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Vitamin C deficiency in the brain impairs cognition, increases amyloid accumulation and deposition, and oxidative stress in APP/PSEN1 and normally-aging mice

Shilpy Dixit¹, Alexandra Bernardo¹, Michelle Jennifer Walker¹, John Andrew Kennard¹, Grace Youngeun Kim^{1,2}, Eric Sean Kessler^{1,2}, and Fiona Edith Harrison¹

¹Division of Diabetes, Endocrinology and Metabolism, Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee, 37232, USA

²Program in Neuroscience, Vanderbilt University, Nashville, Tennessee, 37232, USA

Abstract

Subclinical vitamin C deficiency is widespread in many populations, but its role in both Alzheimer's disease and normal aging is understudied. In the present study we decreased brain vitamin C in the APP_{SWE}/PSEN1_{deltaE9} mouse model of Alzheimer's disease, by crossing APP/PSEN1⁺ bigenic mice with SVCT2^{+/-} heterozygous knockout mice, which have lower numbers of the sodium-dependent vitamin C transporter required for neuronal vitamin C transport. SVCT2^{+/-} mice performed less well on the rotarod task at both 5 and 12 months of age compared to littermates. SVCT2^{+/-} and APP/PSEN1⁺ mice, and the combination genotype SVCT2^{+/-}APP/PSEN1⁺, were also impaired on multiple tests of cognitive ability (olfactory memory task, Y-maze alternation, conditioned fear, Morris water maze). In younger mice, both low vitamin C (SVCT2^{+/-}) and APP/PSEN1 mutations increased brain cortex oxidative stress (malondialdehyde, protein carbonyls, F₂-isoprostanes) and decreased total glutathione compared to wild-type controls. SVCT2^{+/-} mice also had increased amounts of both soluble and insoluble Aβ₁₋₄₂ and a higher Aβ_{1-42/1-40} ratio. By 14 months of age, oxidative stress levels were similar among groups, but there were more amyloid-β plaque deposits in both hippocampus and cortex of SVCT2^{+/-}APP/PSEN1⁺ mice compared to APP/PSEN1⁺ mice with normal brain vitamin C. The data suggest that even moderate intracellular vitamin C deficiency plays an important role in accelerating amyloid pathogenesis, particularly during early stages of disease development, and that these effects are likely modulated by oxidative stress pathways.

Keywords

Vitamin C; oxidative stress; cognition; Alzheimer's disease; amyloid; mouse models

Corresponding author: Fiona E Harrison fiona.harrison@vanderbilt.edu 7465 MRB4, 2213 Garland Avenue, Vanderbilt University Medical Center, Nashville, Tennessee, 37232, USA.

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Introduction

Under normal circumstances vitamin C (ascorbate) is maintained at high concentrations in brain tissue where it is critical for maintenance of oxidative balance¹. Vitamin C is concentrated via a two-step transport system with the sodium-dependent vitamin C transporter (SVCT2); from blood into the cerebral spinal fluid (CSF) at the choroid plexus, and then from extracellular fluid into neurons. Additional recycling pathways exist for retention under conditions of diminished intake, including reduction of the oxidized form (dehydroascorbic acid) to vitamin C (ascorbic acid) within astrocytes. However, brain and CSF levels can decrease under conditions of prolonged deficient intake, which may create a dangerous oxidative imbalance during normal aging, and particularly during inflammatory neurodegenerative diseases such as Alzheimer's disease.

Oxidative stress is a critical component of Alzheimer's disease neuropathology^{2,3}. Several studies have thus sought to define the role for vitamin C in Alzheimer's disease, but population studies have yielded mixed results as to the effectiveness of dietary supplements in older adults⁴⁻⁷. Animal studies of vitamin C supplementation by oral, intraperitoneal, and intravenous administration, have found modest benefits on cognition, oxidative stress markers and amyloid-related pathology in mouse models of aging and Alzheimer's disease⁸⁻¹¹. Vitamin C was also protective against amyloid- β induced apoptosis in cultured SH-SY5Y and diminished amyloid- β secretion from cells¹², and protected against increased intracellular calcium and cell death in PC12 cells¹³. In contrast, in humans under normal or disease conditions, deficiency is likely to be a major contributing factor to pathology. A large portion of the Western world may be deficient in vitamin C, and in several studies lower blood vitamin C correlated with cognitive impairment¹⁴⁻¹⁷. It is therefore critical to determine how prolonged sub-clinical vitamin C deficiency can impact normal aging and neurodegenerative disease from the very earliest stages of disease when pathogenic pathways may be more malleable.

We generated a novel mouse model of vitamin C deficiency in Alzheimer's disease by crossing SVCT2 heterozygous knockout mice¹⁸ with a bigenic mouse carrying two mutations known to cause early-onset Alzheimer's disease (SVCT2^{+/-}APP/PSEN1⁺). These mice have intracellular vitamin C deficiency, but normal circulating levels, since they can synthesize the vitamin in liver. We hypothesized that low vitamin C would induce oxidative stress from an early age and that this would accelerate the development of pathological changes such as amyloid- β production and deposition, as well as the associated cognitive deficits. Accumulation of reactive oxygen species is a natural part of aging and thus we were also interested to study the effects of low vitamin C on normal aging in the non-transgenic mice.

Results and Discussion

Vitamin C is an essential antioxidant that humans must obtain through their diets, and one of which many people have depleted or deficient levels. We predicted that lower brain vitamin C would contribute to an environment of oxidative imbalance that would accelerate Alzheimer's disease neuropathology. We tested this hypothesis by studying cognition,

oxidative stress, and amyloidogenic changes in a mouse model of Alzheimer's disease with partial ablation of vitamin C transport in the brain.

