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**APOLIPOPROTEIN E DEFICIENCY LEADS TO ALTERED  
BRAIN UPTAKE OF ALPHA TOCOPHEROL INJECTED INTO LATERAL  
CEREBRAL VENTRICLES**

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## ABSTRACT

The incorporation of radioactive alpha tocopherol by various brain regions of wild type and apolipoprotein E (apoE) deficient mice was investigated. Labeled tocopherol was injected into the lateral cerebral ventricles of 11 weeks-old, male mice. Radioactive cholesterol injected simultaneously was used as an internal standard to account for experimental variability. Most areas of the brain of apoE deficient mice took up less of alpha tocopherol per mg of protein than wild type animals. However, specific activity of alpha tocopherol was higher in cerebellum, pons, hypothalamus, midbrain and cerebral cortex in apoE deficient brains than the wild type. This could be due to a) the lower levels of alpha tocopherol in apoE deficient brain and b) reductions in the clearance and transport of tocopherol (possibly mediated by apoE). Tocopherol uptake by hippocampus was unusual since it was lower in apoE deficiency whether the data was expressed as specific activity or per mg of protein. Nearly all of the injected alpha tocopherol remained unchanged in the brains of both apoE deficient and wild type animals suggesting low turnover. Overall, the current data reinforces the hypothesis that apoE is a key protein involved with the transport and/or retention of alpha tocopherol in brain.

## INTRODUCTION

A number of reports in the literature suggest that the lipid transport protein, apolipoprotein E (apoE), may play a role in the pathogenesis of Alzheimer's disease (AD). The apo e4 allele has been shown to be associated with the common late onset familial and sporadic form of AD [1]. These authors found that the risk for development of AD increased from 20% to 90% and the mean age of onset of AD decreased from 84 to 68 years with increasing number of apo e-4 alleles in 42 families with late onset AD. This association of the risk for AD with apo e4 allele has been confirmed by many investigations [2]. Therefore, the neurobiology of apoE is an important area of research for understanding the pathogenesis of AD. Numerous studies have been aimed at understanding the basic functions of apoE in brain. A few of the functional roles of apoE include redistribution of lipids within cells of brain, maintaining connections between synaptic regions, and scavenging of toxins (see review [3]).

The role of apoE in lipid transport is well-known and it is conceivable that this molecule may also play a role in tissue handling of the lipid-soluble nutrient alpha tocopherol. ApoE is also capable of modulating the redox status of cells [4] [5]. Therefore, it is reasonable to assume that apoE may be involved in one or more aspects of the neurochemical function of vitamin E, the most potent endogenous antioxidant in membranes. In addition, administration of high doses of vitamin E has shown some promise in the treatment of Alzheimer's disease [6]. Recently, we reported that apoE deficiency was associated with declines in the steady state concentrations of alpha tocopherol in different regions of mouse brain[7]. The extent of decline in concentration was specific to the anatomic site within brain and was also dependent on age. This interaction of apoE with other endogenous molecules such as vitamin E will be important for designing potential strategies for the treatment of neurodegenerative disorders like Alzheimer's disease. To

further elucidate the relationship between vitamin E and apoE we conducted additional experiments where labeled alpha tocopherol was injected into the lateral ventricle of the brain and the incorporation of radioactivity by different parts of the brain was determined. Results of these experiments are presented in this report.

## MATERIALS AND METHODS

Chemicals: The chemicals used were of reagent grade purity from standard sources. Solvents for chromatography were HPLC grade from Fisher Scientific, Itasca, IL, USA. Alpha tocopherol and alpha tocopherolquinone were purchased from Kodak Laboratory Chemicals, Rochester, NY, USA. Absolute ethanol was obtained from Aaper Alcohol and Chemical Company, Shelbyville, Kentucky and was redistilled prior to use. Most of the reagent grade chemicals were from Sigma Chemicals, St. Louis, MO, USA. Alpha tocopherol acetate labeled with tritium in the five position (Roche Pharmaceuticals) was saponified and separated by HPLC prior to injection. Cholesterol,  $^{14}\text{C}$  labeled, was obtained from New England Nuclear (Perkin Elmer).

Animals: The animals were housed in our AALAC-approved facilities following their standards of care; the protocol was approved by the Subcommittee on Animal Studies of the Minneapolis VA Medical Center. Male mice that were 11 weeks old were used. Mice with the *ApoE* gene knocked out (C57BL/6J-*ApoE*<sup>tm1/unc</sup>) were obtained commercially from Jackson Laboratories, Bar Harbor, Maine. Control mice (C57 BL/ 6J) were also obtained from the same

source. The animals were acclimated to our animal care facilities and then placed on normal rodent chow fed ad libitum (Diets Incorporated, PA).

