Ascorbate Deficiency Accelerates Neuropathology in Alzheimer's Disease

By

Shilpy Dixit

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in

Neuroscience

September 30, 2017

Nashville, TN

Approved:

Christine Konradi, Ph.D. (Chair)

Aaron Bowman, Ph.D.

Laura Dugan, M.D.

James May, M.D.

Abstract

Subclinical ascorbate deficiency is widespread in many populations, but its role in both Alzheimer's disease and normal aging is understudied. We decreased brain ascorbate 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 expression of the sodium-dependent vitamin C transporter required for neuronal ascorbate transport. At 6 months, $SVCT2^{+/-}$ and APP/PSEN1 mice and the combination genotype $SVCT2^{+/-}$;APP/PSEN1 were impaired on multiple tests of cognitive ability and exhibited increased oxidative stress in the brain. Increased amyloid plaque burden was observed in $SVCT2^{+/-}$;APP/PSEN1 mice compared to APP/PSEN1 mice at 14 months. At 4 months of age, during what is considered a prodromal stage in mouse models of Alzheimer's disease, mitochondria isolated from $SVCT2^{+/-}$ and APP/PSEN1 mice showed disruptions in oxygen consumption and mitochondrial respiration with opposing directionality indicating that different pathways were being affected. Both groups had significant increases in the production of reactive oxygen species. Additionally, genes commonly associated with Alzheimer's disease pathology and progression were up-regulated in $SVCT2^{+/-}$;APP/PSEN1 mice and APP/PSEN1, with a greater magnitude of gene expression change in $SVCT2^{+/-}$;APP/PSEN1 mice. Furthermore, 5-hydroxymethylcytosine levels, indicative of epigenetic modification, were significantly increased compared to wild type at 4 months. There were differing patterns of gene expression based on $SVCT2^{+/-}$ and APP/PSEN1 status, as well as with age, suggesting an age-related change in gene expression occurs earlier in APP/PSEN+ groups and is exacerbated in combination with ascorbate deficiency. These data support the hypothesis that even moderate ascorbate deficiency plays an important role in accelerating amyloid pathogenesis, particularly during early stages of disease development, and that oxidative stress pathways likely modulate these effects.

Approved by: Dr. Fiona Harrison

Acknowledgements

I would like to express the deepest appreciation to my mentor Dr. Fiona Edith Pfeifer Harrison, who has the attitude and the substance of a genius, and who continually conveyed integrity and enthusiasm with regard to research and scholarship. Without her guidance, this dissertation would not have been possible.

I would like to thank my committee members, Dr. Christine Konradi (Chair), Dr. Aaron Bowman, Dr. Laura Dugan and especially Dr. James May for providing their expertise in my work and for taking an interest in my professional development. Their dedication demonstrated to me that a passion for the science should always accompany the pursuit of research. In addition, I would like to extend a heartfelt thank you to Dr. Joshua Fessel for the crash course in mitochondrial biology, for his expertise in experimental design and for his enduring encouragement.

This work could not have been completed without the resources and support of the Neuroscience Graduate Program at Vanderbilt University and the funding provided by Dr. Harrison.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

4-HNE	4-hydroxynonenal
A2m	Alpha-2-macroglobulin
Ache	Acetylcholinesterase
AD	Alzheimer's disease
AFR	Ascorbate free radical
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
ASC	Ascorbate
Aβ	Amyloid β
BACE1	Beta-site APP cleaving enzyme 1
BACE2	Beta-site APP cleaving enzyme 2
Bdnf	Brain-derived neurotrophic factor
Casp 3	Caspase 3
Casp 4	Caspase 4
Cdk1	Cyclin-dependent kinase 1
Cdk5	Cyclin-dependent kinase 5
Chat	Choline acetyltransferase
Ctsb	Cathepsin B
Ctsd	Cathepsin D
DHA	dehydroascorbate
DNP	2,4-dinitrophenol
Gulo	L-gulono-γ-lactone oxidase
Hsd17b10	Hydroxysteroid (17-beta) dehydrogenase 10
Igf2	Insulin-like growth factor 2
Il1a	Interleukin 1 alpha
Lrp1	Low density lipoprotein receptor-related protein 1
Lrp6	Low density lipoprotein receptor-related protein 6
Lrp8	Low density lipoprotein receptor-related protein 8
Map2	Microtubule-associated protein 2
Mapt	Microtubule-associated protein tau
MDA	malondialdehyde
PSEN1	Presenilin 1
PSEN2	Presenilin 2
ROS	Reactive oxygen species
SVCT1	Sodium-dependent vitamin C transporter, type 1
SVCT2	Sodium-dependent vitamin C transporter, type 2
TMRE	tetramethylrhodamine, ethyl ester

