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Vitamin C deficiency increases basal exploratory activity but decreases scopolamine-induced activity in APP/PSEN1 transgenic mice

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ABSTRACT

Vitamin C is a powerful antioxidant and its levels are decreased in Alzheimer's patients. Even sub-clinical vitamin C deficiency could impact disease development. To investigate this principle we crossed APP/PSEN1 transgenic mice with Gulo knockout mice unable to synthesize their own vitamin C. Experimental mice were maintained from 6 weeks of age on standard (0.33 g/L) or reduced (0.099 g/L) levels of vitamin C and then assessed for changes in behavior and neuropathology. APP/PSEN1 mice showed impaired spatial learning in the Barnes maze and water maze that was not further impacted by vitamin C level. However, long-term decreased vitamin C levels led to hyperactivity in transgenic mice, with altered locomotor habituation and increased omission errors in the Barnes maze. Decreased vitamin C also led to increased oxidative stress. Transgenic mice were more susceptible to the activity-enhancing effects of scopolamine and low vitamin C attenuated these effects in both genotypes. These data indicate an interaction between the cholinergic system and vitamin C that could be important given the cholinergic degeneration associated with Alzheimer's disease.

1. Introduction

Alzheimer's disease is a neurodegenerative disorder that leads to deficits in learning and memory, altered personality, and ultimately death. There are up to 5.2 million sufferers in the United States including half a million under the age of 60 and it is incurable (Alzheimer's Association (2008)). Nevertheless, lifestyle factors such as diet, exercise, and cognitive activity can delay the onset of symptoms (Mattson, 2000). One method suggested to slow the development or progression of the disease is an increase in dietary antioxidants, including vitamin C. Oxidative stress is created by an imbalance in the body's antioxidant defenses and the production of free radicals and is elevated in Alzheimer's disease (Nunomura et al., 2007). Furthermore, plasma levels of the antioxidant vitamin C are lower than normal in Alzheimer's patients, regardless of intake (Charlton et al., 2004; Riviere et al., 1998). Increased vitamin C intake from the diet or supplement form has been shown in some studies to lower the risk of developing Alzheimer's disease (Engelhart et al., 2002; Morris et al., 1998), and Alzheimer's patients with higher baseline CSF/plasma vitamin C ratios exhibit slower disease progression over the course of one year (Bowman et al., 2009). Other studies found no such associations (Gray et al., 2008; Laurin et al., 2004; Luchsinger et al., 2003; Paraskevas et al., 1997).

The brain is particularly susceptible to oxidative stress due to its large oxygen turnover, which leads to the generation of oxygen free radicals. The brain is also rich in polyunsaturated fatty acids and thus especially at risk from the effects of lipid peroxidation as well as oxidative damage to both proteins and oligonucleotides. The putative benefits of Vitamin C to Alzheimer neuropathology are lower oxidative stress in the brain, decreased amyloid aggregation, or both. The neurotoxic amyloid-β (Aβ) peptide is the primary constituent of the neuritic plaques that aggregate in the brains of Alzheimer's patients. Aβ is often considered a primary pathogenic agent in the disease, although greater focus is now also being devoted to other pathogenic features including oxidative stress (Castellani et al., 2006). Aβ is thought to act synergistically with oxidative stress to influence the development of neuritic plaques and plaque-related neuropathology. *In vitro*, oxidative stress has been shown to stimulate β-secretase (BACE1) activity, increasing production of pathogenic $A\beta_{40}$ and $A\beta_{42}$ variants (Tamagno et al., 2008). In turn, small Aβ oligomers act at the mitochondrion to increase production of reactive oxygen species and induce further oxidative damage (Butterfield et al., 2001; Mark et al., 1996; Pappolla et al., 1998; Perluigi et al., 2006; Uberti et al., 2007). Both Aβ and oxidative stress are toxic to neurons (Manelli

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and Puttfarcken, 1995; Resende et al., 2008), and have been shown to impair memory in an acute, drug-like fashion in the absence of Aβ aggregation (McDonald et al., 1994; McDonald et al., 1996; Sweeney et al., 1997). Accordingly, administration of antioxidants such as vitamin C can reverse memory deficits resulting from a number of insults. Dietary vitamin C supplements lowered oxidative stress in the brain in APP/PSEN1 mice, and in combination with a low dose of vitamin E improved cognitive function in the water maze (Harrison et al., 2009c). Vitamin C administered intraperitoneally has been shown to attenuate memory deficits due to age, hippocampal lesion, and the muscarinic receptor antagonist scopolamine (Arzi et al., 2004; de Angelis and Furlan, 1995; Harrison et al., 2009a; Harrison et al., 2009b; Parle and Dhingra, 2003). This may be attributable to the demonstrated ability of vitamin C to inhibit acetylcholinesterase (Dhingra et al., 2006). Pomegranate juice, a rich source of vitamin C and polyphenol antioxidants, improved performance in the water maze and lowered soluble $A\beta_{42}$ and Aβ plaque formation in the hippocampus (Hartman et al., 2006). In addition to reducing the number of apoptotic neurons, the antioxidant melatonin improved cholinergic function, reduced Aβ deposits, and rescued the memory deficits of mice overexpressing amyloid precursor protein (APP) (Feng et al., 2004). These results suggest that vitamin C acts in a common pathway critical to maintaining a good cognitive function, and may be particularly relevant to Alzheimer-related dementia given its interactions with Aβ and cholinergic neurotransmission.

Low Vitamin C levels do not impair cognitive function in mice lacking L-gulono-γ-lactone oxidase (Gulo; EC 1.1.3.8), despite increased oxidative stress in the cortex and cerebellum (Harrison et al., 2008). Unlike wild-type mice, Gulo−/− mice lack the ability to synthesize vitamin C from glucose, and depend on dietary supplements for survival. In the present experiment Gulo−/− mice were bred with APP/PSEN1 transgenic mice overexpressing APP and presenilin 1 (PSEN1), a bigenic mouse model of Aβ plaque formation that normally exhibits elevated oxidative stress (Bernardo et al., 2008). The mice were maintained on dietary ascorbate supplements that were sufficient for healthy growth, or a reduced level that was sufficient to prevent scurvy. Long-term low levels of vitamin C were expected to lead to increased oxidative stress and a concomitant exacerbation of memory deficits and Aβ aggregation in the transgenic mice compared to adequately-supplemented transgenic and non-transgenic mice.

2. Methods

2.1. Subjects

Gulo−/− mice were bred in-house from heterozygous Gulo+/− mice obtained from the Mutant Mouse Regional Resource Centers (www.mmrrc.org, stock #000015-UCD) and were maintained on a C57BL/6J background (Jackson Laboratories, USA stock #000664). APP_{Swe}/PSEN1ΔE9 bigenic mice were obtained from Jackson Laboratory (Bar Harbor, USA; stock #004462) and maintained as double hemizygotes by crossing with wild-type individuals on a B6C3F1/J background strain (Jackson Laboratories stock # 100010). APP/PSEN1 bigenic mice harbor the Swedish double-mutation (K595N/M596L) in the APP gene, and a deletion in exon 9 of the PSEN1 gene. The mice exhibit amyloid aggregation and amyloid-related neuropathology and cognitive decline, and age- and genotype-dependent cholinergic dysfunction (Bernardo et al., 2008; Lalonde et al., 2005; Machova et al., 2008; Reiserer et al., 2007). Gulo−/− mice have normal cognition even when maintained for many weeks on very low (0.033 g/L) levels of vitamin C (Harrison et al., 2008). Mice were group-housed by gender in standard tub cages (26.5×17×12 cm) with fiber bedding under a 12/12-h light/dark cycle (lights on at 0600 h), with free access to food and water. Mice were housed in a colony room in the same suite as the behavioral testing rooms. All procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Breeding schedule

All APP/PSEN1 transgenics were hemizygous for both APP and PSEN1, and all breedings paired a mouse wild-type for both transgenes and a mouse hemizygous for both transgenes. Gulo−/− homozygous mice (APP/PSEN1 wild-type) were bred with APP/PSEN1 mice (wild-type for Gulo). All offspring were therefore Gulo+/− and either APP/PSEN1+ or APP/PSEN−. Breeding pairs were then established with one Gulo+/−APP/PSEN1+ parent and one Gulo+/−APP/PSEN1− parent. Offspring were obtained in the expected Mendelian ratios. Only Gulo−/−APP/PSEN1− (Knockout–Wild–type; KO–WT) and Gulo−/−APP/PSEN1+ (Knockout–Transgenic; KO–Tg) mice were used for behavioral testing. A small number of Gulo+/+APP/PSEN1− (Wild-type–Wild-type; WT–WT) and Gulo+/+APP/PSEN1+ (Wild-type–Transgenic; WT–Tg) mice were kept to assess the effect of the Gulo knockout on oxidative stress. Genotypes were confirmed by polymerase chain reaction analysis of tail biopsies at 21 days of age and again at the end of the experiment with a new tail sample collected after sacrifice. Both the C57BL/6J and B6C3F1/J strains are well characterized both by our lab and others and although the combination of two background strains may bring out characteristics not apparent in either strain individually, all of the mice in the present study were from the same background combinations. Both parents were Gulo+/− and either APP/PSEN1+ or APP/PSEN1−. Therefore, all of the mice used in the study groups were selected from littermates and any strain characteristics balanced across groups.