Intraventricular injection of radioactive compounds: All surgical operations were made under sterile conditions. The mouse was anesthetized using a mixture of ketamine (40 mg/kg) and xylazine (8 mg/kg) and then secured on the stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). An incision was made along the midline (sagittal suture). The injection site was 0.35 to 0.5 mm posterior and 1 mm lateral to the bregma ( See Figures 34 and 35 in [8]). Labeled vitamin E solution (5  $\mu$ l) was used for injection. The solution for injection contained 150,000 dpm of tritiated alpha tocopherol and 75,000 dpm of  $C^{14}$  cholesterol. Radioactive purities of both alpha tocopherol and cholesterol were checked by HPLC prior to injection. The mixture for injection was made in isotonic saline containing 0.5% BSA and 6% DMSO and the injection volume was 5  $\mu$ l. The injection was made slowly at a depth of 2.5 mm using a stopper device at the end of the syringe. After injection, the skin incision was closed with surgical suture and the animal allowed to recover from surgery. Forty eight hours later, the animal was anesthetized and perfused with cold isotonic saline. The brain was taken out and lightly rinsed with cold isotonic saline to remove any adherent isotopic material. The excess saline was removed gently with Kimwipe. The different brain regions were dissected out according to the method of Glowinski and Iversen [9]. The tissues were weighed and homogenized in 0.32 M sucrose, 10 mM HEPES, 1 mM EDTA at pH 7.4 and stored at  $-70^{\circ}C$  and were used for the various assays.

Biochemical assays: Determination of alpha tocopherol: Details of the method have been reported [10]. The samples were saponified at  $60^{\circ}C$  after the addition of 2 ml ethanol

containing 0.025 % (w/v) butylated hydroxytoluene, 0.1 ml of 30 % (w/v) ascorbic acid and 1 ml 10 % (w/v) KOH. The mixture was then extracted with hexane. Part of the hexane extract was evaporated down and the residue was redissolved in mobile phase and analyzed by HPLC for tocopherol using the following conditions: column = ultrasphere ODS, 5 microns, 4.6 X 150 mm from Beckman Instruments; mobile phase = 5.5% water in methanol with final 7.5 mM  $\text{NaH}_2\text{PO}_4$ ; flow rate = 2.5 ml/min. Alpha tocopherol was detected electrochemically utilizing Coulochem 5100 A detector equipped with 5011 analytical cell (detector 1 at -0.25 V and detector 2 at +0.55V) and 5021 conditioning cell at -0.75 V.

The metabolic fate of alpha tocopherol was also followed using this HPLC system. In this experiment the column effluent was collected as one minute fractions at a column flow rate of 2 ml per minute. The radioactivity in the various fractions was counted and the counts in the tocopherol peak were determined using the retention time for standard alpha tocopherol.

Other Biochemical Assays: Samples for radioactive counting were saponified and extracted with hexane. Aliquots of the hexane extract were mixed with Ultima Gold scintillation cocktail (Packard Biosciences, Meriden, CT) and counted on a Packard Tri-Carb 1900-CA liquid scintillation counter. Concentration of total proteins was determined by the Lowry technique as modified by Markwell et al [11].

## RESULTS

The tissue uptake of labeled materials can be studied after oral, intraperitoneal, intramuscular or intravenous administration of the isotopic material. In the first experiment labeled alpha tocopherol was injected into the tail vein. Tritium labeled tocopherol (15  $\mu$  Curies) was employed and the isotope allowed to equilibrate for 72 hours after injection. The animals were anesthetized and perfused with isotonic saline as described under Methods and various tissues were dissected out. The tissue samples were saponified and extracted with hexane. The hexane extractable radioactivity was counted. The results are shown in Figure 1.

Note that the uptake by the different regions of the brain is shown on the right side of the figure using a much smaller scale on the Y-axis. It can be seen that radioactivity in all brain regions was substantially lower than those in the peripheral tissues with most of the radioactivity being associated with organs such as liver and spleen. Even though adrenals showed high levels of activity the total weight of the adrenals is quite small and hence this organ contained only small amounts of the total radioactivity. In short, most of the injected radioactivity did not reach the brain. Therefore, the rest of the experiments involved injection of labeled alpha tocopherol into the lateral cerebral ventricle.

The uptakes of tritiated alpha tocopherol injected into the right lateral ventricle of wild type and apoE deficient mice were then compared. Tissue samples were saponified and the radioactivity extracted by hexane was counted. It was found that the levels of uptake of radioactive alpha tocopherol were quite variable between animals even though the same number of counts was injected into the cerebral ventricle. Hence it would be desirable to simultaneously inject a compound similar to tocopherol as an internal standard, a technique used often in other biochemical assays. It is well-known that alpha tocopherol and cholesterol have many

biochemical properties in common. Therefore, we injected a mixture of tritium labeled alpha tocopherol and  $C^{14}$  labeled cholesterol into the lateral ventricle of the brain as described under methods. The results for the uptake of labeled cholesterol are shown in Figure 2.