Chapter I: Introduction

The multifaceted etiology of Alzheimer's disease

The amyloid cascade hypothesis

Alzheimer's disease (AD) is a significant public health concern in the United States, with projected health care costs reaching trillions of dollars over the next few decades. More than 90% of AD cases occur with no identifiable cause. For many years the leading dogma in the field of AD research, referred to as the "amyloid cascade hypothesis", suggested that the accumulation of amyloid beta (Aβ) and hyperphosphorylated tau peptides are toxic to the brain environment leading to global loss of neurons [1]. These histopathological hallmarks, and mild cognitive impairment in most cases, persist today as the criteria for diagnosis of the disease, but neither has been definitively affirmed as the solely or specifically cause or consequence of the disease. Aβ is produced by cleavage of the transmembrane amyloid precursor protein (APP) by secretase enzymes [2]. Under normal conditions, APP is cleaved by α- and γ-secretase; however, a shift to the amyloidogenic cleavage of APP by β- and γ-secretase results in 38-43 amino acid Aβ peptides that aggregate to form insoluble oligomers, which are toxic to cells [3]. The second major histopathological hallmark of AD is the hyperphosphorylation of tau. This inhibits the ability of the tau proteins to stabilize microtubule assembly and promotes neurofibrillary tangles [2,4], which disrupt intracellular transport [5]. A significant caveat is that some individuals who appear to be at a greater risk for dementia, or already exhibit substantial Aβ plaques and neurofibrillary tangles, never

experience cognitive decline [1,6], suggesting these pathological features alone do not correlate to cognitive impairment [7–9].

The mitochondrial cascade hypothesis

Mitochondria are highly oxidative organelles capable of producing large amounts of reactive oxygen species (ROS), and mitochondrial dysfunction is observed in several neurological diseases [10,11]. According to the "mitochondrial cascade hypothesis" of AD, age-related mitochondrial dysfunction elevates levels of ROS. Along with age-related decreases in the activity of endogenous antioxidant enzymes, this leads to a redox imbalance in the brain capable of driving AD pathology by up-regulating Aβ production and tau phosphorylation [12–15]. The consequences of this dysfunction are a reduction in available energy for proper cellular function and activation of mitochondria-mediated apoptotic pathways. Even with multiple mitochondria within a neuron, oxidative damage can cause a collapse of mitochondrial membrane potential and mitochondrial DNA mutations that compromise complexes I, III, IV of the electron transport chain and ATP synthase [4,10,14], leading to a progressive disruption of energy production through mitochondrial proliferation that emerges before the classical pathological features of AD.

Structural and functional alterations in mitochondria in Alzheimer's disease

Mitochondria have highly specialized structural morphology that dictate both function and productivity, but it is the balance between fusion and fission events that governs health of mitochondria within a cell [16]. Fusion between two mitochondria compensates for deficiencies in function because it allows for the sharing of mitochondrial DNA and membrane

proteins that may have become damaged over time. Conversely, fission facilitates the recycling of irreversibly damaged mitochondria through autophagy of the organelle, or mitophagy. In mouse models of AD, Aβ peptides interact with and alter mitochondrial structure and function before significant plaque deposition is observed [17]. Intracellular accumulation of Aβ peptides localizes with mitochondrial markers and appears to enter mitochondria through translocase machinery on the outer membrane [18]. Choi *et al.* report that hippocampal mitochondria in 7-month-old APP/PSEN1 mice, a mouse model of AD, exhibited bulging outer membranes along with misshapen and absent cristae, but the same was not observed in age-matched wild type mice [19]. Genes associated with mitochondrial biogenesis and replication, and complexes I-IV which are necessary for oxidative phosphorylation were down-regulated in the hippocampi of 3-month-old APP/PSEN1 mice compared to age-matched wild type mice [17], suggesting diminished energy metabolism at very early stages of the disease. Additionally, an increase in fission gene expression and a decrease in fusion gene expression were reported in post-mortem brain specimens from patients with early and late stage AD [20,21] indicating a shift toward mitochondrial fission and increases in mitochondrial DNA oxidation in patients with mild cognitive impairment and early-stage AD [13]. Animal models of AD exhibit similar morphological alterations as well as decreased levels of mitochondrial fusion proteins and increased levels of fission proteins at early stages of disease progression [22]. The accumulation of Aβ peptides by the overexpression of the APP in the Arctic APP mouse model decreased ATP production, impaired mitochondrial membrane potential, and inhibited complex IV activity, in addition to exacerbating oxidative stress [11,23]. While the relationship between mitochondrial dysfunction and AD pathology has yet to be clearly defined, once established this relationship

becomes cyclical: mitochondrial dysfunction increases ROS production and creates an energy deficit for protein degradation leading to an accumulation of toxic Aβ, which then contributes to further oxidative damage capable of inducing cell death.

Contribution of oxidative stress

Technological advances improve our ability to isolate key pathological, cellular and molecular changes that accompany both the inherited and sporadic forms of AD to identify very early stages of disease, particularly in at-risk individuals, sometimes decades before the clinical manifestation of cognitive dysfunction [1,24]. Oxidative stress, which is defined as an imbalance in the homeostatic relationship between oxidation species and antioxidant defenses, manifests as oxidative damage to lipids, proteins and nucleic acids. This oxidative damage contributes to the development and progression of AD pathology and can cause significant alterations to downstream mechanisms involved in energy metabolism and proteolysis, thus compromising cellular repair mechanisms [25–27].