2.3. Ascorbic acid treatments

Gulo−/− mice depend on dietary supplements of vitamin C to maintain levels high enough to prevent scurvy. Drinking water was supplemented with either 0.33 g/L (standard; STD) or 0.099 g/L (reduced, RED) ascorbate. A low supplementation level of 0.033 g/L is sufficient to maintain supra-scorbutic vitamin C levels and allow healthy weight gain with age in C57BL/6J mice (Harrison et al., 2008; Parsons et al., 2006). However, pilot testing with this dose revealed that KO–Tg mice, but not KO–WT mice, lost weight and became sick; thus the vitamin C in the water was increased threefold to 0.099 g/L for the reduced vitamin C group. Treatments were given in deionized water with 20 μL 0.5 M EDTA/L to increase stability of the vitamin C in solution. Water bottles were filled with fresh supplemented water twice each week. Experimental mice were derived from Gulo+/− dams capable of synthesizing their own vitamin C; however, prenatal vitamin C levels may be an important determinant of motor abilities in adulthood (Harrison et al., 2008). Therefore, all breeding pairs received the standard level of vitamin C supplementation to ensure an adequate supply to the mother and pups during all stages of development. All mice up to 6 weeks of age were maintained on the standard treatment level. At 6 weeks of age mice were pseudo-randomly assigned to either STD (0.33 g/L) or RED (0.099 g/L) treatment groups. Behavioral testing was carried out from 4 months of age and cognitive testing began at 6 months of age, with a minimum of 25 KO–WT mice and 15 KO–Tg mice in each treatment group. Approximately equal numbers of male and female mice were used in each group.

3. Behavioural procedures

3.1. Exploratory locomotor activity

Locomotor activity was assessed in commercially-available activity monitors (ENV-510; MED Associates, Georgia, VT), as previously described (McDonald et al., 1998; Siesser et al., 2006). Activity was automatically recorded by the breaking of infrared beams as the mouse explored the chamber, and analyzed using a Windows-based

computer. Each session lasted 10 min, and the chambers were cleaned with a 10% alcohol solution between each session. Four activity sessions were conducted to assess memory for the testing environment, and each mouse was tested in the same activity monitor on every trial. Two sessions were conducted on 2 consecutive days when mice were 4 months old, followed by two sessions on consecutive days at 6 months of age.

Implicit memory for context and procedure can be measured by comparing performance between sequential test sessions using methods modified from the original work on memory savings by Ebbinghaus (1885) and Platel and Porsolt (1982). In the present study, the change in activity over time (habituation) was used to infer memory of the environment. Typically, activity will decrease from one test session to the next until a baseline level of activity is reached. Habituation was calculated as previously described (Harrison et al., 2008). Day-to-day habituation was measured by taking the difference in distance (d) traveled between sessions 1 and 2, and 3 and 4 ($d1-d2$ and $d3-d4$). Memory savings between the second and third test sessions, which were conducted 8 weeks apart, were calculated using the following formula: $[100\cdot(d1-d3)/(d1-d2)]$. Using this formula, the change in activity across test sessions can be compared between groups regardless of initial activity levels. An increase in activity between the second and third sessions indicates a 'loss' of the initial habituation and is reflected in a savings score of <100%. Inversely, equivalent or reduced activity on the third session (savings score of >100%) indicates that all of the habituation processes have been 'saved' and the habituation process is either continuing or baseline activity levels have been reached.

3.2. Anxiety

APP/PSEN1 bigenic mice exhibit slightly decreased anxiety in some studies (Lalonde et al., 2004; Reiserer et al., 2007) but not in others (Bernardo et al., 2008). Ascorbate supplementation did not alter anxiety levels in Gulo−/− mice (Harrison et al., 2008). However, anxiety can significantly confound cognitive tests if one group of animals differs systematically from another (Holmes et al., 2002).

3.2.1. Elevated plus maze

Mice were tested using a standard elevated plus maze to assess anxiety via differential exploratory tendencies in enclosed versus open arms, as previously described (Reiserer et al., 2007). At the beginning of a session, mice were gently placed in one of the open arms facing the central area, and allowed to explore freely for 5 min. Sessions were recorded and analyzed using NIH Image software on a Macintosh computer, running a macro specially written to collect data about the mouse's position on the maze throughout the trial (Miyakawa et al., 2001a). The time spent in closed arms was calculated as the percent of total time on arms, excluding time in the central area. The number of entries into arms and total distance traveled were also recorded.

3.2.2. Locomotor activity monitors

Anxiety was assessed using the activity monitors described above. Mice with higher anxiety tend to stay closer to the walls of the apparatus rather than in the open center. The periphery was defined as the area within 6 cm of each of the side-walls, and the center area the remaining 15×15 cm square. Data from the initial 10 min that the mice spent in the apparatus were reanalyzed to calculate the percent of time that mice spent close to the periphery of the apparatus.

3.3. Learning and memory

3.3.1. Spontaneous alternation

Spontaneous alternation behavior was tested in a standard Y-maze made of clear acrylic tubing, as previously described (Harrison et al., 2008). The number and sequence of arm entries made during a 5-min session were recorded. Alternations were defined as an entry into each arm within three consecutive arm choices (e.g. ABC or BAC). Percent alternation was calculated as the number of alternations divided by the number of total arm entries minus two.

3.3.2. Barnes maze

We have shown that 7-month-old APP/PSEN1 mice are impaired on the spatial learning (hidden target) version of the Barnes maze task, but not on the cued-target control version (Bernardo et al., 2008; Reiserer et al., 2007). As previously described (Bernardo et al., 2008; Harrison et al., 2006; Reiserer et al., 2007), mice were trained to locate an escape box positioned beneath the edge of a 105-cm diam. flat, acrylic disk. The escape box was located under one of 12 equally-spaced escape holes that could be open and closed by means of sliding doors. During the cued version of the task the escape hole was marked by a conspicuous polystyrene cone and its location varied from trial to trial. During the hidden-target version, the hole was always located in the same place relative to the extra-maze cues, but the maze itself was cleaned with a 10% ethanol solution and rotated between trials to eliminate the use of intra-maze cues to locate the target hole. Each trial began with the mouse being released from a black acrylic escape box, and continued until the mouse found the escape hole or to a maximum of 180 s. If the mouse did not find the escape hole within 180 s, it was gently guided to the target location and into the escape box. Upon entering the escape box the mouse was confined for 30 s and then returned to the home cage. Training sessions comprised four trials each, with an intertrial interval (ITI) of approx. 20 min. Five visible and five hidden sessions of four trials each were run on consecutive days. The principal dependent measures studied in the Barnes maze were errors to the first visit to the target hole (primary errors), total errors, and visits to the target without entering the escape box (omission errors). The pattern of errors a mouse makes can be more informative than the number because they represent the strategy used to locate the escape box (Harrison et al., 2006). The use of extra-maze room cues to locate the hidden target is reflected in a direct search strategy. Mice that use the overall shape of the maze to locate the escape hole follow a serial-search strategy, visiting incorrect locations sequentially around the edge of the maze. Trials on which neither of these patterns is evident are designated as mixed. Only the first visit to the escape hole was used to determine escape strategy, i.e., exploration following an omission error was ignored for the purpose of classifying search paths.

3.3.3. Morris water maze

Hidden platform testing was conducted in a 107-cm diam. pool with a circular acrylic platform (10 cm diam.) submerged 1 cm below the surface of the water, as previously described (Bernardo et al., 2007; Harrison et al., 2008). Mice were given four acquisition trials per day for 8 days in a spaced fashion, i.e., each mouse completed its first trial before the first mouse began its second trial. ITIs ranged from 10 to 15 min. The water maze was located in the center of a room with distinct, visual cues fixed to the walls that were clearly visible from the pool. These extra-maze cues remained stationary throughout acquisition and probe test sessions. Sessions were captured by an overhead camera and analyzed in real time using an NIH Image macro on a Macintosh computer written specifically for the water maze task (Miyakawa et al., 2001a,b). Latency and path length to reach the hidden platform were the variables of interest during acquisition. In addition we measured the daily average of the cumulative distance from the platform for each training trial (cumulative search error; (Gallagher et al., 1993)). This measure represents a combination not only of the latency and path length to locate the platform but also the accuracy of the search while the mouse is in the pool. Twenty-four hours following target acquisition a 60-s probe trial was conducted. The time spent in the target and non-target quadrants and average distance from the platform location (search error) were the primary dependent measures derived from the probe trial. Search error may be more sensitive measures of selective search on the probe trial than

time in quadrant (Bernardo et al., 2007; Gallagher et al., 1993). Swim speed and peripheral swimming were also assessed in the water maze, to determine whether differences in performance could be attributed to non-cognitive factors. Peripheral swimming was defined as the percentage of time the mouse spent within 8 cm of the wall of the pool.

3.3.4. Scopolamine-induced locomotion

Scopolamine is a non-selective muscarinic receptor antagonist and is known to increase locomotor activity, presumably due to elevated striatal dopamine induced by increased acetylecholine (ACh) release (Bazalakova et al., 2007; Douglas et al., 2001; Sipos et al., 1999). Scopolamine hydrochloride (S-1013) was obtained from Sigma-Aldrich (St Louis, MO, USA) and dissolved in 0.9% physiological saline and administered intraperitoneally in a volume of 10 mL/kg of mouse. The same activity monitors used to assess context–memory savings were also used for the 4-hour scopolamine challenge; however, each mouse was assigned to a different chamber than in previous sessions. At the beginning of the session mice were left undisturbed for 90 min to permit activity levels to reach a stable baseline level. Three saline injections were then administered at 20-min intervals to monitor and stabilize any change in activity levels as a response to injection. A final injection of 1 mg/kg scopolamine was then administered, and mice were left undisturbed for a further 90 min to assess the effect of the drug on activity over time.

3.3.5. Neurochemistry

Mice were anaesthetized using isoflurane and sacrificed by decapitation. Brains were quickly removed, cut along the midline, and stored following one of the methods described below, depending on the subsequent assay.