Low Vitamin C disrupts memory in wild-type and APP/PSEN1⁺ mice

Olfactory memory—Olfactory memory testing was conducted in 5-month old mice. Data for this test were not normally distributed and therefore a log₁₀ transformation was used on the data. Baseline exploration levels did not vary among the genotypes for either the water or familiar odor trials ($F_s < 3.68$, $p_s > 0.06$). Habituation to the familiar odor (decreased investigation time) on day 2 compared to day 1 was used to index 24-hour recall memory of the familiar odor for each group. A t-test was performed between exploration times of the familiar odor on the two test days for each group. As expected, all mice that did not carry APP/PSEN1 mutations showed habituation to the familiar odor with less exploration recorded on day 2, indicated as a positive preference score (day 1 - day 2 exploration) in Figure 1A, (SVCT2^{+/-} $t(14) = 2.176$, $p = 0.047$; wild-type $t(16) = 4.15$, $p < 0.001$, Fig. 1A (left, open bars)). APP/PSEN1⁺ mice showed a strong but non-significant trend towards habituation of exploration ($t(16) = 2.035$, $p = 0.059$; white hatched bar), whereas no decrease was observed in SVCT2^{+/-} APP/PSEN1⁺ mice ($t(11) = -1.578$, $p = 0.143$; red hatched bar). A secondary index of memory can be derived from differences in greater exploration of the novel smell on day 2 compared to the previously-presented odor. Wild-type mice showed this pattern of behavior ($t(16) = -2.149$, $p = 0.047$ Fig. 1A (right)), but the same differences were not observed for APP/PSEN1⁺ mice ($t(16) = -0.337$, $p = 0.74$), nor for SVCT2^{+/-} mice ($t(14) = 1.18$, $p = 0.258$). SVCT2^{+/-} APP/PSEN1⁺ spent more time exploring the familiar odor than the novel odor ($t(11) = 3.65$, $p = 0.004$). An impairment in olfactory memory may be particularly relevant given that olfactory deficits in mild cognitive impairment and Alzheimer's disease correlate with verbal and visual memory performance¹⁹ and may predict the likelihood of further cognitive decline²⁰.

Spatial memory—Tests of spatial memory in hippocampal-dependent tasks are also very important in Alzheimer's disease in which the hippocampus is so heavily compromised. Alternation behavior in the Y-maze is thought to reflect spatial working memory. At 5 months, mice with APP/PSEN1 mutations made fewer alternations than wild-type mice ($F_{1, 55} = 9.09$, $p = 0.004$, Fig. 1B). Although the effect appeared larger in SVCT2^{+/-} APP/PSEN1⁺ mice, there were no significant effects of SVCT2 genotype ($F_s < 0.87$, $p_s > 0.36$). At 12 months, there were no differences according to APP/PSEN1 genotype ($F_s < 0.58$, $p_s > 0.45$), however, mice with low vitamin C made fewer alternations ($F_{1, 49} = 4.92$, $p = 0.031$, Fig. 1C). Unexpectedly, alternation behavior did not further decline in the older animals compared to the 5-month age group, and the deficit observed in the APP/PSEN1⁺ mice was no longer apparent at the older time point. The number of arm entries decreased with age, from group averages of 21.4–25.2 at 5 months, to 15.9–20.8 in the older mice. The greatest change (a 37% decrease) was observed in the APP/PSEN1⁺ group. It is possible that reducing the number of arms entered, and therefore also increasing the amount of time in each arm per entry, helps to diminish the cognitive demands of this task and therefore leads to improved, or at least less-impaired, performance.

More comprehensive examination of spatial learning and memory was made using the Morris water maze. Decreasing escape latencies across 3 days of cued (visible) platform testing indicated that all mice were physically able to solve the task and learn the rule that the platform led to escape (5M: $F_{2, 114}=90.336$, $p<0.001$; 12M $F_{2, 100}=113.69$, $p<0.001$, *data not shown*). Hidden platform testing was then conducted to test memory for a location within the pool using extra-maze cues. It is important that all mice be given sufficient opportunity to learn the location of the platform in order to make comparisons of memory capacity during the probe trial. We therefore used 8 days of task acquisition training, after which average escape latencies were all under 10 s in the younger mice, and under 15 s in the older mice, indicating learning in all groups (5M: $F_{7, 399}=39.57$, $p<0.001$, Fig. 2A; 12M $F_{7, 336}=34.46$, $p<0.001$, Fig. 2E). At 5 months APP/PSEN1 mutant mice were slightly faster overall to locate the platform ($F_{1, 57}=4.73$, $p=0.034$). This result was likely driven by the slightly poorer performance of the SVCT2^{+/-} mice on the first 4 days of testing, but there were no other significant effects of genotype ($F_s<3.41$, $p_s>0.07$), and all mice were equally quick to locate the maze by the end of training. At 12 months there were no group differences according to genotype ($F_s<2.61$, $p_s>0.07$).

During the 60 s no-platform probe trial, memory is typically assessed through time spent swimming in each of the quadrants (target versus non-target, Fig. 2I). At both ages all groups showed a significant preference for the platform quadrants ($F_s>4.90$, $p_s<0.05$, Figs. 2B,F). *Post hoc* comparisons indicated that at both ages the wild-type mice tended to perform with greater accuracy than the APP/PSEN1 mice, with stronger preferences for the target quadrant over non-target quadrants, and the poorest performance was observed in the SVCT2^{+/-}APP/PSEN1⁺ mice. Goal-directed swimming may be better represented by time spent swimming within a defined radius of the platform edge (20 cm) and number of times the mouse crosses the previous platform location. At 5 months of age, mice carrying APP/PSEN1 mutations spent less time swimming in the target zone than wild-type mice ($F_{1, 57}=9.58$, $p=0.003$), but there was no additional effect of SVCT2 genotype ($F_s<0.58$, $p_s>0.45$; Fig. 2C). At 12 months of age performance was similar across the groups ($F_s<0.93$, $p_s>0.34$, Fig. 2G). Platform crossings did not differ among the groups at 5 months ($F_s<1.14$, $p_s>0.71$, Fig. 2D). At 12 months, mice with low vitamin C levels made 25–50% fewer platform crossings than mice with normal vitamin C, which were still making approximately 4 platform crossings, similar to the young mice, regardless of the presence of APP/PSEN1 mutations (SVCT2 genotype: $F_{1, 48}=6.06$, $p=0.017$, Fig. 2H). There was no main effect of APP/PSEN1 genotype and no interaction ($F_s<0.72$, $p_s>0.40$). Mice with low vitamin C had marginally slower swim speeds overall compared to normal vitamin C mice in the probe trial at 5 months of age ($F_{1, 57}=5.175$, $p=0.027$). This was not the case at 12 months where there were no differences in swim speed ($F_s<2.10$, $p_s>0.15$, *data not shown*), and so poorer performance at this age cannot be attributed to physical ability. These data are in line with previous reports in mice of this genotype which found similar, or even larger, cognitive impairments in APP/PSEN1⁺ mice to those reported here in both water maze and Y-maze tasks²¹, although other reports have failed to show deficits in the Y-maze at 7 months²². The APP/PSEN1 mouse line was originally developed on a hybrid background²³, and reports in mice that have been backcrossed for multiple generations to the C57Bl/6

background used here, typically show fewer deficits at early age points (e.g. 6–8 months^{24, 25}).

