are best explained by a combination of discrimination for *RRR-α-tocopherol* in the infant brain and the source of  $\alpha$ -tocopherol consumed in the diet.

The predominance of *RRR-α-tocopherol* in the infant brain reported here might be explained by the presence of  $\alpha$ -TTP and tocopherol-associated protein (TAP) in the human brain. Extra-hepatic stereoisomer-specific discrimination has been previously demonstrated in the human fetus/fetal-placental unit<sup>(28,29)</sup>

and is believed to be at least partially explained by placental expression of  $\alpha$ -TTP<sup>(30)</sup>. The human cerebellum expresses  $\alpha$ -TTP<sup>(31)</sup>, and multiple regions of the human brain express relatively high levels of TAP<sup>(32)</sup>. TAP has a high degree of binding-site sequence homology to  $\alpha$ -TTP, and similar to  $\alpha$ -TTP is known to possess differential affinity for tocopherol structural isomers<sup>(33)</sup>. These observations suggest that TAP may be capable of discrimination among  $\alpha$ -tocopherol stereoisomers.

In conclusion, these first-of-kind data reveal a clear, preferential accumulation of the naturally occurring stereoisomer *RRR-α-tocopherol* in the human infant brain. This is a surprising observation given the extensive exposure of the decedents to *all-rac-α-tocopherol*. Our data are consistent with discrimination among the *2R* stereoisomers of *all-rac-α-tocopherol* in favour of *RRR-α-tocopherol*. We believe this finding raises important questions, as biological discrimination often reflects biological importance. Emerging evidence suggests that *RRR-α-tocopherol* and *all-rac-α-tocopherol* may have differential cellular effects mediated through gene expression<sup>(34)</sup>. It is not known whether there is an advantage of increased *RRR-α-tocopherol*, or if accumulation of synthetic stereoisomers is unfavourable for brain development. However, as vitamin E is essential for the integrity of cerebellar Purkinje neurons<sup>(21,35,36)</sup> through the action of  $\alpha$ -TTP, a stereo-selective protein, we believe this is an important question for future research.

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None of the authors has any conflicts of interest to declare.

### Supplementary material

For supplementary material/s referred to in this article, please visit <http://dx.doi.org/doi:10.1017/S0007114516001719>

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