3.3.6. Vitamin C

Vitamin C levels were measured in cortex, cerebellum, hippocampus, and liver of seven to 19 mice per group. Samples were quickly dissected on ice from fresh tissue, frozen in liquid nitrogen, and stored at −80 °C until assayed. Vitamin C concentrations were measured by ion pair HPLC (Pachla and Kissinger, 1979) and electrochemical detection as previously described (Harrison et al., 2008; May et al., 1998), with tetrapentyl ammonium bromide used as the ion pair reagent.

3.3.7. Lipid peroxidation

Lipid peroxidation was assessed via malondialdehyde levels. Malondialdehyde was measured in 5 to 11 cortical samples per group by homogenizing small, weighed tissue samples in 1 mL 5% TCA solution. Samples were centrifuged at 13,000 rpm at room temperature for 5 min. 250 µL of sample was reacted with the same volume of 0.02 M thiobarbituric acid for 35 min at 95 °C followed by 10 min at 4 °C. Malondialdehyde was then specifically measured using HPLC with inline fluorescence detection of the malondialdehyde–thiobarbituric acid adduct. Malondialdeyhde levels were also assessed in Gulo+/+APP/PSEN1+ (WT–Tg) and Gulo+/+APP/PSEN1− (WT–WT) mice in order to ascertain whether Gulo knockout genotype alone contributed to increased oxidative stress.

In order to assess β-amyloid aggregates, hemi-brains from 5 to 8 KO–Tg mice per treatment were placed in a 10% formalin solution for three days as a fixative and then stored in phosphate-buffered saline. Sections 30-µm thick were cut using a freezing microtome, and mounted on slides for histology. Thioflavin S staining was conducted and analyzed as described previously (Bernardo et al., 2008; Bernardo et al., 2007). Digital images of the dorsal hippocampus and overlying cortex were taken using a fluorescent imaging microscope with an Axiocam high-resolution camera (Carl Zeiss Microimaging Inc., Thornwood, NY, USA) at a magnification of 40×. The area of the hippocampus and overlying cortex 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 experimenter who was blind to the treatment condition of mice. Plaque coverage was calculated as percent of total region measured in pixels. Three sections were assayed per mouse for hippocampus and cortex, and the mean value used to calculate percent coverage for each area.

3.3.8. Statistical analyses

The comparisons of interest in the present experiment were the effect of genotype at each treatment level and the effect of treatment within each genotype. Therefore, univariate and Repeated-Measures Analysis of Variance (ANOVA and RMANOVA) were conducted with Genotype and Treatment as between-groups variables. Planned follow-up comparisons were conducted using Bonferroni-corrected *t*-tests with or without a significant omnibus ANOVA. No post-hoc tests were needed.

Savings is measured as a percent change from baseline exploration levels, the meaning of which differs depending on whether it is an increase or a decrease. Because this renders the data susceptible to skewing by a small number of animals, analyses were conducted using $\log_{10}$-transformed data. Mice that explored more in the second session than the first were excluded from the analysis, as this represents an abnormal habituation process that could skew the data (3 of 28 KO–WT STD; 8 of 22 KO–WT RED; 1 of 15 KO–Tg STD; and 2 of 13 KO–Tg RED). Mice that explored more on the third than the original test session were given a savings score of 0% saved. The four locomotor activity sessions were analyzed using a 2 Session × 2 Trial RMANOVA.

4. Results

4.1. Survival

Survival rates varied between the genotypes on the initial low supplementation level [$\chi^2 = 12.098$, $P < .001$; Fig. 1], as mentioned above. There was a significantly higher death rate in KO–Tg mice compared to KO–WT mice on the LOW vitamin C (0.033 mg/L) supplementation ($P < .001$) but not in RED- (0.099 mg/L) or STD- (0.33 mg/L) treated mice. Age of death in the LOW-treated mice ranged from 10 weeks to 22 weeks. The average age of death was approximately 14 weeks, i.e., following 8 weeks of LOW supplementation. Only one KO–Tg mouse and none of the KO–WT mice on the STD treatment died before the end of the experiment.

4.2. Anxiety

Mice in all four groups spent 70–80% of the time in the periphery of the locomotor activity chamber during the first 10-min activity session. Percent time spent in periphery and ambulatory velocity did not differ among groups [$Fs_{1,77} < 3.07$; $Ps > .084$, data not shown]. Behaviors on the elevated plus maze also did not differ by group, indicating that exploration and anxiety were not affected by genotype or treatment. Total distance, percent time spent in closed arms, and percent entries

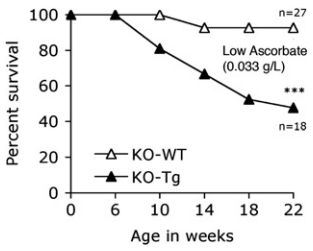

Fig. 1. Survival. KO–Tg mice receiving the initial 0.033 mg/L low level of vitamin C supplementation were more likely to die than KO–WT mice on the same supplementation. Fig. 1 shows percentage survival rates by age in weeks. Based on these data the reduced vitamin C supplementation level for the experiment was increased to 0.099 mg/L vitamin C. Low-treated KO–Tg versus KO–WT ***$P < .001$.

into closed arms were all similar among groups [$Fs_{(1, 76)} < 2.917$, $Ps > .092$, data not shown].

4.3. Locomotor activity and contextual memory savings

Overall, APP/PSEN1 mice traveled farther in the locomotor activity chambers than wild-type mice [$F_{1, 78} = 14.458$, $P < .001$], and RED-treated mice were more active than STD-treated mice [$F_{1, 78} = 4.403$, $P = .039$, Fig. 2a]. However, a significant Genotype × Treatment interaction [$F_{1, 78} = 10.085$, $P = .002$] highlighted a more interesting pattern. RED-treatment increased activity in KO–Tg ($P = .001$) but not KO–WT mice ($P < .644$).

Locomotor activity tends to decrease across successive trials as mice habituate to the testing environment. Significant effects of session and trial showed that this was the case in the present study [Fig. 2a; $Fs > 26.565$, $Ps < .001$]. There was also a significant Session × Trial × Treatment interaction [$F = 6.085$, $P = .016$]. Follow-up analyses revealed that although the pattern of decreasing activity between trials held for all groups of mice during session 1 ($P < .001$), there was no difference between trial 1 and trial 2 in session 2 for STD-treated mice ($P = .152$). This indicates that these mice, but not RED-treated mice ($P < .001$), had fully habituated to the testing context. The abnormal pattern of activity in the RED-treated KO–Tg mice is more clearly revealed in the scores for saved habituation (Fig. 2b). There was no main effect of genotype on savings score [$F_{1, 65} = 1.929$; $P = .17$], but treatment had a significant effect on saved habituation between the two test sessions [Treatment $F_{1, 65} = 8.379$; $P = .005$, Genotype × Treatment $F_{1, 65} = 5.805$; $P = .019$]. Savings did not differ according to genotype in STD-treated mice ($P = .443$), but RED-treated KO-Tg mice had lower savings scores than KO–WT mice given the same supplement level ($P = .014$). The effect of treatment was restricted to the KO–Tg mice; KO–WT mice had similar savings scores regardless of supplementation status ($P = .708$).

4.4. Y-maze

Mice that completed 10 or fewer arm entries (one mouse from each group) were excluded from Y-maze analyses. Exploration in the Y-maze varied among groups [genotype $F_{1, 75} = 7.34$, $P = .008$; treatment $F_{1,75} = .154$, $P = .696$; Genotype × Treatment $F_{1, 75} = 4.285$, $P = .042$]. KO–Tg mice on the RED treatment made more arm entries than KO–WT mice on the same supplementation ($P = .002$) and KO–WT mice on the STD supplementation ($P = .038$, data not shown). KO–Tg and KO–WT mice on STD treatments did not differ ($P = .635$), and vitamin C supplementation did not affect arm entries in KO–Tg mice ($P = .305$). Alternation behavior in the Y-maze did not differ according to genotype or treatment [62%–68% alternation for each group; $Fs_{1, 75} < 1.472$, $Ps > .229$] indicating intact spatial working memory on this task in all groups of mice.

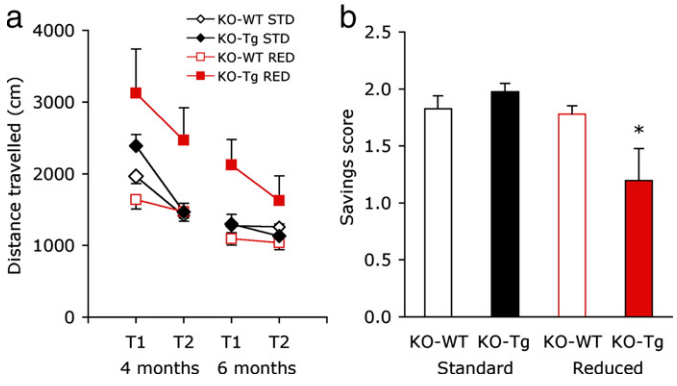

Fig. 2. Locomotor activity. Mice were given two 10-min sessions in locomotor activity chambers on consecutive days at 4 months and 6 months of age (a) KO–Tg RED-treated mice were initially hyperactive in the locomotor activity chambers and (b) had a lower savings score ($P < .05$), indicating impaired habituation processes. Data represent mean + 1 S.E.M. *$P < .05$ different from KO–WT mice on equivalent vitamin C supplementation.