Alterations in gene expression occur with age [28]; however, even greater changes in expression of genes involved in oxidative stress, amyloid beta production and inflammation are observed in AD patients compared with controls [29]. Studies show that oxidative stress increases the expression of presenilin-1 (PSEN1), and BACE1, both of which are major components of the two secretase enzymes responsible for amyloidogenic cleavage of the APP [30–32]. Aβ peptides are produced and degraded under normal conditions throughout life; however, increasing evidence has implicated Aβ oligomers in the neurotoxicity associated oxidatively modified molecules [33,34]. Li *et al.* reported hippocampal neurons treated with glycated Aβ, a result of protein oxidation, showed decreased viability, increased

tau hyperphosphorylation and apoptosis compared to neurons treated with Aβ alone [35]. Consistent with evidence that chronic neuroinflammation is observed in AD, ROS can also trigger the release of inflammatory cytokines, which themselves contribute to oxidative stress, fueling a damaging feedback cycle [36,37].

Lipid peroxidation refers to the degradation of lipids following oxidative attack. Clinical studies indicate lipid peroxidation is elevated as a result of age, with even greater increases observed in AD brains compared with age-matched controls [38,39]. Lipid peroxidation end products, such as isoprostanes, malondialdehyde (MDA), and 4-hydroxynonenal (4-HNE) are highly reactive and disrupt normal cellular function [40]. Aβ-induced cell death also appears to be mediated through 4-HNE [31], by activation of the caspase cascade and mitochondria-mediated apoptotic pathways [41,42]. Several studies show that 4-HNE is able to bind to and inhibit membrane components such as glucose and glutamate transporters [43,44], and Na/K ATP-ase [45], resulting in structural alterations, loss of function and resistance of dysfunctional proteins to degradation [27]. Redox proteomics studies in AD brains have identified oxidatively modified proteins associated with ATP production and proteasome activity [46]. These findings are consistent with the decreased energy metabolism and accumulation of damaged proteins due to deficient degradation observed in the brains of AD patients [6].

Vitamin C: a therapeutic strategy for protection

Current interventions do not address the role of oxidative stress in AD pathogenesis or progression, but preventative strategies to delay pathological decline by attenuating oxidative stress may delay age of onset of cognitive decline. Interventions after the onset of clinical

symptoms, such as impaired memory and reasoning, are far more challenging due to the extent of damage, whereas preventative strategies to delay pathological decline would also delay age of onset of cognitive decline. The activity of endogenous antioxidant enzymes and levels of nutrient-based antioxidants are significantly reduced in early and late stages of AD [12]. While little can be done to increase levels of endogenous antioxidants in AD patients, proper diet and supplementation can easily and efficiently reinforce a depleted antioxidant barrier. Vitamin C, known as ascorbate (ASC) when referring to activity or biosynthesis within the body, is an extraordinarily powerful, water-soluble antioxidant capable of reducing most oxidant species due to its the low reduction potential [47]. The abbreviation "ASC" will be used to described vitamin C moving forward in this document. ASC scavenges aqueous free radicals, such as ROS and reactive nitrogen species, which cause oxidative damage to proteins, nucleic acids and lipids. ASC also recycles vitamin E, a lipophilic antioxidant, thereby supporting the overall redox balance in the body and preventing oxidative stress.

ASC transport and metabolism

ASC is absorbed into the bloodstream from ingested food through the sodium-dependent Vitamin C transporter, type 1 (SVCT1) which is primarily expressed in the intestines and kidneys. ASC is taken up by most tissues on the ubiquitously expressed sodium-dependent vitamin C transporter, type 2 (SVCT2), which has a higher affinity for ASC [48]. SVCT2 transports ASC from the bloodstream into cerebrospinal fluid at the choroid plexus and then into neurons. In humans, typical plasma concentrations range from 40-60μM and cerebrospinal fluid concentrations range from 100-200μM [48]. Tissue concentrations, diagrammed in **Figure 1**, differ based on the type and amount of transporter expressed,

which is ultimately determined by specific tissue requirements. The highest concentrations of ASC in the body are found in the brain, which is particularly vulnerable to oxidative damage because of its enriched lipid composition and significant oxygen consumption [36]. The brain is highly metabolically active compared to the periphery, which generates higher concentrations of ROS, however the brain has lower concentrations of enzymatic antioxidants compared to the periphery, supporting the requirement for additional antioxidant resources [12]. Neuronal concentrations (2-10mM) are as much as ten-fold greater than glial concentrations (1mM) and concentrations across different brain areas also differ, presumably due to higher rates of metabolism [47,49,50].