The older cohort of mice was also tested using conditioned fear chambers. Twenty-four hours after training, similar levels of freezing were observed among the groups in the original test chamber ($F_s < 0.77$, $p_s > 0.39$, Fig. 1D). However, when tested in a novel environment with the conditioned stimulus (tone), APP/PSEN1 mutant mice were significantly impaired, showing less freezing, compared to wild-type mice ($F_{1, 46} = 4.458$, $p = 0.04$, Fig. 1E). There was no effect of SVCT2 genotype ($F_s < 0.19$, $p_s > 0.67$). There were no differences among the groups in shock-threshold; all mice flinched or jumped, and vocalized at shocks of 0.35mA or lower, which was below the test stimulus of 0.5 mA.

We also assessed locomotor activity and anxiety in the mice because impairments in either of these areas could confound data from the tests of cognitive ability. Mice were tested in locomotor activity chambers for 15 min per day on two consecutive days. At both 5 and 12 months all mice showed expected decreases in distance traveled on day 2 compared to day 1 ($F_{1, 59} = 52.2$, $p < 0.001$; $F_{1, 50} = 118.89$, $p < 0.001$ Fig. 3A; B), a pattern of habituation that reflects memory for the testing context. Taken in combination with the results described above, these data suggest that cognitive impairments were not limited to behaviors dependent on the integrity of the hippocampal formation, and also that the impairments observed were not reflective of global dysfunction. Both low vitamin C and APP/PSEN1 genotype, separately or in combination, led to poorer performance in mice under conditions of more active memory demands (e.g. alternation in Y-maze, probe trial in water maze), but not when recall of the testing context was more passive (context-dependent freezing, habituation to locomotor activity chambers).

Vitamin C deficiency is associated with mild hyperactivity at 12 months but does not alter anxiety

At 12 months, low vitamin C (SVCT2^{+/-}) mice were slightly hyperactive compared to mice with normal vitamin C ($F_{1, 50} = 6.79$, $p = 0.012$), but there were no other differences according to genotype at either age. As a measure of anxiety in a novel environment we performed ‘open field’ analyses on time spent in the center of the chamber during the first 5 min of the first trial in the locomotor activity chambers. There were no differences according to group on this measure of anxiety ($F_s < 1.96$, $p_s > 0.17$, *data not shown*). At 5 months there were no differences in exploration of the elevated zero maze ($F_s < 2.47$, $p_s > 0.12$, Fig. 3C), but at 12 months the SVCT2^{+/-} and the SVCT2^{+/-}APP/PSEN1⁺ mice showed further evidence of mild hyperactivity in that they traveled further in the maze than mice with normal vitamin C levels ($F_{1, 52} = 5.81$, $p = 0.19$, Fig. 3D). There were no differences on the time spent in the closed zones at either age ($F_s < 0.72$, $p_s > 0.40$, *data not shown*). Increased exploration was not observed in Y-maze arm entries in the low vitamin C mice, neither were differences detected in investigation time in the olfactory learning task. In combination with the lack of differences in anxiety measures, it is not likely that this mild difference affected cognitive behavior. Mild hyperactivity has been reported in this Alzheimer’s disease mouse model, but is not thought to be a determinant of cognitive deficits^{26,27}, and in a related study, vitamin C supplementation given in drinking water (1.0 g/L) improved a hyperactivity

deficit in female J20 mice (bearing Swedish and Indiana mutations of APP) in the Y-maze spontaneous exploration task¹¹. Extreme vitamin C deficiency leads to severe lethargy and low activity²⁸, but agitation and motor disturbances are features of Alzheimer's disease that may be being modeled by the hyperlocomotion in mouse models^{26, 29,30} and it is therefore interesting that the modest decrease in vitamin C contributed to this increased activity in the older cohort of mice.

Low vitamin C impairs performance on the rotarod

We next assessed procedural learning and neuromuscular ability using the rotarod, with 3 trials conducted on each of 2 consecutive days. This task is known to be sensitive to effects of normal aging and as expected, 12 month old mice performed more poorly than younger mice, with shorter latencies to fall or rotate on the equipment. At both ages mice showed improvements on day 2 compared to day 1, indicating intact procedural learning ($F_{s>10.68}$, $p<0.002$). A significant impairment was observed in low vitamin C mice at both ages compared to normal vitamin C mice (5M $F_{1,54}=6.81$, $p=0.012$ Fig. 1E; 12 M: $F_{1,44}=10.096$, $p=0.003$ Fig. 1F). The SVCT2 is expressed³¹ in muscle fibers, and thus transporter deficiency could conceivably lead to weakness. We did not specifically measure the decrease in vitamin C in muscle although the heterozygous mutation, although data from brain and other organs in these mice³², suggests the mutation would result in a similar vitamin C decrease of 30–50%. Muscular weakness in aging is likely a combination of muscular atrophy and neuronal changes, particularly at the neuromuscular junction³³. Low vitamin C in the *gulo*^{-/-} model that cannot synthesize its own vitamin C, and is therefore vulnerable to even greater decrease in vitamin C based on dietary intake, has been shown to impact motor abilities in rotarod and also water maze in mice younger than 5 months of age, further supporting a major role for maintaining vitamin C to support optimal muscular strength^{34, 35}. These mice are not known to have major cognitive deficits, although testing has mostly been limited to mice younger than 5 months of age^{34, 35}. The deficits observed in strength and coordination on the rotarod in the younger cohort of mice suggests that vitamin C during middle age could be critical to maintaining good muscular health in aging.

Low vitamin C and APP/PSEN1 mutations enhance development of lipid peroxidation and decrease antioxidant potential

As expected, cortex vitamin C level was determined by SVCT2 transporter expression, and was up to 30% lower in SVCT2^{+/-} mice in both 6 and 14 month old mice ($F_{1,59}=18.14$, $p<0.001$; $F_{1,42}=30.88$, $p<0.001$, Fig. 4A). APP/PSEN1 genotype had no effect on brain vitamin C level at either age ($F_{s<3.78}$, $p>0.057$). A modest 20–30% decrease in brain vitamin C is likely to be present in a significant number of humans with decreased dietary intake or vitamin C loss. Although direct comparisons with human brain levels are not possible, we have shown that mice on a low, but non-scorbutic, vitamin C deficiency schedule can have much larger decreases of up to 75% from normal brain, with almost undetectable levels in serum and liver, without suffering ill health³⁶. Clinical and population studies of plasma vitamin C routinely report levels in the depleted and deficient range ($<28\text{ }\mu\text{M}$) in 10–15% of subjects, with clinical scurvy reported in some populations^{37, 38}.