4.5. Barnes maze

During Barnes maze testing, when the target hole was marked by a conspicuous, visible cue, all mice quickly learned to locate the target and escape from the maze. The latency and number of errors to target all decreased over the initial five cued sessions [$Fs_{4, 256} > 16.976$, $Ps < .001$] and did not differ according to treatment or genotype [$Fs < 1.202$, $Ps > .310$; data not shown]. When mice relied on distal spatial cues to locate the escape hole all mice improved further in primary and total errors and latency over the five sessions [$Fs_{4, 256} > 22.626$, $Ps < .001$]. KO–Tg mice made more primary errors [$F_{1, 64} = 5.999$, $P = .017$], and a higher number of total errors [$F_{1, 64} = 8.601$, $P = .005$; Fig. 3a] than KO–WT mice, reflecting poorer spatial learning in transgenic mice. STD- and RED-treated mice did not differ in primary or total errors and there was no Genotype × Treatment interaction [$Fs_{1, 64} < 1.104$, $Ps > .297$]. Consistent with impaired learning of the Barnes maze, KO–Tg mice took slightly longer to locate the escape hole [primary latency, $F_{1, 64} = .3.099$, $P = .083$] and to enter the escape box [total latency, $F_{1, 64} = 5.627$, $P = .021$] compared to KO–WT mice (data not shown). There were no main effects of treatment or Genotype × Treatment interactions [$Fs_{1, 64} < 1.753$, $P > .190$] on latency measures in the Barnes maze.

Occasionally a mouse will visit the target hole but not enter the escape box. The number of these omission errors is generally low although such errors are more common during early test sessions. Omission errors typically decline as mice habituate to the maze and any anxiety responses associated with entering the escape hole, or motivation to further explore the maze have declined. Given the generally low numbers of omission errors that are made during hidden-target training the group differences become clearer when omission errors are summed across trials. During hidden-target trials KO–Tg mice made more omission errors than the KO–WT mice [$F_{1, 64} = 5.169$, $P = .026$] and the RED-treated mice made more errors than STD-treated mice [$F_{1, 64} = 3.992$, $P = .050$]. There was no interaction [$F_{1, 64} = 1.111$, $P = .296$]. Despite the significant effects of genotype and supplementation status, most mice made fewer than two omission errors across all 5 days of hidden-target testing (Fig. 3b).

The way a mouse solves the Barnes maze may reveal whether they are making use of distal room cues or using non-spatial information such as the shape of the maze, to locate the escape hole (Harrison et al., 2006). Among all groups combined, the types of strategy used differed across sessions [$F_{2, 128} = 52.5$, $P < .001$]. This reflected the fact that the use of direct (spatial) searches increased and the proportion of non-spatial (serial and mixed) strategies decreased ($Ps < .001$), as mice learned the location of the escape hole. However, strategy use varied between KO–WT and KO–Tg mice across sessions, regardless of vitamin C supplementation status [$F_{2, 128} = 3.358$, $P = .038$]. KO–WT mice made significantly more direct searches than KO–Tg mice indicating stronger spatial learning abilities in these mice ($P = .048$, Fig. 3c). The number of serial searches ($P = .082$) and mixed searches ($P = .396$) did not differ between genotypes. Strategy use was not affect by vitamin C supplementation [$F_{1, 64} = 1.0$, $P = .321$].

4.6. Water maze

Search error for all groups combined decreased across the eight acquisition sessions in the Morris water maze [$F_{7, 546} = 132.66$, $P < .001$]. Consistent with our previous data, transgenic mice did not learn as quickly as the wild-type groups [Genotype $F_{1,78} = 5.92$, $P = .017$; Genotype × Session $F_{7, 546} = 3.113$, $P = .003$; Fig. 3d]. The effect of ascorbate treatment was not significant [$F_{1, 78} = .036$, $P = .851$]. Escape latency and path length data showed similar genotype [$Fs_{1,78} > 5.02$, $Ps < .029$] and interaction effects [$Fs > 2.23$,

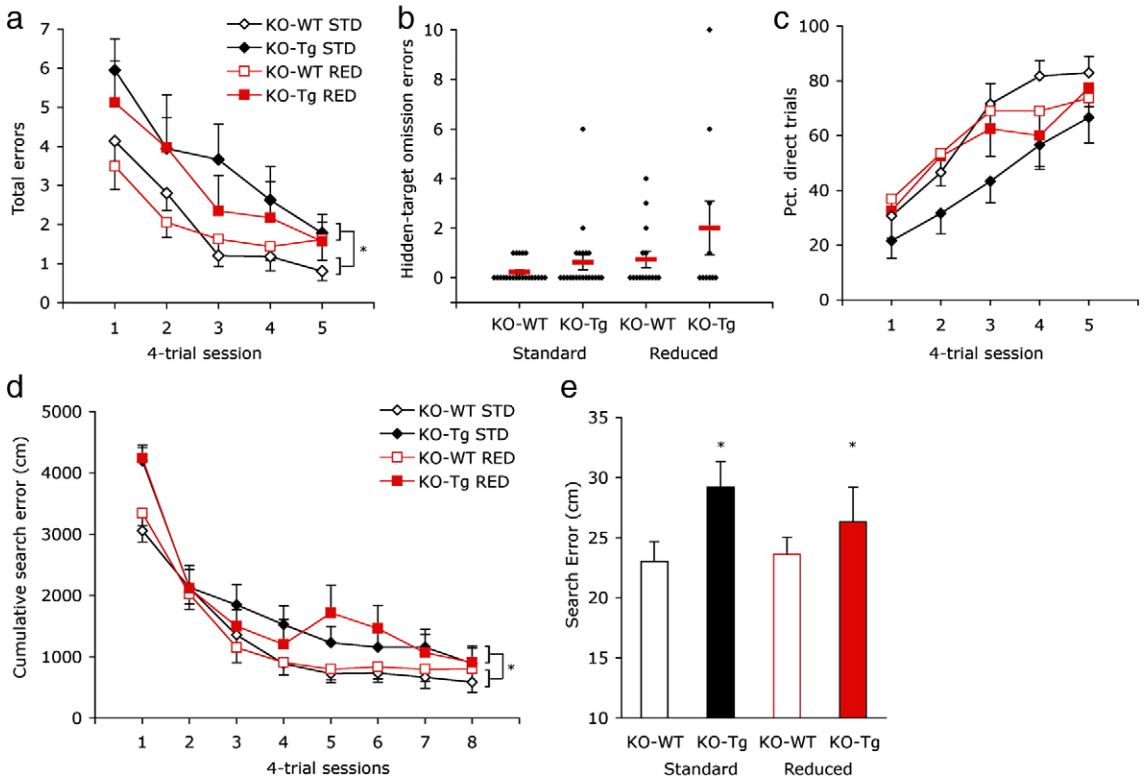

Fig. 3. Learning and memory. At 8 months of age mice were trained for 10 days on the Barnes maze (5 cued-target and 5 hidden-target sessions) followed by 8 acquisition sessions and a probe trial in the Morris water maze. (a) During Barnes maze hidden-target training KO–Tg mice made significantly more errors than KO–WT mice ($P<.05$). (b) Omission errors—visits to the target hole but not entering it — during hidden-target acquisition were higher in KO–Tg than KO–WT mice ($P<.05$) and higher in mice receiving RED vitamin C supplementation mice than in mice on STD treatments ($P<.05$). Individual scores shown, group mean indicated by red horizontal bar. (c) KO–WT mice were more likely than KO–Tg mice to use a direct escape strategy to locate the target box ($P<.05$), but strategy use was not affected by vitamin C supplementation. All mice learned to locate the hidden platform across the 8 days of training in the Morris water maze. (d) Cumulative search error during training indicated greater search accuracy in KO–WT than KO–Tg mice but no effect of vitamin C. (e) KO–Tg mice were less accurate in their platform search during the probe trial as shown by a greater search error. Data represent mean + 1 S.E.M. *$P<.05$ different from KO–WT mice.

$Ps<.032$]; data not shown. Neither escape latency nor path length was affected by ascorbate treatment [$Fs_{1,\ 78}<.054$, $Ps>.817$]. During the 60-s probe trial all four groups showed preferential search, spending more time in the target quadrant than the non-target quadrants [$Fs>10.69$; $Ps<.006$]. However, the measure of search error revealed greater accuracy in KO–WT compared to KO–Tg mice [$F_{1,\ 78}=10.179$, $P=.002$; Fig. 3e]. There was no effect of treatment on search error [$F_{1,\ 78}<.032$, $Ps>.859$]. Groups did not differ in time spent in the perimeter, path length, or swim speed during the probe trial [$Fs_{1,\ 78}<1.029$, $Ps>.314$].

4.7. Scopolamine and activity

Scopolamine-induced activity was assessed in a subset of animals ($n=6$–16 mice per group). Following a 90-min habituation period during which mice were left undisturbed in the locomotor activity chambers, three saline injections were administered at 20-min intervals. During this time all mice continued to decrease in locomotor activity [$F_{5,\ 185}=6.32$, $P<.001$] and there were no differences according to genotype or treatment following saline injections [$Fs_{1,\ 37}<2.58$, $Ps>.12$, Fig. 4]. Importantly, there were also no group differences in the first 10 min of the session [$Fs_{1,\ 37}<1.39$, $Ps>.25$], showing that the hyperactivity observed in the 4-month-old KO–Tg mice receiving low levels of ascorbate had abated by the time the scopolamine challenge was administered. Following administration of a 1 mg/kg dose of scopolamine there were significant changes over time as all mice experienced an immediate increase in activity that tailed off with time [$F_{8,\ 296}=33.512$, $P<.001$]. All mice demonstrated an initial increase in activity immediately following the scopolamine injection [$F_{1,\ 37}=143.66$, $P<.001$]. This increase was greater in STD-treated mice than RED-treated mice [treatment $F_{1,\ 37}=9.58$, $P=.004$, treatment × time $F_{1,\ 37}=6.04$, $P=.019$]. Although all groups except the KO–Tg STD mice appeared to be approaching pre-scopolamine levels of activity by the final two 10-min periods of the session, the significant differences in activity levels according to genotype and treatment remained [Genotype $F_{1,\ 37}=6.60$, $P=.014$; Treatment $F_{1,\ 37}=4.40$, $P=.043$; Genotype × Treatment interaction $F_{1,\ 37}=1.95$, $P=.17$].