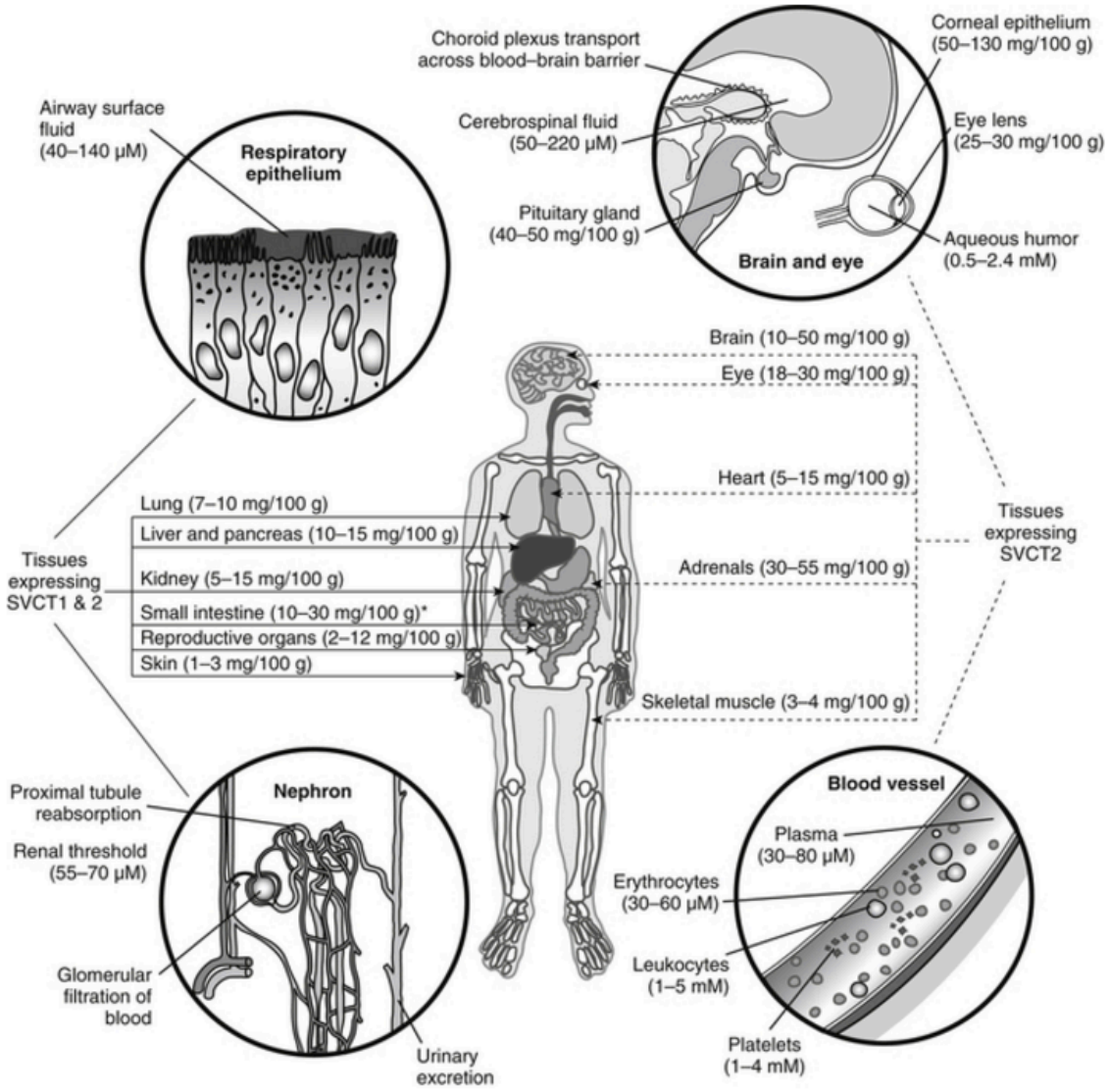


Figure 1. ASC concentrations are determined by SVCT distribution. Michels, A., Frei, B. (2017, Feb. 26) Vitamin C.

Retrieved from www.basicmedicalkey.com/vitamin-c/

Several intracellular recycling mechanisms exist in the brain in order to maintain such high concentrations [47] (**Fig. 2**). Ascorbic acid is oxidized to the ascorbate free radical (AFR) by scavenging ROS and reactive nitrogen species, or through enzymatic activity. NADH/NADPH-dependent enzymes and glutathione can reduce AFR, and AFR molecules preferentially react with one another to produce a reduced ascorbic acid molecule and a twice-oxidized dehydroascorbate (DHA) molecule via a dismutation reaction. DHA has a half-life of only 6 minutes at physiological pH, which accounts for the gradual loss of ascorbic acid stores in the body. DHA can be transported in and out of cells on the GLUT transporters and reduced to ascorbic acid by NADPH-dependent enzymes and glutathione. In fact, astrocytes do not express SVCT2 endogenously, but accumulates ascorbic acid by taking up and recycling DHA. Astrocytes exhibit a synergistic hetero-exchange of glutamate uptake and ascorbic release, presumably to maintain redox balance at the level of the synapse [48].

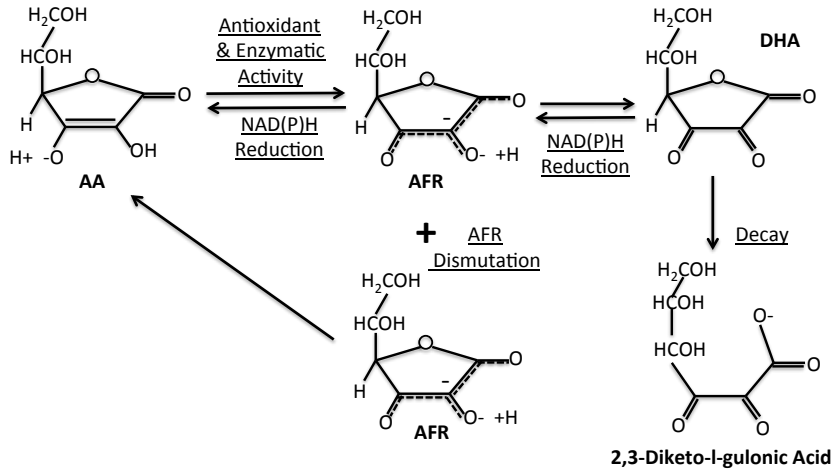


Figure 2 Ascorbic acid oxidation and reduction. Ascorbic acid is recycled from the ascorbate free radical (AFR) and dehydroascorbate (DHA) through enzymatic and dismutation reactions.