The pattern of uptake of alpha tocopherol and cholesterol were remarkably similar confirming the basis for our selection of cholesterol as an “internal standard” because of the known similarity of biochemical properties of the two compounds (See also Figure 3 that follows). Next, we calculated the uptake of alpha tocopherol using cholesterol uptake data to normalize alpha tocopherol counts. An average value for cholesterol counts in each area was calculated first. Then the cholesterol counts for the specific area from an individual animal were divided by the mean count and a ratio was calculated. The measured count of alpha tocopherol radioactivity was then multiplied by this ratio in order to obtain the corrected alpha tocopherol counts for each sample. The calculated counts of radioactive alpha tocopherol in different regions of brains are shown in Figure 3.

The variability in the final results was considerably reduced by using the correction ratios. The results show that the uptake of alpha tocopherol was reduced in the apoE deficient animals in hypothalamus, striatum, hippocampus and midbrain. Cerebellum and pons showed an increase in uptake in the apoE deficient animals. Similar grouping of the different brain regions was observed in our earlier study which demonstrated changes in tissue alpha tocopherol concentrations in apoE deficiency [7]. In short, apoE deficiency was associated with altered uptake of alpha tocopherol from the brain extracellular fluid which is in equilibrium with cerebrospinal fluid (CSF).

The uptake of tocopherol may also be dependent upon the level of alpha tocopherol present in the tissue. This can be examined by expressing the uptake of radioactivity as a function of the endogenous levels of alpha tocopherol. These calculations were done and the results are shown in Figure 4.

The tocopherol uptakes by most areas of the brains of apoE deficient mice were higher than that of the wild type animals. This change in relationships between the wild type and apoE deficient animals is partly due to the lower concentrations of alpha tocopherol in the apoE deficient animals. However, note that the hippocampus was unique in being the only site with higher uptake of radioactive tocopherol by the wild type compared with the apoE deficient mice as seen in both Figures 3 and 4. Therefore, handling of tocopherol within the hippocampus may have some additional unique features. In general, data presented so far show that apoE deficiency alters the dynamics of alpha tocopherol uptake by different areas of the brain (Figures 3 and 4).

Radioactive tocopherol was present in very small amounts in peripheral tissues. Figure 4 (right side) shows the amount of radioactivity present in liver and serum. Firstly, these results confirm that the normal routes of absorption of CSF into the systemic circulation were intact in all animals. Secondly, the specific activities of alpha tocopherol in both liver and serum were lower in apoE deficient compared with wild type animals. It should be noted that the concentrations of endogenous alpha tocopherol in serum and liver were higher in the apoE deficient than in wild type animals: serum  $5.62 \pm 0.25$  and  $19.5 \pm 0.83$  (S.E.) nmoles per ml: liver  $17.5 \pm 0.6$  and  $19.90 \pm 0.76$  (S.E.) nmoles per gram, for wild type and apoE deficient mice,

respectively. Since these concentrations appear in the denominator during calculations of specific activities it would undoubtedly have an effect upon the final results.

The metabolic fate of radioactive alpha tocopherol in brain regions during the experimental period of 48 hours after intraventricular injections was also examined. In this experiment the brain samples from both control and apoE deficient animals were dissected out, and the samples saponified. Hexane extracts were prepared and processed by HPLC as usual. The eluents from the column were collected into individual fractions and the radioactivity in each fraction was counted. The radioactive counts in the alpha tocopherol peak as a percent of the total activity were determined. The percent radioactivity recovered in the alpha tocopherol peaks were as follows: a) cerebellum, hypothalamus, striatum, hippocampus and cerebral cortex –  $96.1 \pm 0.97$  and  $94.9 \pm 0.5$  (mean  $\pm$  SEM, n = 5) for wild type and apoE deficient animals respectively; liver –  $102.5 \pm 8$  and  $95.6 \pm 5$  (mean  $\pm$  SEM, n = 3) for wild type and apoE deficient animals respectively. There were no statistically significant differences between wild type and apoE deficient animals. The data show that nearly all of the radioactive alpha tocopherol remained unchanged in the brains of both wild type and apoE deficient animals. This agrees with previous observations that the rate of metabolism of alpha tocopherol is quite low in brain. Very small amounts of alpha tocopherol quinone were found at its retention time. Oxidation of tocopherol during the processing of samples could account for the small amounts of activity in the quinone peak. In addition, our data also show that apoE deficiency did not have any influence on the slow turnover of alpha tocopherol in brain suggesting that apoE may be involved in only the dynamics of transport of alpha tocopherol and not in its metabolism.

## DISCUSSION

Intravenous injection of alpha tocopherol: Compounds can be administered through various routes in order to study its uptake by brain. A few of the most commonly used methods are oral, intramuscular or intravenous delivery. In the first experiment we studied the uptake of labeled alpha tocopherol after intravenous administration. Our data showed that the level of radioactivity (per gram tissue) in the different areas of the brain was less than a few percent of that found in liver or adrenals (Figure 1). Gallo-Torres [12] has summarized his extensive studies on the tissue distribution of radioactive tocopherol after intragastric and intravenous routes of administration. He observed that only 0.03% of total radioactivity was found in brain twelve hours after oral dosing. When administered intravenously the brain was found to contain only 0.22% of the radioactivity injected. Thus our study and that of Gallo-Torres indicate that the uptake of tocopherol radioactivity by brain is quite low. Interestingly, the steady state concentration of alpha tocopherol in brain is very similar to that of other tissues such as the liver even though uptake by brain is much smaller than that by liver. This suggests that tocopherol entering brain has a slow turnover rate compared with other organs. Neither oral nor intravenous administration of radioactive tocopherol result in significant uptake of activity by the brain and the techniques are associated with substantial wasting of the radioactive compound. This led us to the use of intracerebroventricular injection of labeled alpha tocopherol.