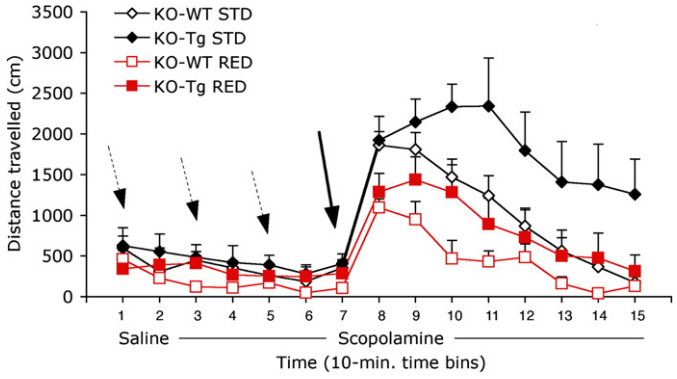

Fig. 4. Scopolamine and locomotor activity. Following all learning and memory testing a subset of mice were given an additional 4-hour long locomotor activity session. Following a 90-min habituation in the activity chambers, mice were given 3 saline injections at 20-min intervals (broken arrows) and finally a single 1 mg/kg i.p. injection of scopolamine (solid arrow). RED-treated mice were less active following scopolamine administration than their STD-treated counterparts ($P<.01$), and KO–Tg mice were more active than KO–WT mice ($P<.05$). Data represent mean + 1 S.E.M.

4.8. Biochemical measures

4.8.1. Vitamin C

In order to assess the effects of prolonged low levels of vitamin C supplementation on stored vitamin C levels, samples were analyzed from the cortex, hippocampus, cerebellum and liver. The liver is the site of vitamin C synthesis in *Gulo* wild-type mice and reflects any changes in circulating antioxidant levels. In the liver, mice receiving reduced dietary ascorbate had lower levels of stored vitamin C, compared to mice on the standard diet [$F_{1,\ 53} = 12.944$, $P = .001$, Fig. 5a]. There was no effect of genotype and no Genotype × Treatment interaction in the liver [*F*s.096, *P*s>.758]. In each of the brain areas assayed, treatment with the RED-supplement led to stored vitamin C levels that were significantly lower than those in STD-supplemented groups [cortex $F_{1,\ 43} = 8.57$, $P = .005$; hippocampus $F_{1,\ 43} = 15.20$, $P < .001$; cerebellum $F_{1,\ 43} = 6.74$, $P = .012$; Fig. 5a]. There were no genotype differences and no Genotype × Treatment interactions in any of the brain tissues assayed [*F*s<2.75, *P*s>.10].

4.8.2. Oxidative stress

Malondialdehyde levels were measured in cortical samples from standard- and reduced-treated KO–WT and KO–Tg ($n = 5$–11 per group) mice and also in WT–WT and WT–Tg mice ($n = 4$ per group) maintained on unsupplemented water in order to assess the role of knocking out the *Gulo* enzyme on oxidative stress. When standard-treated Gulo−/− mice were compared with water-fed Gulo+/+ mice there were no differences in cortical MDA levels according to Gulo genotype (or water treatment) or APP/PSEN1 genotype [*F*s<4.0, *P*s>.06, Fig. 5b]. When Gulo−/− mice on different water treatments were compared, RED-treated mice had greater levels of malondialdehyde in the cortex than STD-treated mice [$F_{1,\ 26} = 9.32$, $P = .005$, Fig. 5b]. There was no significant main effect of genotype and no significant interaction between the factors [*F*s<2.18, *P*s>.15]. However, planned comparisons revealed that the effect of treatment was due to differences between KO–Tg mice ($P = .008$) and not KO–WT mice ($P = .21$), indicating that transgenic animals were more susceptible to the effects of low ascorbate.

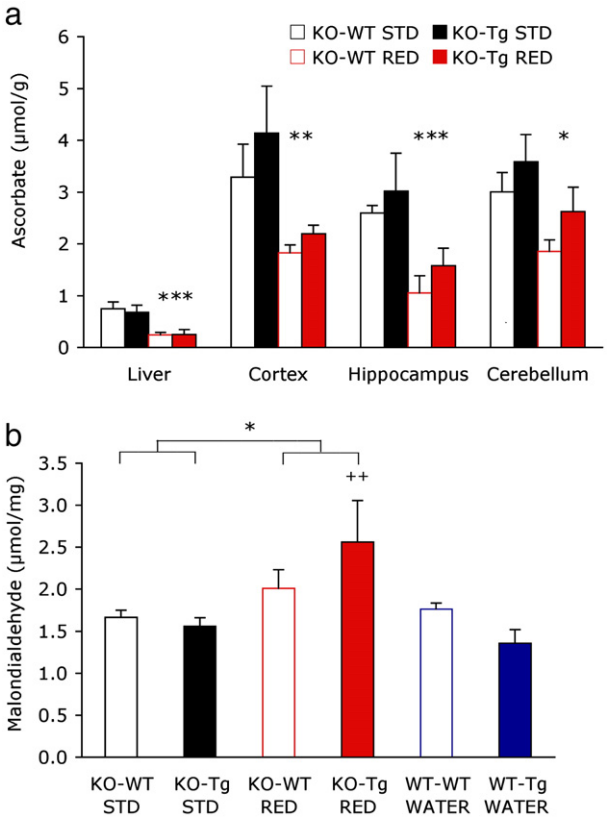

Fig. 5. Vitamin C and oxidative stress. Vitamin C levels were measured in the liver, cortex, hippocampus and cerebellum. (a) Vitamin C levels were significantly lower in RED-treated mice in liver and each of the brain areas assayed. Malondialdehyde, a marker for lipid peroxidation, was used as an indicator of oxidative stress in the brain. (b) Malondialdehyde levels in STD-treated Gulo−/− mice did not differ from water-fed Gulo+/+ mice. Mice in the RED-treated groups had increased malondialdehyde levels relative to standard-treated mice and Gulo wild-type mice. There was no effect of genotype in STD-treated mice but malondialdehyde levels were higher in RED KO–Tg mice than KO–WT mice on the same supplementation level. Data represent mean + 1 S.E.M. *$P < .05$; **$P < .01$; ***$P < .001$, different from STD-treated mice. $^{++}P < .01$ KO–Tg different from KO–WT mice.

4.8.3. β-amyloid deposition

β-amyloid plaques were quantified in five KO–Tg RED-treated and eight KO–Tg STD-treated mice. No gender differences were observed [*F*s<1, *P*s>.34] so data were collapsed and analyzed together. There were no differences in percent area covered with plaque or mean size of plaques between mice given reduced and standard vitamin C supplements in either the hippocampus or cortex [$Fs_{1,11} < 2.25$, *P*s>.16; data not shown].

5. Discussion

We have shown that APP/PSEN1 transgenic mice lacking Gulo are more sensitive to low levels of vitamin C than Gulo knockouts that do not carry the mutant transgenes. Using a supplementation level previously shown to maintain good health in Gulo−/− mice, half the double-transgenics lacking Gulo died within 6 months. Thus, dietary supplements were increased to avoid the survival bias that would have otherwise arisen in the data. The mice survived when provided with a 3-fold higher concentration of vitamin C, but exhibited significant behavioral abnormalities compared to the other three groups. Impaired cognition in the KO–Tg mice under reduced supplementation was associated with reduced habituation, and accompanied by increased neocortical oxidative stress. These results suggest that APP/PSEN1 mice are more susceptible to the deleterious effects of vitamin C deficiency.

Transgenic mice were impaired on the measures of learning and memory in the present study, as has been shown previously in ours and others' labs (Bernardo et al., 2008; Ding et al., 2008; Lalonde et al., 2005; Reiserer et al., 2007). In the Barnes maze, KO–Tg mice took longer to locate the escape hole beneath the maze and visited more non-target holes before escaping. They were also less likely than KO–WT mice to use a direct/spatial search strategy. Similarly, KO–Tg mice took longer to locate the escape platform in the water maze and were not as accurate in their swim search during the probe trial. Reduced vitamin C led to greater numbers of omission errors by KO–Tg mice during hidden-target Barnes maze testing. An omission error is defined as a failure to enter the Barnes maze escape box the first time it is encountered on a particular trial. This type of behavior can result from a number of non-mnemonic processes. Like the water maze, the Barnes maze is an aversively-motivated task. Mice tend to avoid brightly-lit open spaces, presumably because this leaves them vulnerable to predators (e.g., owls). However, other factors may influence behavior to keep the mouse on the surface of the maze. For example, mice may also be afraid of predators (e.g., snakes) lurking in the dark escape tunnel. In addition, mice may have an innate tendency to explore novel environments, a behavior that generally habituates with repeated trials. Both of these factors are more prevalent early in training, when the maze and its features are relatively new. Consistent with this, omission errors are common during the first few days of training, i.e., during cued-target testing, but are generally negligible during the later hidden-target (spatial) stage of testing (Reiserer et al., 2007). The persistent omission errors during spatial training on the Barnes maze cannot be attributed to abnormal anxiety, as there were no group differences on any of the anxiety measures assessed. It is possible that KO–Tg RED-treated mice may have deficient exploratory habituation processes. Such a possibility is reinforced by the finding that, unlike the other three groups, RED-treated KO–Tg mice

failed to fully habituate to the open field chambers across the first four testing sessions and had much lower savings scores (Fig. 2a–b). Although scorbutic guinea pigs have been shown to exhibit decreased locomotor behaviors, this is likely attributable to ill health (Kaufmann et al., 1986). The health and weight of the mice in the present study was constantly monitored and although vitamin C intake was decreased, the animals were otherwise healthy. These deficiencies likely represent a change in habituation processes rather than in long-term spatial memory because these mice did not show greater learning or memory deficits than standard-treated counterparts in the Barnes or water mazes. The reduced vitamin C level used here was three times higher than necessary to maintain good health in Gulo null mice (Harrison et al., 2008) and the behavioral effects of decreased supplementation in the transgenic mice would likely have been much greater had they been able to survive on the lower supplementation level.