ASC as an enzymatic co-factor

ASC has an established function as a co-factor in 2-oxoglutarate-dependent dioxygenases enzymes, which require 2-oxoglutarate and iron to incorporate oxygen into molecules. It is proposed that ASC prevents inhibitory self-oxidation in these enzymatic reactions [51]. This family of enzymes are involved in several biochemical pathways including synthesis of essential molecules and catecholamines, cell signaling and gene transcription, and DNA demethylation [52]. ASC supplementation reduced DNA damage following carcinogenic or mutagenic exposure [53]. ASC concentrations in the brains of mice and rats are highest perinatally [50,54], and several studies have shown that cellular reprogramming via demethylation is impacted by the availability of ASC [55–58]. The addition of ASC to stem cells and mouse embryonic fibroblasts promotes DNA demethylation and histone modification suggesting an important epigenetic role during development.

ASC and oxidative stress

As little as a 30% deficiency of ASC in brain induces oxidative stress in mice whether from decreased expression of SVCT2, the sole ASC transporter expressed in the central nervous system [59,60], or decreased dietary intake in mice that lack the ability to synthesize AA in the liver (gulo-/-) [61,62]. Our research group first established elevated markers of oxidative stress and sensorimotor deficits in gulo-/- mice even after supplementation to restore physiological ASC levels [60,62], indicating lasting changes to physiology. In a follow-up study conducted by Harrison *et al.*, ASC fell precipitously in the cortex of non-supplemented gulo-/- pups between P1 (5.5umol/g) and P18 (1.5umol/g), indicating significant ASC turnover in the brain. Interestingly, no significant difference in MDA levels, a

marker of lipid peroxidation, was observed in the cortex of gulo-/- pups compared with littermate controls, which may account for the rapid decrease in ASC concentration. One caveat to consider is that genetic manipulation, though a powerful experimental tool, does not account for changes to synergistic mechanisms that occur through evolution. Guinea pigs, like humans, cannot synthesize ASC due to an evolutionary mutation in the gene for L-gulono-γ-lactone oxidase (gulo). This model may resemble homeostatic regulation of ASC and deficiency in humans more closely than the gulo-/- mouse model. Paidi *et al.* investigated the effects of pre- and post-natal ASC deficiency and repletion on MDA levels in the brains of gestationally-depleted guinea pigs [63]. Brain and plasma ASC concentrations were significantly lower, while MDA levels were significantly higher in the depleted condition compared with the repleted condition. Importantly, no significant difference was observed on any measure in repleted animals compared with control, which highlights mechanistic differences between the guinea pig and mouse models. Guinea pigs may have better oxidative damage repair mechanisms in repleted conditions that co-evolved with L-gulono-γ-lactone oxidase inactivation.

In human studies, ASC supplementation of at least 250mg per day increased plasma concentrations of ASC and lowered markers of systemic lipid peroxidation in all participants [64]. Additionally, studies in which ASC supplementation resulted in plasma concentration that were consistently greater than 50μmol/L decreased markers of oxidative damage to DNA such as chromosomal abnormalities, DNA adducts, and DNA strand breaks, while increasing markers of DNA repair mechanisms. These data support a direct relationship between oxidative stress and ASC deficiency, and the protective role ASC has in the body. ASC supplementation provides a potential strategy to slow the progression of AD by attenuating

the damaging effects of oxidative stress and may prove to be a valuable addition to current therapies.

ASC attenuates Alzheimer's disease pathology

Clinical studies suggest nutrient-based antioxidant supplementation, including vitamins C and E, confers some protection against cognitive decline associated with AD [65–68]. A meta-analysis evaluating oxidative stress biomarkers confirmed decreased antioxidant capacity in the plasma of patients with AD, suggesting that antioxidant defenses are being consumed more rapidly in these individuals because of increased oxidative stress associated with AD [39]. In *in vitro* studies, Aβ-induced cell death is mediated via oxidative stress [41,42], tying the etiological pathways together. Animal studies offer more definitive evidence that ASC not only ameliorates oxidative stress, but also inhibits the production of Aβ [69–73]. Acute administration of ASC improved cognitive deficits in AD mouse models without affecting plaque formation [74,75]. 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 hypoxia [76–79]. Similarly, vitamin E supplementation to Tg2576 mice decreased oxidative stress in the brain and decreased $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ 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 months [80]. Six months of a medical food cocktail containing vitamins C and E, among several other constituents, also decreased soluble and insoluble $A\beta_{1\text{-}40}$ as well as soluble $A\beta_{1\text{-}42}$ in Tg2576 mice [81]. 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 $A\beta_{1-40}$ and $A\beta_{1-42}$ in APP/PSEN1^{+} mice at 6 months of age [82].