Malondialdehyde (MDA) levels followed a significant inverse relationship with brain vitamin C levels such that MDA was higher in mice with low vitamin C in the brain compared to mice with normal vitamin C at both 6 and 14 months ($F_{1,58}=9.89$, $p=0.003$; $F_{1,44}=6.47$, $p=0.015$, Fig. 4B). At 6 months MDA levels were also higher in APP/PSEN1⁺ mice than wild-type ($F_{1,58}=5.19$, $p=0.026$), and although this effect was likely driven by the low value in the wild-type mice compared to the three other groups, there was no interaction between the genotypes ($F_{1,58}=2.52$, $p=0.12$). At 6 months protein carbonylation was also higher in all mutant groups than in wild-type mice as indicated by an SVCT2 x APP/PSEN1 genotype interaction ($F_{1,37}=5.29$, $p=0.027$, Fig. 4C). There were no main effects of either SVCT2 or APP/PSEN1 mutation alone at 6 months ($F_{s1,37}<2.49$, $ps>0.12$) and there were no differences among groups at 14 months ($F_{s1,29}<0.88$, $ps>0.36$). At 6 months, F₂-isoprostanes were highest in APP/PSEN1 mutant mice ($F_{1,37}=4.56$, $p=0.039$, Fig. 4D) with no further effect of SVCT2 genotype ($F_s<0.12$, $ps>0.73$). At 14 months, there were no differences among the groups ($F_s<1.16$, $ps>0.29$). Total glutathione (GSH+GSSG) was measured in cerebellum and was higher in wild-type mice than in other groups at 6 months (interaction $F_{1,35}=7.46$, $p=0.010$; main effects of SVCT2 and APP/PSEN1 alone $F_{s1,35}<0.85$, $ps>0.36$, Fig. 4E). There were no differences among groups in 14-month old mice ($F_{s1,26}<2.28$, $ps>0.14$). The ratio between GSH:GSSG did not differ among the groups at either age ($F_s<1.52$, $ps>0.23$, *data not shown*). Finding adverse changes in this wide range of markers of antioxidant/oxidative stress profile is strongly indicative that oxidative stress is an important driving feature of the other pathological changes observed in the mice. However, it is not the only mechanism that could be impacted by the lower vitamin C in the brain. Inflammatory response, including astrocytic activation, is another pathological change associated with Alzheimer's disease. In this study, inflammatory response was indexed by measuring GFAP in hippocampus by semi-quantitative Western blot in 4 to 8 mice per group. At 6 months there were no differences in GFAP expression among the groups ($F_{s1,14}=1.04$, $ps>0.33$). By 14 months, a larger inflammatory response was observed in the APP/PSEN1 mutant mice, which had greater GFAP protein expression ($F_{1,19}=4.28$, $p=0.05$, Fig. 4F), but there were no further effects of SVCT2 genotype ($F_s<0.40$, $ps>0.54$). Similarly, Murakami et al.¹¹ reported decreased carbonyls following vitamin C supplementation to normal mice, and increased GSH, but no change in GFAP expression.

Low vitamin C enhances amyloid accumulation and deposition

Our initial hypothesis was that elevated oxidative stress would contribute to acceleration of other pathological features of Alzheimer's disease. Total A β_{1-40} and A β_{1-42} levels in hippocampus were very low overall in the younger mice. Nonetheless, some differences were noted according to vitamin C level. Where assumption of equal variances was violated, non-parametric tests (Mann Whitney U test) were employed instead of a two-tailed t-test. Soluble and insoluble A β_{1-40} did not vary solely according to SVCT2 genotype ($ps>0.077$, Fig. 5A), but soluble and insoluble A β_{1-42} were both higher in SVCT2^{+/-}-APP/PSEN1⁺ mice than in APP/PSEN1⁺ mice with normal vitamin C levels (soluble: Mann Whitney U=10, $p=0.04$; insoluble: $t(13)=2.38$, $p=0.033$, Fig. 5C). This increase in A β_{1-42} was also reflected in the ratio of total A $\beta_{1-42/1-40}$, which was increased in SVCT2^{+/-}-APP/PSEN1⁺ mice (Mann Whitney U=7, $p=0.014$, Fig. 5E). Thioflavin-S positive plaque deposits were extremely low at 6 months, and in many mice, none was visible in hippocampus or cortex. Accordingly,

there was no difference according to SVCT2 genotype in either area ($p>0.87$, Fig. 5G, I). At 14 months, $A\beta_{1-40}$ and $_{1-42}$ levels were greatly increased from the previous age point but no longer differed according to SVCT2 genotype ($p>0.53$, Fig. 5B,D), possibly indicating that disease processes had advanced far enough to make it harder to tease apart relatively subtle differences that were evident at an earlier age. The ratio of total $A\beta_{1-42/1-40}$ was also similar between the two groups ($t(10)=0.62$, $p=0.55$). However, there were significantly more thioflavin-S positive plaques observed in SVCT2^{+/-}APP/PSEN1⁺ mice than in APP/PSEN1⁺ mice in both hippocampus ($t(19)=2.66$, $p=0.015$) and cortex ($t(20)=2.42$, $p=0.025$, Fig. 5H, I) indicating that the earlier increase in $A\beta_{1-42}$ may have contributed to more robust amyloid seeding.