Uptake of radioactive alpha tocopherol by different regions of the brain after intracerebroventricular injection: First the uptake data from wild type animals (filled bars in Figure 3) will be considered. The different regions of the brain incorporated varying amounts of radioactive alpha tocopherol that was injected into the lateral ventricle. A variety of factors determine the level of incorporation of radioactivity by different regions with two physical

factors playing major roles. Firstly, the larger the ventricular surface area that is in contact with CSF the higher will be the uptake by that region. In fact, this may be one reason for the high levels of uptake by hippocampus. Interestingly, the latter region is important on behavioral grounds since it is involved with memory function.

The path of flow of CSF is the next physical factor to be considered. We observed that after hippocampus the next highest level of incorporation of radioactive alpha tocopherol was in the midbrain. This may be explained partially by the fact that CSF flows to the third ventricle and the cerebral aqueduct from the lateral ventricles. At this point CSF is still in contact with internal surfaces of the brain parenchyma where the CSF flows into interstitial space across ependymal cells which have leaky gap junctions permeable to macromolecules [13]. Ultimately, CSF flows to the fourth ventricle and then out via the foramina of Magende and Luschka into the basilar cisterns. This may account for the moderate levels of uptake by pons and cerebellum. Distribution of label into the subarachnoid space surrounding the spinal cord and finally the subarachnoid space overlaying brain surfaces occurs last. Samples of cortex which contact CSF only at the brain surface showed low alpha tocopherol uptake. Biomolecules in the subarachnoid space diffuse directly across pial surfaces and through Virchow-Robin spaces into the interstitial space [14]. Frontal cortex showed one of the lowest levels of uptake since this will be the region exposed to CSF towards the end of its path through brain prior to its absorption on the superior surface of the brain at the arachnoid granulations.

Role of apoE in the transport and uptake of tocopherol from CSF into the brain: The above two paragraphs takes into account only physical factors that may control uptake of radioactive tocopherol by different areas of the brain. Obviously, there will be receptor-

mediated as well as passive transport events involved in the uptake. CSF is in equilibrium with the extracellular fluid of the brain. Therefore, substances injected into the ventricular fluid would encounter only small barriers for transport into brain. For example, studies involving radionuclide cisternography show that absorption of CSF can occur throughout the brain parenchyma at numerous sites [15]. It should also be stressed that apoE is a major apolipoprotein in CSF and its CSF concentration is about 3% of the plasma concentration [16]. Thus apoE is well suited to be involved with tocopherol transport between brain cells and the extracellular fluid. Entry of labeled tocopherol from extracellular fluid into neurons and glia could be mediated by transporters and/or by diffusion across plasma membrane. Studies show that transport of plasma lipids from blood to brain may involve fast dissociation of lipid from protein complexes followed by cellular uptake [17]; such processes may be operating in the transfer of protein-bound tocopherol from CSF to brain parenchyma. The final pattern of uptake of labeled tocopherol after ventricular injection would be expected to depend upon one or more of the factors noted so far.

Next, one can consider the tocopherol uptake data that was converted to specific activity by expressing radioactivity per nanomoles of alpha tocopherol (Figure 4). A comparison of this data with those where uptake data are expressed per mg protein shows some common features and a few differences (See Figures 3 and 4). The apoE deficient animals incorporated more tocopherol in cerebellum and pons whereas the uptake by hippocampus was lower when compared with the wild type animals in both Figures 3 and 4. It is unknown whether different modes of uptake of alpha tocopherol occur in hippocampus compared with cerebellum and pons. Note that the radioactive uptake calculated as dpm per nanomole of alpha tocopherol in most regions of the brain except hippocampus and striatum show an increase in alpha tocopherol

specific activity in apoE deficient mice when compared with the wild type animals (Figure 4). It can be safely assumed that all physical factors that could affect uptake remain the same for both the wild type and apoE deficient mice. One factor that would cause the specific activity to go up in the case of apoE deficient tissue is the lower concentrations of alpha tocopherol in the apoE deficient brains as reported by us [7].