A study by Murakami *et al*. investigated chronic ASC administration and Aβ pathology in the AβPP transgenic AD mouse model. In this study, mice were treated with ~1000mg of ASC daily through *ad libitum* access to supplemented drinking water for six months [69]. At twelve months of age, the mice were assessed for differences in oxidative stress and AD pathology, with particular interest in Aβ assembly. As expected, the ASC-treated group showed decreased levels of protein carbonylation, as a marker of oxidative stress, and increased glutathione, an endogenous antioxidant capable of recycling ASC. Interestingly, ASC-treated mice showed a decrease in total soluble Aβ42 (ELISA), Aβ dimers (ELISA, Western blot) and phosphorylated tau protein (Western blot) compared with vehicle-treated mice. Congruent with studies employing acute ASC administration, chronic ASC supplementation did not alter plaque formation in AβPP mice (Murakami, 2011). The ASC-treated AβPP group performed more like wild-type controls on Y-maze tasks than did the vehicle-treated AβPP group; with no observed improvement on memory tasks (Murakami, 2011). Kook *et al.* reported similar histopathological results using a gulo-/-;5XFAD transgenic AD mouse model [70]. In this study, higher ASC supplementation (3.3g/L) reduced overall amyloid plaque burden. This finding is inconsistent with the studies discussed above; however, the mice in this study began supplementation much earlier and carry a greater mutation burden for a more severe phenotype. The authors from either study did not venture to characterize a mechanism by which ASC is protective; however the presence of oxidants has been shown to induce Aβ and neurofibrillary tangle assembly. Thus, ASC may prevent AD pathology by scavenging reactive species before they impact Aβ and tau pathologies.

ASC at the subcellular level

SVCT2 co-localizes with several organelle markers and compartmentalized increases in ASC concentration indicate a specific requirement for ASC at the subcellular level [reviewed in 76]. As discussed previously, ASC plays a role in differentiation and epigenetic modification based on its role as an enzymatic co-factor, many of which are localized to the nuclear compartment. SVCT2 also co-localized with proteins of the endoplasmic reticulum, which also utilizes 2-oxoglutarate-dependent dioxygenases enzymes in protein biosynthesis [84]. Of particular interest, the localization of SVCT2 on mitochondria isolated from guinea pig liver was first identified in a study published in 1982 [85]. This study not only reported that mitochondrial membranes were permeable to DHA in the presence of the ascorbate oxidase enzyme, as determined by HPLC, but also that ASC concentrations in isolated mitochondria reflected the ASC concentration in which they were incubated suggesting that DHA transport and recycling was not the primary mode ASC accumulation. Recent reports show SVCT2 expression in mitochondria maintains kinetics similar to SVCT2 in plasma membranes in cell lines [86,87]. With regard to the relationship between mitochondria and AD pathogenesis, in addition to reducing plaque burden, Kook et al. found pronounced morphological changes in mitochondria from gulo-/-;5XFAD mice on low ASC supplementation compared with the high supplementation group suggesting ASC is necessary for maintaining mitochondrial integrity by mitigating oxidative damage to the organelle [70]. *What is still unknown is how ASC deficiency can compromise cellular function at the subcellular level within the context of disease outcomes.*

Significance

Alzheimer's disease is a balance of risk and benefit

Risk factors most closely associated with increased risk of developing AD include age, genetic markers [88], family history [13] and environmental or lifestyle choices. Low socioeconomic status, which is associated with increased AD risk factors, such as decreased access to education and increased reports of chronic stress, have also been shown to correlate with poor nutrition from development throughout life [89]. In contrast, years of education, avoiding social isolation and regular exercise are strongly associated with decreased risk. Adherence to the Mediterranean diet, which emphasizes high intake of fruits and vegetables and unsaturated fatty acids, reduces risk of developing AD, indicating that nutrition plays an important role in modifying risk [88,90–92]. The "threshold to disease" concept is often applied to conditions in which no single etiology has been identified: it is the additive effect of risk factors and risk modifiers that ultimately confers individual risk for disease and the impact that pathological changes have on cognitive function. This balancing of risk factors and risk modifiers suggests that alternative pathways may be the driving force behind degeneration observed in Alzheimer's disease. ASC is essential to maintaining normal biological function and the cellular redox balance; therefore, life-long maintenance of adequate ASC concentration can act as a risk modifier for AD.