The scopolamine challenge was performed at the end of the behavioral battery (at 10 months) and the other tasks were begun earlier (at 4 and 6 months). By the time scopolamine testing was conducted mice showed similar activity levels during the habituation period of the task, further supporting the proposal that the increased exploration observed in the earlier locomotor activity sessions was attributable to differences in early habituation processes rather than persistent hyperactivity. Scopolamine increases locomotor activity through an interaction between the cholinergic and dopaminergic systems (Fink and Morgenstern, 1980; Mathur et al., 1997). When challenged with scopolamine, transgenic mice on both treatments showed greater increases in activity than wild-type mice, and both RED-treated groups were less active than the STD groups, with no interaction between the factors. The demonstration of a differential effect on RED-treated mice indicates an interaction between ascorbate and the cholinergic and/or dopaminergic systems. APP/PSEN1 mice have impaired cholinergic function with onset between 5 and 7 months of age (Goto et al., 2008; Machova et al., 2008), which may affect their response to scopolamine. Dopaminergic function in these mice has not yet been explored. However, differing patterns of activity between supplementation groups may reflect differences in striatal or accumbens dopamine induced by varying brain vitamin C levels. Vitamin C is an essential co-factor in the synthesis of norepinephrine from dopamine and vitamin C deficiency in guinea pigs has been shown to increase brain dopamine level in proportion to the change in brain ascorbate (Deana et al., 1975; Hoehn and Kanfer, 1980; Saner et al., 1975). Vitamin C is released in the striatum with behavioral activation (O'Neill and Fillenz, 1985) and both locomotor activity and vitamin C release increase in response to dopamine agonists (Pierce and Rebec, 1990).

The tissue vitamin C levels reported here in standard-treated mice are similar to levels previously reported in Gulo −/− and APP/PSEN1 mice (Harrison et al., 2009c; Harrison et al., 2008). Lower vitamin C treatments in the triple-mutant (KO–Tg) mice led to significantly lowered (40–60%) vitamin C in cortex, hippocampus, and cerebellum. Striatal vitamin C levels were not measured specifically in this case; however, we have found previously that striatal levels in Gulo−/− mice are similar to levels in the cortex (Harrison et al., 2008). Gulo−/− mice are entirely dependent on dietary vitamin C supplements to maintain tissue levels high enough to prevent scurvy. For Gulo−/− mice on a pure inbred C57BL/6J background, a low supplementation level of 0.033 g/L is sufficient to maintain supra-scorbutic vitamin C levels and to allow healthy weight gain with age (Harrison et al., 2008; Maeda et al., 2000). However, during preliminary testing for the present study it became apparent that this level was not sufficient to maintain KO–Tg mice. The cause of death in these animals is not clear. Regular monitoring of weight and appearance make it unlikely that the premature deaths can be attributed solely to scurvy. Given the discrepancy in the death rates between the genotypes it is likely that Gulo−/−APP/PSEN1 needed higher amounts of vitamin C to maintain health, perhaps in part to counteract the elevated oxidative stress levels in these mice.

Oxidative stress has been reported to be higher in APP/PSEN1 mice relative to wild-types (Bernardo et al., 2008; Harrison et al., 2009c). In the present study malondialdehyde levels were elevated in reduced-vitamin-C-treated mice, and mice carrying the APP/PSEN1 transgenes had the highest levels detected. Brain vitamin C levels were similarly reduced by treatment regardless of genotype, but the effect on both malondialdehyde levels and behavioral function was greater in KO–Tg mice. These data suggest that low levels of vitamin C can induce oxidative stress and that its effects are more pronounced where there is a preexisting genetic susceptibility. The lower levels did not appear to affect spatial learning but did alter habituation processes and may also have disrupted normal cholinergic and dopaminergic signaling. It is possible, therefore, that a level of vitamin C that is sufficient to maintain basic health in a normal mouse is not sufficient to maintain optimal cognitive health in a mouse model of Alzheimer's disease. If these conclusions can be extrapolated to humans, they reinforce the need to maintain sufficient daily dietary vitamin C levels. Furthermore, dietary deficiency may be even more dangerous in those carrying genetic risk factors for developing Alzheimer's disease (Morris et al., 2002). In the present study only vitamin C levels were restricted, and although this is a major antioxidant in the body, it is possible that production or sequestration of other antioxidants (such as glutathione) was increased to maintain oxidative homeostasis in the mice. However, in human disease, increased oxidative stress and reduced antioxidant intake are likely to occur together due to poor diet or other environmental factors.

Although Aβ deposition is often considered an endpoint to a neuropathological cascade, it is correlated with levels of small oligomers that are toxic *in vitro*. Soluble Aβ induces oxidative stress and can have an acute, drug-like amnestic effect in the absence of amyloid aggregation (Cleary et al., 2005; McDonald et al., 1994; McDonald et al., 1996; Sweeney et al., 1997). No changes were observed in Aβ plaque deposition in the present study, despite differing brain vitamin C levels. Similarly, hyperhomocysteinemia exacerbated spatial memory deficits in the Tg2576 APP-overexpressing mouse line without altering Aβ deposition (Bernardo et al., 2007). These results suggest that processes related to oxidative stress and cell death may not affect initial Aβ seeding or the rate of aggregation. Although this is the first study examining the effects of vitamin C deficiency on plaque formation in APP-overexpressing mice, other dietary antioxidants have been investigated with mixed results (Lim et al., 2001; Quinn et al., 2007; Stackman et al., 2003; Yang et al., 2008). In one study, vitamin E was administered in the diet to Tg2576 APP-overexpressing mice, which normally start to develop plaques from approximately 10 months of age (Sung et al., 2004). When treatment was initiated at 5 months of age, significantly reduced plaque was observed in 13-month-old mice; in contrast, there was no effect of vitamin E on Aβ aggregation when administered from 14 to 20 months of age. These results may be attributable to the age of the mice, the duration of treatments, or the amount of Aβ in the brain. Although APP levels are stable throughout life in these mice, Aβ levels increase exponentially starting at around 9 months of age (Kawarabayashi et al., 2001). A similar exponential increase begins at 4-5 months of age in APP/PSEN1 double-transgenics (Garcia-Alloza et al., 2006; Gordon et al., 2002). In the present study vitamin C levels were reduced from 6 weeks of age, long before Aβ starts to aggregate, and although transgenic mice maintained on the 0.099 mg/kg dose treatment level had increased oxidative stress and exhibited significantly greater exploratory locomotor activity, plaque levels did not vary among the groups. These results suggest that modest reductions in vitamin C and elevated malondialdehyde levels do not affect the oligomerization or initial aggregation of Aβ, and that aggregated amyloid was unlikely to be responsible for the abnormal habituation processes in the RED-treated transgenic mice.

A key feature of Alzheimer's disease neuropathology is neuronal death. This is not typically seen in APP/PSEN1 mice, and a valid criticism of this model is that it does not recapitulate this important feature of the disease. Oxidative stress is a common inducer of neuronal apoptosis in a number of neurodegenerative conditions, and both oxidative stress and

cell death are reduced by antioxidant treatment (melatonin) in senescence-accelerated mice (Caballero et al., 2009). Further work is now needed on the role of vitamin C deficiency in these processes.

Given the selective nature of the behavioral deficits in reduced-vitamin C groups, our data suggest that stored vitamin C levels, oxidative stress, and global cognitive functioning do not share a simple relationship. There was a clear reduction in vitamin C levels in RED-treated mice in all brain areas assayed as well as in the liver, indicating that the mice can survive long-term with low vitamin C intake and appear physically healthy even as low antioxidant levels are affecting brain biochemistry and function. However, there is considerable potential for reducing stored levels when intake is three-to-four times lower than normal requirements. This window of potential change for brain vitamin C, during which time normal bodily functioning can be maintained, has the potential to be damaging long term. While the current recommended daily vitamin C intake for adults is 75 mg for females and 90 mg for males, the clinical signs of scurvy will be avoided if just 10 mg per day in ingested. Nevertheless, there is clearly a great difference in physical, biochemical, and cognitive function between individuals with sufficient Vitamin C and those with sub-clinically deficient levels. It is likely that a far greater proportion of the general public than expected exists at deficient vitamin C levels without any apparent sign of ill health (Hampl et al., 2004; Johnston et al., 1998; Johnston and Thompson, 1998) and these low levels of vitamin C may have a critical impact on cognitive ability and brain health.

There are two routes by which ascorbate can enter the brain. Ascorbate may pass via Sodium Dependent Vitamin C Transporters or as oxidized dehydroascorbic acid (DHA) via glucose transporters (Harrison and May, 2009). The latter is not a primary route due to short half-life of DHA in physiological buffers (approx. 6 min) (Bielski et al., 1981) and the low amount of DHA circulating in the blood. Circulating DHA concentrations in are very low human plasma (<2 μM) relative to those of ascorbate con (40–50 μM) (Dhariwal et al., 1991). However, when infused in high quantities DHA will enter the brain where it can be recycled to ascorbate (Minamizono et al., 2006). Thus it is possible that infusions of DHA could provide an acute method of increasing low ascorbate to normal, or even increasing relatively normal ascorbate levels in those under higher risk of oxidative stress and Alzheimer's disease. Such treatments are already under investigation to treat oxidative damage following ischemic stroke and may have increased benefits in hyperglycemic animals where excess glucose blocks the route of entry of DHA into the brain (Bemeur et al., 2005; Minamizono et al., 2006).