Six months of vitamin C supplementation lowered soluble $A\beta_{1-42}$ and the $A\beta_{1-42/1-40}$ ratio, in 12-month old J20 mice, which the authors attributed to an effect on oligomerization¹¹. Our mice had a lifelong decrease in vitamin C, and we noted changes from a much earlier stage of amyloid accumulation, at 6 months, although our data still fit the hypothesis that vitamin C affects amyloid oligomerization. *In vitro*, vitamin C suppressed reactivity of the amyloid- β A11 antibody that recognizes a particular conformation of toxic, prefibrillar $A\beta$ oligomers³⁹. Less specific changes in oxidative stress can also influence factors in the pathways for over-production of amyloid- β (e.g. BACE1 enzymatic function)⁴⁰. Familial forms of Alzheimer's disease are more typically associated with increased amyloid- β production, whereas sporadic Alzheimer's disease is more likely to implicate failed clearance mechanisms. The altered $A\beta_{1-42/40}$ ratio in young mice and increased plaques at 12 months suggest that vitamin C could be involved in both processes. Gulo^{-/-} mice lack the ability to synthesize vitamin C, and like humans, require supplementation for survival⁴¹. In Gulo^{-/-} mice crossed with the 5XFAD Alzheimer's model, high dose (3.3g/L) supplemented mice showed less plaque deposition, and less GFAP immunoreactivity than mice with a lower, although not deficient, supplementation level (0.66g/L)¹⁰. 5XFAD mice had disrupted cerebral capillaries in the vicinity of plaques, but this effect was lessened with the very high vitamin C supplementation. *In vitro* vitamin C tightens endothelial cell junctions⁴², which may be another mechanism by which vitamin C is beneficial in Alzheimer's disease, and one that requires closer attention given the co-morbidity with cardiovascular disease and dementia.

One further potential role for vitamin C deficiency in the cognitive decline observed in this study is that SVCT2^{+/-}APP/PSEN1⁺ mice appear more susceptible to pharmacologically-induced seizures and have a higher mortality rate than the other genotypes⁴³. There is a known association between APP mutations and seizures⁴⁴⁻⁴⁶. Seizures can independently induce additional β -amyloid production, as well as cognitive deficits, and also significantly increase oxidative stress⁴⁷⁻⁵⁰. It is, therefore, possible that unobserved seizures while in the home-cage could have contributed to the pathologies reported here, but such a relationship would have to be determined specifically in future studies.

In our study, mice with low vitamin C, whether carrying APP/PSEN1 mutations or not, were exposed to potential oxidative imbalance levels for their entire lives. We found detectable oxidative stress increases by 6 months of age. The same changes were evident in wild-type mice by 14 months of age, representing a much shorter duration of oxidative imbalance in

those mice. The finding of learning and memory deficits in normally-aging SVCT2^{+/-} mice, with deficits starting at just 5 months of age, also suggests that avoiding deficiency may be more useful in the prevention of cognitive decline, but does not rule out a role for supplementation to maintain a maximal or optimal level during aging. It is likely that different pathways are implicated in the damage seen in the mice with and without amyloid accumulation, however, it is likely that each pathway involves oxidative imbalance, either directly or indirectly. The extent to which specific changes are synergistic, or additive, within the pathological framework of Alzheimer's disease rather than normal aging, remains to be established. Dietary treatments with vitamins C, E and other antioxidants have been shown to rescue memory impairments in rodents with oxidative stress and learning deficits due to APP and PSEN1 mutations, melamine treatment, and hypoxia⁵¹⁻⁵⁴. Similarly, vitamin E supplementation to Tg2576 mice decreased oxidative stress in the brain and decreased A β ₁₋₄₀ and A β ₁₋₄₂ levels, but the latter effects were found only when supplements were started before 6 months of age, not in an older cohort that were treated from 14 months⁵⁵. Six months of a medical food cocktail containing vitamins C and E, among several other constituents, also decreased soluble and insoluble A β ₁₋₄₀ as well as soluble A β ₁₋₄₂ in Tg2576 mice⁵⁶. Three months of a combination diet combining antioxidants (including vitamins C and E), plus a number of items specifically designed to stimulate synaptic membrane formation, decreased both A β ₁₋₄₀ and A β ₁₋₄₂ in APP/PSEN1⁺ mice at 6 months of age⁵⁷. Finally, the antioxidant resveratrol decreased plaque deposition in Tg19959 mice when treatments were begun at 45 days⁵⁸.

The range of behavioral measures used in the present study was designed to tap into a number of specific brain areas; hippocampal tasks of spatial learning, amygdala-dependent cue-testing in the conditioned fear task, striatal-dependent locomotor activity, and cerebellar-dependent procedural learning on the rotarod. Although the focus of Alzheimer's disease-related studies more typically center on hippocampal and cortical tasks, owing to the concentration of amyloid pathology in those areas, vitamin C is high, and preferentially preserved, in each of these brain areas. The behavioral and biochemical data suggest that antioxidant status is critical across a number of brain areas, and that each may contribute to the behavioral changes observed in aging and Alzheimer's disease. Given that not all brain areas under oxidative stress are also associated with high amyloid load, the lack of function cannot solely be attributed to increased amyloidogenesis. Other potential causes of functional decline include cell death, impaired synaptogenesis and neurotransmitter function, and the specific role of vitamin C deficiency in each of these has yet to be shown.

Data from human clinical trials of antioxidant supplementation seldom show clear efficacy against the clinical manifestation of Alzheimer's disease (such as A β ₁₋₄₂ levels, or cognitive decline⁵⁹, although supplements do increase vitamin levels (C and E) and decrease measures of oxidative stress in CSF after only one month of supplementation^{60, 61}. Unfortunately, such studies seldom make comparisons between deficient and replete states; they are typically conducted in subjects already suffering from mild to moderate Alzheimer's disease, and are often limited in the number of measures that may be taken to assess cognition and biochemical changes. That dietary antioxidants can ameliorate the oxidative state *in vivo* has been born out many times^{60, 61}, but the effects of prolonged non-

scorbutic deficiency of vitamin C (and other antioxidants) beginning before or with disease development, has not yet been adequately tested in clinical populations. The findings reported here suggest that greater impetus needs to be given to dietary control to avoid deficiency in early to mid-adulthood, rather than late-life supplementation when disease processes are much more firmly established. Amyloid- β is detectable in brains of cognitively normal individuals as early as their mid-thirties⁶².

Summary and Conclusions

Combined, these data suggest that chronic hypovitaminosis for vitamin C may accelerate the development of oxidative stress in the brain during normal aging, and also has a role in amyloid production, oligomerization and/or deposition. Of particular note is that the greatest effects of both APP/PSEN1 mutations and low vitamin C on oxidative stress and amyloid- β were observed before 6 months of age. In APP/PSEN1 mice this represents a very early stage of disease pathogenesis, before significant amyloid production or accumulation, and before a large inflammatory response has been triggered. By 14 months, group differences in oxidative stress levels were less distinguishable, presumably eclipsed by normal changes due to aging that occur even in the wild-type mice. We conclude therefore that vitamin C deficiency can play a critical role in protection against both Alzheimer's neuropathology and normal aging, but that greater attention should be paid to nutritional intakes in early middle-age rather than waiting for later life interventions.