Another possible reason for the increase in specific activity in apoE deficient animals is that the lack of apoE may be impeding the normal mode of transport of tocopherol within the brain resulting in lower clearance of labeled tocopherol in most areas of the brain. Reports suggest that nascent lipoprotein particles secreted by astrocytes contain apoE and it has been proposed that they take part in cholesterol transport within the brain [18]; tocopherol transport may follow similar routes. If this were the case, tocopherol injected as an albumin complex would be cleared from the interstitial fluid to a lesser extent in apoE deficient mice than in controls. Consequently, we would expect tocopherol specific activity to be higher in many areas of the apoE deficient brains and this was what we observed (Figure 4). Interestingly, hippocampus in apoE deficient animals had lower uptake of labeled tocopherol whether the data was expressed per mg protein (Figure 3) or as specific activity (Figure 4). Hence hippocampus may be utilizing a more complex process for handling tocopherol. With all brain regions it is also possible that molecules that normally accept alpha tocopherol at the plasma membrane site may have been altered in concentration, composition or structure in response to apoE deficiency. One example of such a molecule is scavenger receptor BI; a deficiency of this receptor has been shown to cause declines in brain levels of tocopherol [19].

Accumulation of radioactivity in the periphery is also altered by apoE deficiency (Figure 4). As expected, the level of activity was quite small both in liver and serum when compared with the activity in brain. Interestingly, the specific activity levels in the apoE deficient animals were lower than those in the wild type. This trend is opposite of that found in most of brain regions. It is noteworthy that the concentrations of endogenous alpha tocopherol go up in the periphery in response to apoE deficiency whereas the opposite is the case with brain regions. Regardless of the direction of change, the data show that apoE deficiency was associated with an alteration in the dynamics of alpha tocopherol uptake in peripheral tissues as well.

Conclusions: Intravenously injected alpha tocopherol entered the brain to a much lesser extent than the peripheral tissues such as liver. Nonetheless, brain alpha tocopherol levels at steady state were comparable to those of peripheral tissues indicating that tocopherol turnover rate in brain is much lower than in peripheral tissues. Our finding that most alpha tocopherol injected into brain remained unchanged without the production of quinone or other species in both wild type and apoE deficient animals also suggest low rates of turnover of the compound. Earlier studies using very different techniques have also shown low turnover rate of tocopherol in the brain [20]. Furthermore, apoE deficiency did not change the metabolism of alpha tocopherol. In the current investigation, labeled tocopherol was injected into the lateral cerebral ventricles since the uptake after intravenous injection was quite low. Data from radioactive cholesterol injected simultaneously were used as internal standard to reduce the experimental variability during ventricular injection. Examination of data from control animals showed that a) areas with larger surface areas in contact with CSF took up larger amounts of alpha tocopherol (e.g., hippocampus) and b) areas that are close to the lateral ventricle also took up more activity possibly because these regions are more proximal in the pathway of flow of CSF (e.g.,

hippocampus). ApoE deficiency resulted in increases in uptake and retention of radioactive vitamin E when data was expressed as dpm per nanomole of alpha tocopherol. This indicates that apoE may be involved in the normal processing of vitamin E in brain and apoE deficiency causes an impaired clearing of alpha tocopherol. In any case, the uptake and retention of alpha tocopherol by different regions of the brain were clearly modulated by apoE deficiency. Alpha tocopherol was incorporated less in the hippocampus from apoE deficient brains whether the uptake was expressed as units per mg protein or per nanomole of alpha tocopherol. This suggests that the handling of tocopherol by the hippocampus may have some unique features. Using literature reports and the data presented we propose that a complex of proteins including apoE may be involved in the transport of vitamin E across the brain cellular membranes. The current data along with our previous publication [7] provide additional evidence illustrating a functional interaction between apolipoprotein E and vitamin E in mammalian brain.

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## FIGURE LEGENDS

Figure 1. Incorporation of radioactive alpha tocopherol by different tissues after injection of 15 micro curies of tritium labeled compound. Male mice, 11 weeks old, were injected with tritium labeled alpha tocopherol. After 72 hours the mice were anesthetized, perfused with isotonic saline and tissues were dissected out. The tissues were saponified, extracted with hexane and the radioactivity in the hexane extract was determined. The error bars show stand error.

Figure 2. Comparison of the uptakes of labeled cholesterol injected into the right lateral cerebral ventricle of control and apoE deficient mice. Tritiated alpha tocopherol (150,000dpm) and C<sup>14</sup> labeled cholesterol (75,000) were injected into the right lateral ventricles of 11 weeks-old mice of the respective group. After 48 hours the animals were sacrificed, brains perfused, tissues dissected out, saponified, extracted with hexane; the C<sup>14</sup> radioactivity in the hexane extracts were counted. Error bars show S.D. of means from ten animals in each group.

Figure 3. Comparison of the corrected uptakes of labeled alpha tocopherol injected into the right lateral cerebral ventricles of normal and apoE deficient mice. Using the incorporation of isotopic cholesterol uptake as an internal standard the alpha tocopherol uptake data were normalized (see Results for the method of calculation). Error bars indicate S.E. of means from ten animals in each group. Statistical significance is indicated in the figure using stars over each pair of data from wild type and apoE deficient animals: \* P <0.05, \*\* P <0.005.