ASC deficiency is a public health issue

The most severe form of ASC deficiency is most commonly associated with ocean voyages and sailors developing scurvy, a disease in which collagen is destabilized by the lack of ASC. The earliest descriptions of the disease were documented in records as far back

as Hippocrates, but it was not until the 18th century that scurvy prevention was linked to diet, specifically to ascorbic acid [93]. While a very low daily dose (10mg/d) is sufficient to prevent scurvy [94,95], ASC deficiency is common due to advanced disease (e.g. cancer and neurodegenerative diseases), lifestyle choice and food insecurity, which is defined by the USDA as anything from reduced quality or variety of nutritious food to disrupted or inadequate food intake. Affordable, shelf stable foods are often calorie dense but do not provide adequate nutrition. ASC depletion (defined as lower than 28 μM) is typically found in 10-30% of populations, and cases of extreme deficiency (<11 μM) are also reported, with low-income and elderly individuals being at even greater risk [96–99]. GWAS studies report that SVCT polymorphisms can alter dietary ASC absorption and are associated with lower concentrations in biological fluids [100–102]. While the brain can preferentially store and recycle ASC under conditions of inadequate intake, in extreme deficiency conditions, or under prolonged periods of deficiency, these mechanisms are unable to maintain sufficient ASC concentrations. ASC deficient individuals are vulnerable to oxidative stress, which renders these individuals more vulnerable to developing disease with more rapid decline and poorer outcomes.

Contributions to the field

The research described herein was designed to investigate the role of ASC deficiency in impaired mitochondrial function and oxidative stress, and how these events contribute to sub-cellular damage and genetic modification associated with AD-related neurodegeneration. To date, very few studies have addressed deficiency, rather opting for ASC supplementation in attenuating disease pathology. We are well-equipped to conduct this research because we

are the only lab to maintain colonies of all known mouse models of ASC deficiency and altered transporter expression, whereas supplementation studies have been critically limited by the use of rodent models that cannot become deficient due to conserved ASC biosynthesis and transport. Using a genetic modification to disrupt ASC transport in the brain ($SVCT2^{+/-}$), we have modeled the effects of human ASC deficiency in AD using the $APP_{SWE}/PSEN1_{dE9}$ (APP/PSEN1) mouse model that predisposes mice to the development Aβ pathologies. Chapter III highlights a direct relationship between ASC deficiency and the oxidative stress and cognitive decline associated with AD pathology. Chapter IV addresses the effects of ASC deficiency and Aβ pathologies on mitochondrial activity: specifically, oxygen consumption, mitochondrial membrane potential and the generation of ROS. Lastly, Chapter V details the role of ASC deficiency in the expression of AD-related genes. The ultimate goal of this work is to establish adequate ASC intake as an effective and inexpensive path to life-long brain health and a necessary preventative strategy against neurodegenerative disease, as well as offer new avenues for targeted interventions.

Chapter II: Materials and Methods

Animals

All animals were housed in a temperature and humidity controlled vivarium and were kept on a 12:12 hour light cycle. Female C57Bl/6J wild-type mice (http://jaxmice.jax.org/strain/000664.html) and male bigenic $APP_{SWE}/PSEN1_{\Delta E9}$ 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 $SVCT2^{+/+}$ littermates [59]. 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. SVCT2 transgenic mice ($SVCT2^{Tg}$) used in Chapter 4 were developed for our research group, and exhibit a global increase in SVCT2 expression and concomitant increases in ASC concentration [103]. 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). *All procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee.*

Methods for Chapter 3

For Chapter 3, the total numbers of mice available for behavioral and biochemical studies for each group are presented in **Table 1.**

Table 1. Total numbers of mice included in behavioral and biochemical studies

	5 months – behavior 6 months - neurochemistry		12 months – behavior 14 months - neurochemistry	
	APP/PSEN1-	**APP/PSEN1+**	**APP/PSEN1-**	**APP/PSEN1+**
SVCT2$^{+/-}$	**"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$^{+/+}$	**"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.

Behavioral testing

All behavior testing was undertaken using facilities of the Vanderbilt Murine Neurobehavioral Core. The experimental design is shown in **Figure 3**. 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).