Methods

Animals—All animals were housed in a temperature and humidity controlled vivarium and were kept on a 12:12 hour light cycle. All procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee. Female C57Bl/6J wild-type mice (<http://jaxmice.jax.org/strain/000664.html>) and male bigenic APP_{SWE}/PSEN1^{E9} mice (<http://jaxmice.jax.org/strain/005864.html>) were obtained from Jackson Laboratories and used to found the colonies used in this study. SVCT2^{+/-} mice have decreased expression of the SVCT2 transporter and 20–30% decreased brain vitamin C levels although they retain the ability to synthesize vitamin C, and peripheral SVCT2-dependent tissue contents are within 50–90% of SVCT2^{+/+} littermates¹⁸. These mice were originally obtained from Dr. Robert Nussbaum. They were backcrossed at least 10 generations to the C57Bl/6J strain and maintained on that same background. The total numbers of mice available for behavioral and biochemical studies for each group are presented in Table 1. For simplicity we use the term “wild-type” to denote mice that do not carry the mutations APP and PSEN1. Mice that are wild-type for SVCT2 will be described using “SVCT2^{+/+}” or by describing their vitamin C levels (normal vitamin C versus low vitamin C in the SVCT2^{+/-} mice).

Behavioral testing

All behavior testing was undertaken using facilities of the Vanderbilt Murine Neurobehavioral Core. The experimental design is shown in Figure 6. In addition to the planned cognitive testing, a series of control tasks for anxiety (elevated plus maze), locomotor activity, and neuromuscular ability (rotarod), were also performed. If behavior in

these control tasks were affected either by APP/PSEN1 mutations, or by vitamin C deficiency, it could confound interpretation of learning and memory tasks (e.g. extreme anxiety and hypolocomotion would limit exploration of a novel area and diminish learning potential).

Elevated Zero Maze—Anxiety was measured using a standard Elevated Zero Maze (San Diego Instruments, CA). A single 5-minute trial was filmed from above, and the exploration paths in open and closed zones were analyzed using AnyMaze (Stoelting Co. IL).

Locomotor activity—Activity was measured on two consecutive days in standard locomotor activity chambers (approx. 30 x 30 cm, ENV-510; MED Associates, Georgia, VT, USA). Activity was recorded automatically for 15 minutes by the breaking of infrared beams.

Rotarod—Motor coordination and balance were tested using a commercially available accelerating rotarod (Ugo Basile model 7650; Stoelting Co., Wood Dale, IL, USA) as described³⁴. The time taken for the mouse to rotate on the rod (clinging to the rod and rotating along with it instead of remaining on top) and/or to fall, were recorded with a maximal trial duration of 300 s. Three trials were given per day, on 2 consecutive days.

Y-maze—Spontaneous alternation was measured in a single 5-minute trial in a standard Y-maze made of clear acrylic tubing, with arms 32 cm long³⁴. Alternation was defined as consecutive entries into three different arms (e.g. ABC, BCA).

Olfactory learning—Olfactory learning was undertaken to assess 24-hour recall of a familiar scent, based on the methods described in⁶³. On the initial test day mice were given two 3-minute trials in which they were exposed to a 2.5 X 2.5 cm square of filter paper moistened with either water or a scent (designated the ‘familiar’ odor). Olfactory cues were cherry, almond, or vanilla (diluted 1/400 in water, McCormick & Co., Inc, MD). On the second day of testing mice were again given two trials, one with the familiar odor and one with a novel odor. Olfactory cues and test order were randomized. Each trial was recorded and later scored by two trained observers for number of visits to the scented paper, and the time spent investigating the paper. Decreasing investigation time of the familiar odor on day 2 compared to day 1, and preference for the novel odor over the familiar odor on day 2 were used to index memory of the familiar odor. This task was only used at 5 months due to lower exploratory activity and the potential for loss of olfactory ability in the older mice.

Water maze—Water maze testing was conducted in a 107-cm diam. pool with a circular, acrylic platform (10 cm diam.) in equipment as described³⁴. For cued-platform testing the platform surface was visible above the water and a marker, visible to the mice while swimming, was inserted into the platform. For hidden platform testing the water was rendered opaque through the addition of non-toxic white paint and the platform was submerged 1 cm below the water. Mice were given four acquisition trials per day (max. 60 s each) for cued trials (3 days), and hidden platform acquisition (8 days). Sessions were captured by an overhead camera and analyzed using AnyMaze (Stoelting Co. IL). Twenty-four hours following the final training trial a 60-s probe trial was conducted. The time spent

in the target and non-target quadrants, number of crosses of the platform location, and time spent within 20 cm of the platform edge were the primary dependent measures derived from the probe trial. Swim speed and peripheral swimming (time within 10 cm of the pool wall) were also assessed to determine whether differences in performance could be attributed to non-cognitive factors. This protocol was employed to ensure that mice from both age groups, and all genotypes, would have sufficient opportunity to learn the platform location. Anxiety associated with this task can impact learning ability⁶⁴, and pre-training with the visible version helps to reduce anxiety through repeated exposure, and also serves to introduce the animals to the rule that escape from the maze is possible following location of a platform. The 8-days of acquisition testing ensures that even at the older age group, mice are able to learn the spatial task, and thus, probe testing for memory is possible (which it is not if all mice are not given sufficient opportunity to learn the location of the platform).

Fear Conditioning—Fear conditioning was carried out with two specialized chambers and computer software (Med Associates Inc. USA). Mice were placed in conditioning chambers that had a plexiglass door, metal walls and a metal grid floor through which a shock could be delivered. These were housed within sound attenuating chambers. During the initial training trial mice learned to associate a 30 s shock with a 2 s electric shock (0.5 mA). There were three tone-shock pairings during the 8-minute trial. Twenty-four hours later, mice received a context-retrieval trial in which they were placed in the same testing chamber as was used the day before and left undisturbed for 4 minutes before being returned to the home cage. One hour later, the context was altered by placing a white plastic, curved ‘wall’ and floor into the chambers, along with a dish containing 1 ml of vanilla flavoring (McCormick, USA). Mice were tested in the chamber that they had not previously been tested in. For each trial cameras mounted to the inside of the door of the outer containment box and computer software scored the mice for the amount of time spent freezing (remaining immobile). As a final control measure to ascertain whether genotypes were equally sensitive to the shocks, mice underwent shock threshold testing. They were exposed to a series of 1 second shocks of increasing intensity (0.075 to 0.5 mA). Their response (flinch, run, jump and vocalize) was noted for each shock value. The trial was ended and no further shocks were given once the mouse had vocalized at a particular shock strength.