Figure 4. Comparison of the corrected uptakes of alpha tocopherol injected into the lateral ventricle of wild type and apoE deficient mice with the radioactivity expressed per nanomoles of

endogenous alpha tocopherol. Error bars indicate S.E. Statistical significance is indicated in the figure using stars over each pair of data from wild type and apoE deficient animals: \* P <0.05, \*\* P <0.005.

ACCEPTED MANUSCRIPT

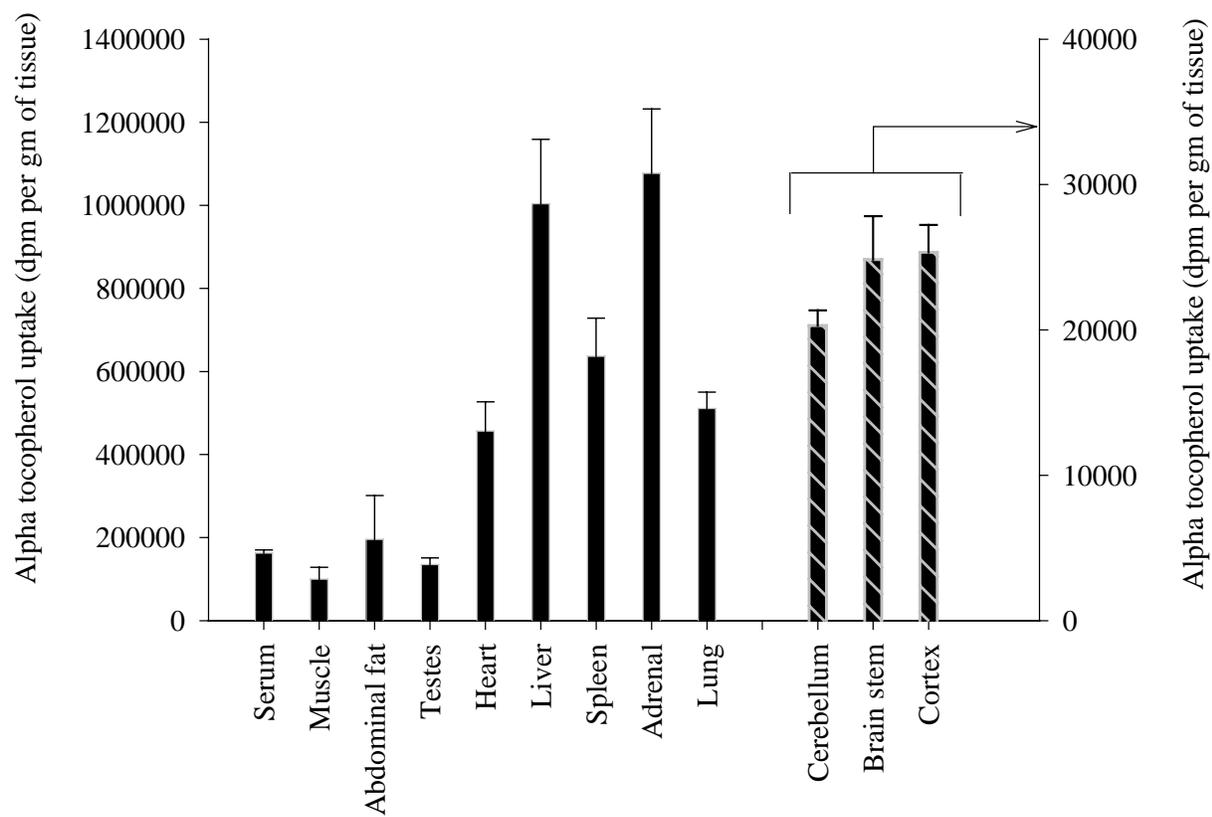


Figure 1

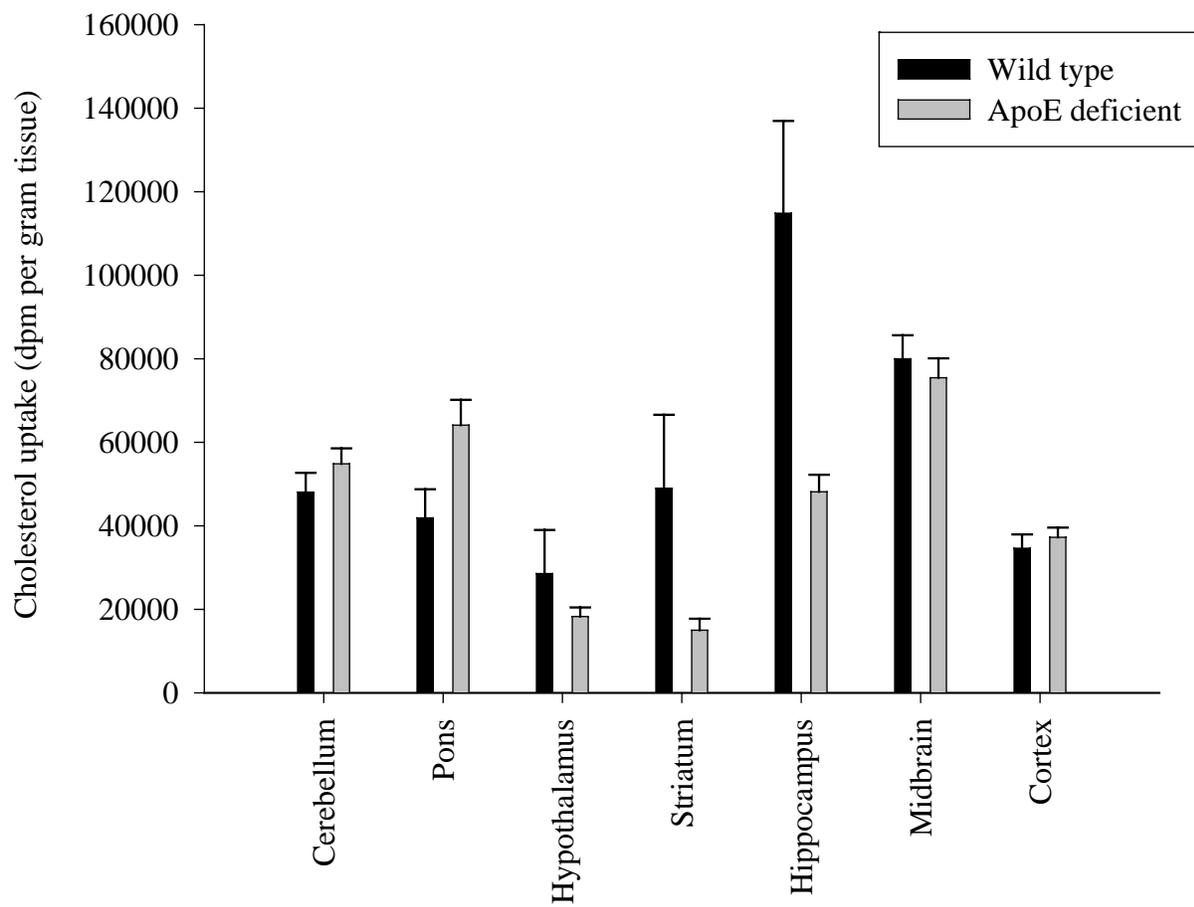


Figure 2

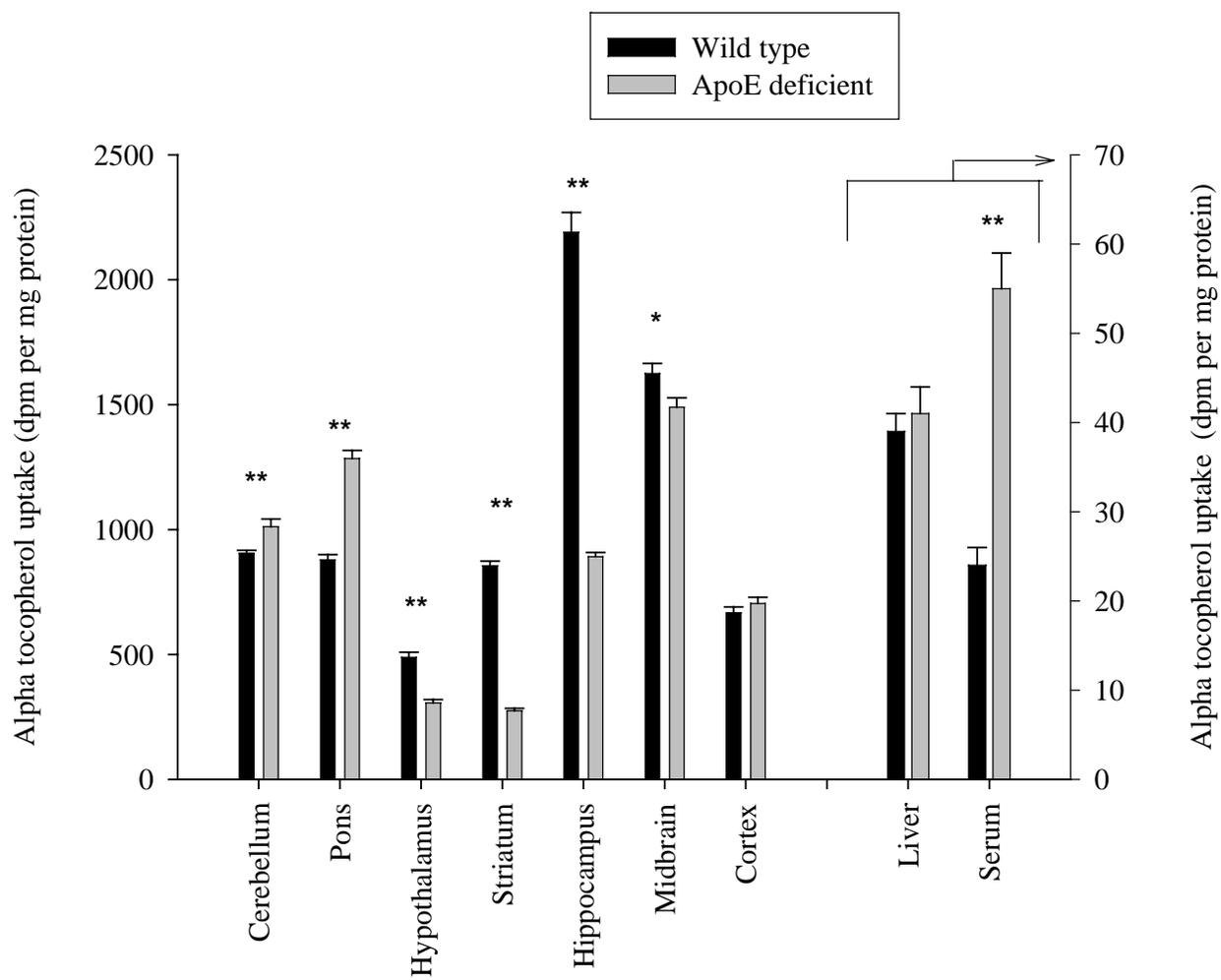


Figure 3

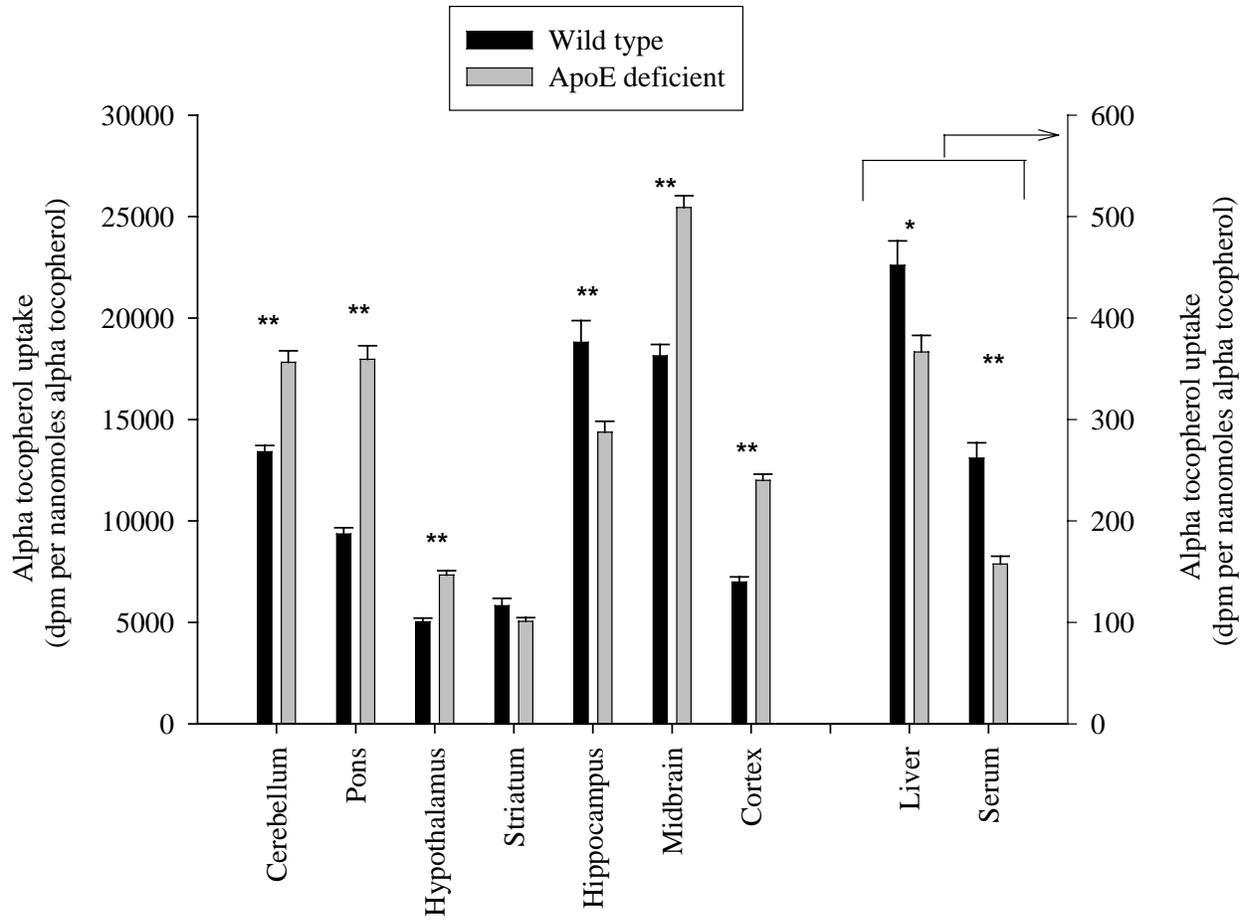


Figure 4