A considerable body of evidence exists to suggest that low antioxidant status is associated with development of Alzheimer's disease, and that antioxidant therapies may reduce risk. Our original hypothesis was that lowering vitamin C intake would increase oxidative stress and concomitantly increase amyloid burden and memory deficit. However, amyloid burden was not affected despite increased oxidative stress in the reduced-vitamin-C group. Overall, the reduced vitamin C had two major effects in the transgenic mice—to disrupt normal habituation processes (in the activity chambers and in the Barnes maze) and to increase oxidative stress—effects that may become more pronounced at an advanced age. Given the differential reactions to scopolamine on locomotor activity, it is plausible that vitamin C may be exerting its effects by modulating cholinergic and/or dopaminergic systems. This is an area that warrants further investigation.

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References

Alzheimer's Association. Alzheimer's disease facts and figures. Alzheimers Dement 2008;4:110–33.
Arzi A, Hemmati AA, Razian A. Effect of vitamins C and E on cognitive function in mouse. Pharmacol Res 2004;49:249–52.
Bazalakova MH, Wright J, Schneble EJ, McDonald MP, Heilman CJ, Levey AI, et al. Deficits in acetylcholine homeostasis, receptors and behaviors in choline transporter heterozygous mice. Genes Brain Behav 2007;6:411–24.
Bemeur C, Ste-Marie L, Desjardins P, Vachon L, Butterworth RF, Hazell AS, et al. Dehydroascorbic acid normalizes several markers of oxidative stress and inflammation in acute hyperglycemic focal cerebral ischemia in the rat. Neurochem Int 2005;46:399–407.
Bernardo A, Harrison FE, McCord M, Zhao J, Bruchey A, Davies SS, et al. Elimination of GD3 synthase improves memory and reduces amyloid-beta plaque load in transgenic mice. Neurobiol Aging 2008.
Bernardo A, McCord M, Troen AM, Allison JD, McDonald MP. Impaired spatial memory in APP-overexpressing mice on a homocysteinemia-inducing diet. Neurobiol Aging 2007;28:1195–205.
Bielski BH, Allen AO, Schwarz HA. Mechanisms of disproportionation of ascorbate radicals. J Am Chem Soc 1981;103:3516–8.
Bowman GL, Dodge H, Frei B, Calabrese C, Oken BS, Kaye JA, et al. Ascorbic acid and rates of cognitive decline in Alzheimer's disease. J Alzheimers Dis 2009;16:93–8.
Butterfield DA, Drake J, Pocernich C, Castegna A. Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid beta-peptide. Trends Mol Med 2001;7:548–54.
Caballero B, Vega-Naredo I, Sierra V, Huidobro-Fernandez C, Soria-Valles C, De Gonzalo-Calvo D, et al. Melatonin alters cell death processes in response to age-related oxidative stress in the brain of senescence-accelerated mice. J Pineal Res 2009;46:106–14.
Castellani RJ, Lee HG, Zhu X, Nunomura A, Perry G, Smith MA. Neuropathology of Alzheimer disease: pathognomonic but not pathogenic. Acta Neuropathol 2006;111:503–9.
Charlton KE, Rabinowitz TL, Geffen LN, Dhansay MA. Lowered plasma vitamin C, but not vitamin E, concentrations in dementia patients. J Nutr Health Aging 2004;8:99-107.
Cleary JP, Walsh DM, Hofmeister JJ, Shankar GM, Kuskowski MA, Selkoe DJ, et al. Natural oligomers of the amyloid-beta protein specifically disrupt cognitive function. Nat Neurosci 2005;8:79–84.
de Angelis L, Furlan C. The effects of ascorbic acid and oxiracetam on scopolamine-induced amnesia in a habituation test in aged mice. Neurobiol Learn Mem 1995;64:119–24.
Deana R, Bharaj BS, Verjee ZH, Galzigna L. Changes relevant to catecholamine metabolism in liver and brain of ascorbic acid deficient guinea-pigs. International journal for vitamin and nutrition research. Internationale Zeitschrift fur Vitamin- und Ernahrungs-forschung 1975;45:175–82.
Dhariwal KR, Hartzell WO, Levine M. Ascorbic acid and dehydroascorbic acid measurements in human plasma and serum. Am J Clin Nutr 1991;54:712–6.
Dhingra D, Parle M, Kulkarni SK. Comparative brain cholinesterase-inhibiting activity of *Glycyrrhiza glabra*, *Myristica fragrans*, ascorbic acid, and metrifonate in mice. J Med Food 2006;9:281–3.
Ding Y, Qiao A, Wang Z, Goodwin JS, Lee ES, Block ML, et al. Retinoic acid attenuates beta-amyloid deposition and rescues memory deficits in an Alzheimer's disease transgenic mouse model. J Neurosci 2008;28:11622–34.
Douglas CL, Baghdoyan HA, Lydic R. M2 muscarinic autoreceptors modulate acetylcholine release in prefrontal cortex of C57BL/6 J mouse. J Pharmacol Exp Ther 2001;299:960–6.
Ebbinghaus H. Memory: a contribution to experimental psychology. New York: Teachers College; 1885. Translated by Henry A. Ruger & Clara E. Bussenius (1913).
Engelhart MJ, Geerlings MI, Ruitenberg A, van Swieten JC, Hofman A, Witteman JC, et al. Dietary intake of antioxidants and risk of Alzheimer disease. Jama 2002;287:3223–9.
Feng Z, Chang Y, Cheng Y, Zhang BL, Qu ZW, Qin C, et al. Melatonin alleviates behavioral deficits associated with apoptosis and cholinergic system dysfunction in the APP 695 transgenic mouse model of Alzheimer's disease. J Pineal Res 2004;37:129–36.
Fink H, Morgenstern R. Scopolamine-induced hypermotility in rats is mediated via a dopaminergic system. Acta Biol Med Ger 1980;39:903–10.
Gallagher M, Burwell R, Burchinal M. Severity of spatial learning impairment in aging: development of a learning index for performance in the Morris water maze. Behav Neurosci 1993;107:618–26.
Garcia-Alloza M, Robbins EM, Zhang-Nunes SX, Purcell SM, Betensky RA, Raju S, et al. Characterization of amyloid deposition in the APPswe/PS1dE9 mouse model of Alzheimer disease. Neurobiol Dis 2006;24:516–24.
Gordon MN, Holcomb LA, Jantzen PT, DiCarlo G, Wilcock D, Boyett KW, et al. Time course of the development of Alzheimer-like pathology in the doubly transgenic PS1 + APP mouse. Exp Neurol 2002;173:183–95.
Goto Y, Niidome T, Hongo H, Akaike A, Kihara T, Sugimoto H. Impaired muscarinic regulation of excitatory synaptic transmission in the APPswe/PS1dE9 mouse model of Alzheimer's disease. Eur J Pharmacol 2008;583:84–91.
Gray SL, Anderson ML, Crane PK, Breitner JC, McCormick W, Bowen JD, et al. Antioxidant vitamin supplement use and risk of dementia or Alzheimer's disease in older adults. J Am Geriatr Soc 2008;56:291–5.
Hampl JS, Taylor CA, Johnston CS. Vitamin C deficiency and depletion in the United States: the Third National Health and Nutrition Examination Survey, 1988 to 1994. Am J Public Health 2004;94:870–5.
Harrison FE, May JM. Vitamin C function in the brain: vital role of the ascorbate transporter SVCT2. Free Radic Biol Med 2009.
Harrison FE, Reiserer RS, Tomarken AJ, McDonald MP. Spatial and nonspatial escape strategies in the Barnes maze. Learning & memory, vol. 13. 2006. p. 809–19. Cold Spring Harbor, N.Y.
Harrison FE, Yu SS, Van Den Bossche KL, Li L, May JM, McDonald MP. Elevated oxidative stress and sensorimotor deficits but normal cognition in mice that cannot synthesize ascorbic acid. J Neurochem 2008;106:1198–208.