Biochemical testing—Following terminal anesthesia with isoflurane and cervical dislocation, the tip of the tail was cut off and saved for genotyping. Mice were then decapitated and brains were quickly removed and hemisected sagittally. One hemisphere was immersion-fixed in 10 % formalin for 3 days, then removed to a 10 % sucrose solution and stored at 4°C. The remaining brain was dissected into cortex, hippocampus, and cerebellum. All samples were frozen on dry ice and stored at –80°C.

Ascorbic acid—Vitamin C (ascorbic acid) was measured by HPLC with electrochemical detection as described previously³⁴. Values were calculated per gram tissue wet weight.

Malondialdehyde (MDA)—MDA was analyzed as thiobarbituric-reactive substances as described previously⁶⁵. Values were calculated per milligram tissue wet weight.

Isoprostanes—Isoprostanes were determined by GC-MS in the Vanderbilt Eicosanoid Core Facility, using previously described methods ⁶⁶.

Protein carbonyls—Protein carbonyls were determined by reaction with DNPH using previously described methods ^{67, 68} with values calculated per mg protein.

Glutathione—Total glutathione (reduced glutathione (GSH) and oxidized glutathione (GSSG)) were measured using previously described methods ⁶⁹.

ELISA (soluble/insoluble A β ₁₋₄₀ and A β ₁₋₄₂)—A β levels were quantified using anti-human A β ₁₋₄₀ and A β ₁₋₄₂ sandwich ELISA kits, according to the manufacturer's instructions (Invitrogen Corporation, Camarillo, CA; cat. # KHB3481 & KHB3441).

Western Blotting—GFAP was detected using previously described methods ⁷⁰. Incubation with primary antibody - 1:1,000 anti-GFAP (Cat.#MAB360, Millipore, Bedford, MA) diluted in blocking buffer (5% milk, TBS-0.1% Tween 20), occurred overnight at 4°C with shaking. The membrane was then washed with TBS-Tween-20 and incubated with secondary antibody – 1:20,000 anti-mouse IgG-HRP (Promega, Madison, WI) for 1 hour at room temperature before detection with chemiluminescence (Perkin Elmer, Waltham, MA).

Thioflavin S—Sections (30 microns thick) were cut from the formalin-fixed hemi-brain using a benchtop sliding microtome (Leica) on which the brains were frozen with dry ice. Sections were floated in 24-well plates containing 1xPBS and then mounted on gelatin-coated, charged glass slides. Three to five sections per mouse, containing hippocampus and cortex and spaced approximately 100 microns apart were chosen for quantification of thioflavin-S (Sigma Aldrich, USA) positive plaques as described previously ^{8, 71}. Digital images of the hippocampus and overlying cortex were taken using a fluorescent imaging microscope (EVOSfl, AMGmicro) at a magnification of 4X. Separate images were stitched together in Adobe Photoshop and the area of the hippocampus and overlying cortical areas occupied by amyloid plaques was determined using the freely-available Image J software (National Institute of Health, Bethesda, MD, USA). Quantification was performed by an experienced researcher who was blind to the genotype of the mice. Plaque coverage was calculated as percent of total region measured, in pixels.

Statistics—Data were first checked for normality, skew and outliers (greater than two standard deviations above or below the mean). Where necessary, data were transformed with log₁₀ transformation, or analyzed using non-parametric analyses as described in results. All analyses were first run with sex as a fixed variable. There were no significant differences according to sex so all data were collapsed and analyzed together. Normality testing, ANOVA and t-test were analyzed using SPSS 19.0 for MAC. Single factor (2x2) ANOVA was conducted with SVCT2 genotype (SVCT2^{+/-}, SVCT2^{+/+}), and APP/PSEN1 genotype (wild-type, APP/PSEN1⁺) as the between-groups variables. Behavioral tests with multiple trials were analyzed with Repeated Measures ANOVAs with the same between-groups factors as above. Non-parametric testing was done in Graphpad Prism 5 for Mac OS X.

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Abbreviations

SVCT	sodium-dependent vitamin C transporter
APP	amyloid precursor protein
PSEN1	presenilin 1
MDA	malondialdehyde

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Figure 1. Learning and memory tasks

At 5 months of age (5M) SVCT2^{+/-}-APP/PSEN1⁺ mice were the only group to show no decrease in interest of the familiar odor on the second day of testing (24 hour recall) (A, left). Only wild-type mice showed evidence of a significant preference for the novel over the familiar odor on day 2. In contrast, SVCT2^{+/-}-APP/PSEN1⁺ mice spent significantly less time exploring the novel odor than the familiar odor (A, right). At 5 months of age, mice carrying APP/PSEN1 mutations were impaired on the Y-maze alternation task regardless of vitamin C status (B), whereas at 12 months of age (12M) impairments were seen in SVCT2^{+/-} and SVCT2^{+/-}-APP/PSEN1⁺ mice compared to mice with normal vitamin C levels (C). The older cohort of mice was also tested in the conditioned fear paradigm. All groups spent similar time freezing during the 4-minute trial in the original training context (D). However, mice carrying APP/PSEN1 mutations spent less time freezing in a new context, when they heard the tone that had been the conditioned stimulus, indicating impaired cue recall in these mice (E). Data shown are mean +S.E.M. $p < 0.05$ t-test between exploration of odors (time in seconds, log₁₀ transformed) on two separate trials (left: familiar on day 1 versus day 2, right: novel versus familiar on day 2); * $p < 0.05$, main effect of SVCT2^{+/-} compared to SVCT2^{+/+}, or main effect of APP/PSEN1⁺ compared to APP/PSEN1⁻.

Figure 2. Morris water maze learning

Mice were trained on the hidden version of the water maze for 8 days to ensure that all mice had been given sufficient trials to learn the location of the hidden platform (**A, E**).

Swimming locations during the no-platform probe trial were recorded and analyzed by group according to quadrant (target versus non-target), time spent within 20 cm of the platform edge, and crosses of the platform location, as depicted in (**I**). At both 5 months (5M) and 12 months (12M), each group showed some degree of preference for swimming in the target quadrant, although these preferences were clearer in mice that did not carry APP/PSEN1 mutations (**B, F**). Time spent in the 50 cm-diameter zone around the platform (20 cm from platform edge) was lower in APP/PSEN1 mutant mice at 5 months (**C**), but did not

differ according to group at 12 months (**G**). Number of crosses of the platform location was similar across groups at 5 months (**D**), but was significantly lower in all mice with low vitamin C (SVCT2^{+/-}) at 12 months of age (**H**). Data shown are mean +S.E.M. $p < 0.05$ non-target quadrants compared to target quadrant for each group; * $p < 0.05$, Main effect of SVCT2^{+/-} compared to SVCT2^{+/+}, or main effect of APP/PSEN1⁺ compared to APP/PSEN1⁻.

Figure 3. Activity, anxiety and neuromuscular co-ordination

Locomotor activity was tested in two 15-minute sessions on two consecutive days (D1, D2). At 5 months of age (5M) there were no differences according to genotype (**A**), but at 12 months (12M) SVCT2^{+/-} mice were slightly hyperactive compared to SVCT2^{+/+} mice with normal vitamin C levels (**B**). Performance on the elevated zero maze was similar among groups at 5 months (**C**), but at 12 months SVCT2^{+/-} mice traveled further than SVCT2^{+/+} mice (**D**). Performance on the accelerating rotarod, tested across 2 days (D1, D2), was significantly poorer in SVCT2^{+/-} mice than SVCT2^{+/+} at both 5 months (**E**) and 12 months of age (**F**). Data shown are mean +S.E.M. * $p < 0.05$, ** $p < 0.01$, main effect of SVCT2^{+/-} compared to SVCT2^{+/+}.

Figure 4. Measures of oxidative stress, antioxidant status and neuroinflammation

Mice that had undergone behavioral testing were sacrificed at 6 months (6M: “5-month” group) and 14 months of age (14M: “12-month” group). Lacking one copy of the SVCT2 successfully lowered vitamin C in the brains of SVCT2^{+/-} mice (A). Low vitamin C significantly increased MDA at both ages, and at 5 months, MDA was also higher in APP/PSEN1⁺ mice (B). Protein carbonyls were also higher in all groups compared to wild-type mice at 6 months, but by 14 months of age the levels of carbonyls were similar regardless of genotype (C). F₂-isoprostanes were increased in mice carrying APP/PSEN1 mutations, but only in 6-month old mice (D). Total glutathione levels were higher in wild-type mice than all other groups at 6 months, but no further differences were seen at 14 months (E). APP/PSEN1 mutations increased GFAP expression as detected by Western blot in 14-month old mice, although no differences were apparent in the younger cohort (F). Data shown are mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001, Main effect of SVCT2^{+/-} compared to SVCT2^{+/+}; +p<0.05, main effect of APP/PSEN1⁺ compared to APP/PSEN1⁻; p<0.05 different from wild-type controls according to pairwise comparisons following significant interaction between genotypes in the Univariate ANOVA (comparisons between APP/PSEN1⁺ and wild-type at each level of SVCT2, and between SVCT2^{+/-} and SVCT2^{+/+} within APP/PSEN1⁺ or wild-type mice).

Figure 5. Measurement of amyloid-β

Amyloid-β levels are only reported in mice that carry APP/PSEN1 mutations. At 6 months of age (6M), soluble and insoluble amyloid-β₁₋₄₀ and amyloid-β₁₋₄₂ were very low, and insoluble amyloid proteins of both lengths were more numerous in mice with low vitamin C (**A**, **C**). This relationship was confirmed by the higher ratio of total amyloid-β₁₋₄₂ /amyloid-β₁₋₄₀ in SVCT2^{+/-}APP/PSEN1⁺ mice with low brain vitamin C (**E**). In the older cohort that was sacrificed at 14 month of age (14M), amyloid-β₁₋₄₀ and amyloid-β₁₋₄₂ levels were much higher than in the younger mice, but did not differ according to group (**B**, **D**). Neither did the ratio of total amyloid-β₁₋₄₂ /amyloid-β₁₋₄₀ differ according to vitamin C level at that age (**F**). Thioflavin-S-positive plaques were infrequent and small in 6-month old mice and coverage did not vary according to vitamin C level (**G**). By 14 months of age plaque deposits were far more numerous and were also significantly greater in SVCT2^{+/-}APP/PSEN1⁺ mice compared to APP/PSEN1⁺ mice with normal vitamin C transport (**H**). Data shown in panels **A** to **F** are mean +S.E.M. Data shown in panels **G** and **H** are medians,

+quartiles, with maximum and minimum group values represented by whiskers. $*p<0.05$, SVCT2^{+/-}APP/PSEN1⁺ compared to SVCT2^{+/+}APP/PSEN1⁺ mice. Example images of thioflavin-S stained sections are given in (**I**). Panels Ii and Iii show representative sections of the average percent coverage values for SVCT2^{+/-}APP/PSEN1⁺ and SVCT2^{+/+}APP/PSEN1⁺ mice at 6 months of age. Panels Iiii and Iiv depict representative sections of the average coverage for 14-month-old mice.

Figure 6. Experimental design

Behavioral testing was begun at 5 months, or 12 months of age. Mice were sacrificed for biochemical measures following behavioral testing, at 6 months, or 14 months of age.

Table 1

Total numbers of mice included in behavioral studies.

	5 months – behavior 6 months - neurochemistry		12 months – behavior 14 months - neurochemistry	
	APP/PSEN1-	APP/PSEN1+	APP/PSEN1-	APP/PSEN1+
SVCT2^{+/-} Low vitamin C	“SVCT2 ^{+/-} ” 8 male, 7 female	“SVCT2 ^{+/-} APP/PSEN1 ^{+/+} ” 7 male, 5 female	“SVCT2 ^{+/-} ” 6 male, 5 female	“SVCT2 ^{+/-} APP/PSEN1 ^{+/+} ” 7 male, 5 female
SVCT2^{+/+} Normal vitamin C	“Wild-type” 7 male, 12 female	“APP/PSEN1 ^{+/+} ” 10 male, 8 female	“Wild-type” 8 male, 13 female	“APP/PSEN1 ^{+/+} ” 9 male, 5 female

Group names are given above group distributions. Not all mice were included in all biochemical tests due to quantity of brain samples available for analyses.