Harrison FE, Hosseini AH, Dawes SM, Weaver S, May JM. Ascorbic acid attenuates scopolamine-induced spatial learning deficits in the water maze. Behav Brain Res 2009a;205(2):550–8.
Harrison FE, Hosseini AH, McDonald MP, May JM. Vitamin C reduces spatial learning deficits in middle-aged and very old APP/PSEN1 transgenic and wild-type mice. Pharmacol Biochem Behav 2009b;93:443–50.
Harrison FE, Allard J, Bixler R, Usoh C, Li L, May J. M., & McDonald, M. P. Antioxidants and cognitive training interact to affect oxidative stress and memory in APP/PSEN1 mice. Nutr Neurosci 2009c;12:203–18.
Hartman RE, Shah A, Fagan AM, Schwetye KE, Parsadanian M, Schulman RN, et al. Pomegranate juice decreases amyloid load and improves behavior in a mouse model of Alzheimer's disease. Neurobiol Dis 2006;24:506–15.
Hoehn SK, Kanfer JN. Effects of chronic ascorbic acid deficiency on guinea pig lysosomal hydrolase activities. J Nutr 1980;110:2085–94.
Holmes A, Wrenn CC, Harris AP, Thayer KE, Crawley JN. Behavioral profiles of inbred strains on novel olfactory, spatial and emotional tests for reference memory in mice. Genes Brain Behav 2002;1:55–69.
Johnston CS, Solomon RE, Corte C. Vitamin C status of a campus population: college students get a C minus. J Am Coll Health 1998;46:209–13.
Johnston CS, Thompson LL. Vitamin C status of an outpatient population. J Am Coll Nutr 1998;17:366–70.
Kaufmann P, Wiens W, Dirks M, Krehbiel D. Changes in social behavior and brain catecholamines during the development of ascorbate deficiency in guinea pigs. Behav Processes 1986;13:13–28.
Kawarabayashi T, Younkin LH, Saido TC, Shoji M, Ashe KH, Younkin SG. Age-dependent changes in brain, CSF, and plasma amyloid (beta) protein in the Tg2576 transgenic mouse model of Alzheimer's disease. J Neurosci 2001;21:372–81.
Lalonde R, Kim HD, Fukuchi K. Exploratory activity, anxiety, and motor coordination in bigenic APPswe + PS1/DeltaE9 mice. Neurosci Lett 2004;369:156–61.
Lalonde R, Kim HD, Maxwell JA, Fukuchi K. Exploratory activity and spatial learning in 12-month-old APP(695)SWE/co + PS1/DeltaE9 mice with amyloid plaques. Neurosci Lett 2005;390:87–92.
Laurin D, Masaki KH, Foley DJ, White LR, Launer LJ. Midlife dietary intake of antioxidants and risk of late-life incident dementia: the Honolulu–Asia Aging study. Am J Epidemiol 2004;159:959–67.
Lim GP, Chu T, Yang F, Beech W, Frautschy SA, Cole GM. The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. J Neurosci 2001;21:8370–7.
Luchsinger JA, Tang MX, Shea S, Mayeux R. Antioxidant vitamin intake and risk of Alzheimer disease. Arch Neurol 2003;60:203–8.
Machova E, Jakubik J, Michal P, Oksman M, Iivonen H, Tanila H, et al. Impairment of muscarinic transmission in transgenic APPswe/PS1dE9 mice. Neurobiol Aging 2008;29:368–78.
Maeda N, Hagihara H, Nakata Y, Hiller S, Wilder J, Reddick R. Aortic wall damage in mice unable to synthesize ascorbic acid. Proc Natl Acad Sci U S A 2000;97:841–6.
Manelli AM, Puttfarcken PS. Beta-amyloid-induced toxicity in rat hippocampal cells: *in vitro* evidence for the involvement of free radicals. Brain Res Bull 1995;38:569–76.
Mark RJ, Blanc EM, Mattson MP. Amyloid beta-peptide and oxidative cellular injury in Alzheimer's disease. Mol Neurobiol 1996;12:211–24.
Mathur A, Shandarin A, LaViolette SR, Parker J, Yeomans JS. Locomotion and stereotypy induced by scopolamine: contributions of muscarinic receptors near the pedunculopontine tegmental nucleus. Brain Res 1997;775:144–55.
Mattson MP. Existing data suggest that Alzheimer's disease is preventable. Ann N Y Acad Sci 2000;924:153–9.
May JM, Qu ZC, Mendiratta S. Protection and recycling of alpha-tocopherol in human erythrocytes by intracellular ascorbic acid. Arch Biochem Biophys 1998;349:281–9.
McDonald MP, Dahl EE, Overmier JB, Mantyh P, Cleary J. Effects of an exogenous beta-amyloid peptide on retention for spatial learning. Behavioral Neural Biol 1994;62:60–7.
McDonald MP, Overmier JB, Bandyopadhyay S, Babcock D, Cleary J. Reversal of beta-amyloid-induced retention deficit after exposure to training and state cues. Neurobiol Learn Mem 1996;65:35–47.
McDonald MP, Wong R, Goldstein G, Weintraub B, Cheng SY, Crawley JN. Hyperactivity and learning deficits in transgenic mice bearing a human mutant thyroid hormone beta1 receptor gene. Learning & Memory, vol. 5. 1998. p. 289–301. Cold Spring Harbor, N.Y.
Minamizono A, Tomi M, Hosoya K. Inhibition of dehydroascorbic acid transport across the rat blood–retinal and –brain barriers in experimental diabetes. Biol Pharm Bull 2006;29:2148–50.
Miyakawa T, Yamada M, Duttaroy A, Wess J. Hyperactivity and intact hippocampus-dependent learning in mice lacking the M1 muscarinic acetylcholine receptor. J Neurosci 2001a;21:5239–50.
Miyakawa T, Yared E, Pak JH, Huang FL, Huang KP, Crawley JN. Neurogranin null mutant mice display performance deficits on spatial learning tasks with anxiety related components. Hippocampus 2001b;11:763–75.
Morris MC, Beckett LA, Scherr PA, Hebert LE, Bennett DA, Field TS, et al. Vitamin E and vitamin C supplement use and risk of incident Alzheimer disease. Alzheimer Dis Assoc Disord 1998;12:121–6.
Morris MC, Evans DA, Bienias JL, Tangney CC, Bennett DA, Aggarwal N, et al. Dietary intake of antioxidant nutrients and the risk of incident Alzheimer disease in a biracial community study. JAMA 2002;287:3230–7.
Nunomura A, Moreira PI, Lee HG, Zhu X, Castellani RJ, Smith MA, et al. Neuronal death and survival under oxidative stress in Alzheimer and Parkinson diseases. CNS & neurological disorders drug targets 2007;6:411–23.
O'Neill RD, Fillenz M. Circadian changes in extracellular ascorbate in rat cortex, accumbens, striatum and hippocampus: correlations with motor activity. Neurosci Lett 1985;60:331–6.
Pachla LA, Kissinger PT. Analysis of ascorbic acid by liquid chromatography with amperometric detection. Methods Enzymol 1979;62:15–24.
Pappolla MA, Chyan YJ, Omar RA, Hsiao K, Perry G, Smith MA, et al. Evidence of oxidative stress and *in vivo* neurotoxicity of beta-amyloid in a transgenic mouse model of Alzheimer's disease: a chronic oxidative paradigm for testing antioxidant therapies *in vivo*. Am J Pathol 1998;152:871–7.
Paraskevas GP, Kapaki E, Libitaki G, Zournas C, Segditsa I, Papageorgiou C. Ascorbate in healthy subjects, amyotrophic lateral sclerosis and Alzheimer's disease. Acta Neurol Scand 1997;96:88–90.
Parle M, Dhingra D. Ascorbic acid: a promising memory-enhancer in mice. J Pharmacol Sci 2003;93:129–35.
Parsons KK, Maeda N, Yamauchi M, Banes AJ, Koller BH. Ascorbic acid-independent synthesis of collagen in mice. Am J Physiol 2006;290:E1131–9.
Perluigi M, Joshi G, Sultana R, Calabrese V, De Marco C, Coccia R, et al. *In vivo* protective effects of ferulic acid ethyl ester against amyloid-beta peptide 1-42-induced oxidative stress. J Neurosci Res 2006;84:418–26.
Pierce RC, Rebec GV. Stimulation of both D1 and D2 dopamine receptors increases behavioral activation and ascorbate release in the neostriatum of freely moving rats. Eur J Pharmacol 1990;191:295–302.
Platel A, Porsolt RD. Habituation of exploratory activity in mice: a screening test for memory enhancing drugs. Psychopharmacology 1982;78:346–52.
Quinn JF, Bussiere JR, Hammond RS, Montine TJ, Henson E, Jones RE, et al. Chronic dietary alpha-lipoic acid reduces deficits in hippocampal memory of aged Tg2576 mice. Neurobiol Aging 2007;28:213–25.
Reiserer RS, Harrison FE, Syverud DC, McDonald MP. Impaired spatial learning in the APPSwe + PSEN1DeltaE9 bigenic mouse model of Alzheimer's disease. Genes Brain Behav 2007;6:54–65.
Resende R, Ferreiro E, Pereira C, Resende de Oliveira C. Neurotoxic effect of oligomeric and fibrillar species of amyloid-beta peptide 1-42: involvement of endoplasmic reticulum calcium release in oligomer-induced cell death. Neuroscience 2008;155(3):725–37.
Riviere S, Birlouez-Aragon I, Nourhashemi F, Vellas B. Low plasma vitamin C in Alzheimer patients despite an adequate diet. Int J Geriatr Psychiatry 1998;13:749–54.
Saner A, Weiser H, Hornig D, Da Prada M, Pletscher A. Cerebral monoamine metabolism in guinea-pigs with ascorbic acid deficiency. J Pharm Pharmacol 1975;27:896–902.
Siesser WB, Zhao J, Miller LR, Cheng SY, McDonald MP. Transgenic mice expressing a human mutant beta1 thyroid receptor are hyperactive, impulsive, and inattentive. Genes Brain Behav 2006;5:282–97.
Sipos ML, Burchnell V, Galbicka G. Dose–response curves and time-course effects of selected anticholinergics on locomotor activity in rats. Psychopharmacology 1999;147:250–6.
Stackman RW, Eckenstein F, Frei B, Kulhanek D, Nowlin J, Quinn JF. Prevention of age-related spatial memory deficits in a transgenic mouse model of Alzheimer's disease by chronic *Ginkgo biloba* treatment. Exp Neurol 2003;184:510–20.
Sung S, Yao Y, Uryu K, Yang H, Lee VM, Trojanowski JQ, et al. Early vitamin E supplementation in young but not aged mice reduces Abeta levels and amyloid deposition in a transgenic model of Alzheimer's disease. Faseb J 2004;18:323–5.
Sweeney WA, Luedtke J, McDonald MP, Overmier JB. Intrahippocampal injections of exogenous beta-amyloid induce postdelay errors in an eight-arm radial maze. Neurobiol Learn Mem 1997;68:97-101.
Tamagno E, Guglielmotto M, Aragno M, Borghi R, Autelli R, Giliberto L, et al. Oxidative stress activates a positive feedback between the gamma- and beta-secretase cleavages of the beta-amyloid precursor protein. J Neurochem 2008;104:683–95.
Uberti D, Bianchi I, Olivari L, Ferrari-Toninelli G, Canonico P, Memo M. Pramipexole prevents neurotoxicity induced by oligomers of beta-amyloid. Eur J Pharmacol 2007;569:194–6.
Yang X, Yang Y, Li G, Wang J, Yang ES. Coenzyme Q10 attenuates beta-amyloid pathology in the aged transgenic mice with Alzheimer presenilin 1 mutation. J Mol Neurosci 2008;34:165–71.