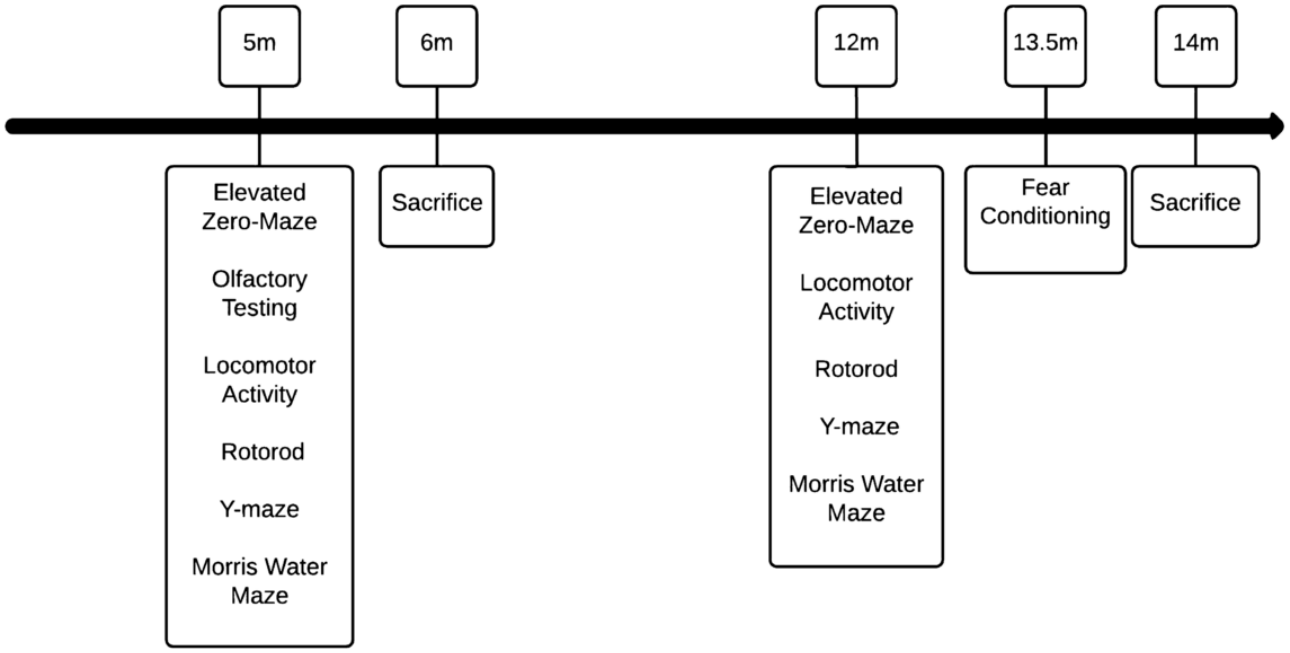


Figure 3 Behavioral testing experimental design at 5 and 12 months of age. Mice were sacrificed for biochemical measures following behavioral testing at 6 and 14 months.

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 described [62]. Acceleration was from 4 to 40 revolutions per minute, over the course of 4 minutes. 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 long [62]. 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 in [104]. 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 described [62]. 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 ability [105], 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 a maximum of 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 DNA extraction to confirm genotype. 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. Concentrations were measured by ion pair HPLC and electrochemical detection as previously described [62], except that tetrapentyl ammonium bromide was used as the ion pair reagent. Tissue ascorbic acid was extracted as follows. Tissue samples were weighed and wet tissue was homogenized in a 1.5 ml microfuge tube with a combination of two solutions, 25% (w/v) aqueous metaphosphoric acid and 100 mM sodium phosphate buffer containing 5 mM EDTA (pH 8.0), mixed together in a ratio of 2:7. A total of 10 μl of buffer solutions was used for each mg of tissue. The samples were then centrifuged at 13,600 g for 4 min at 3° C, and aliquots of the clear supernatant were taken for assay of

ascorbic acid as described above following appropriate dilution with HPLC mobile phase.

Malondialdehyde. Malondialdehyde was measured by homogenizing small, weighed tissue samples in 1 ml 5% TCA solution. Samples were centrifuged at 13 000 rpm at room temperature for 5 mins. 250 µl of sample was reacted with the same volume of 20 mM thiobarbituric acid for 35 minutes at 95 °C, followed by 10 minutes at 4 °C. Malondialdehyde was then specifically measured using HPLC with inline fluorescence detection of the malondialdehyde-thiobarbituric acid adduct [74].

Isoprostanes. Isoprostanes were determined by GC-MS in the Vanderbilt Eicosanoid Core Facility. Briefly, esterified F4-neuroprostanes were quantified by stable isotope dilution negative ion chemical ionization gas chromatography mass spectrometry (GC/MS) using [2H4]-15-F2t-isoprostane as an internal standard [106].

Protein carbonyls. Protein carbonyls were determined by reaction with DNPH using previously described methods from [107] with values calculated per mg protein.

Glutathione. Total glutathione (reduced glutathione (GSH) and oxidized glutathione (GSSG)) were measured using previously described methods from [108]. Briefly, cerebella were weighed and homogenized per protocol. Glutathione reductase (GSH) was oxidixed using 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), forming yellow derivative 5′-thio-2-nitrobenzoic acid (TNB). Glutathione disulfide (GSSG) is recycled back to GSH using NADPH. Absorbance was measured at 412 nm using Synergy™ H4 Hybrid microplate reader (Biotek Instruments, USA).

ELISA (soluble/insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$). Aβ levels were quantified using anti-human $A\beta_{1-40}$ and $A\beta_{1-42}$ 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 [74,109]. 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.

Statistical analyses. 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 $\log_{10}$ 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.

Methods for Chapter 4

Table 2. Total numbers of 4-month-old mice included in mitochondrial studies

	APP/PSEN1-	**APP/PSEN1+**
$SVCT2^{+/-}$	**"$SVCT2^{+/-}$"** (n=18) *Respirometry, TMRE/DHF, ATP/ADP	**"$SVCT2^{+/-}$ $APP/PSEN1^{+}$"** (n=6) *Respirometry
$SVCT2^{+/+}$	**"Wild-type"** (n=31) *Respirometry, TMRE/DHF, ATP/ADP	**"$APP/PSEN1^{+}$"** (n=23) *Respirometry, TMRE/DHF, ATP/ADP
$SVCT2^{Tg}$	**"$SVCT2^{Tg}$"** (n=9) * TMRE/DHF	(NOT TESTED)

High-resolution respirometry. Fresh cortical tissue was mechanically homogenized in a buffer consisting of 200mM sucrose, 5mM HEPES, 1mM EGTA and 0.05mM saponin and protease

inhibitors. Sequential centrifugation was used to obtain a mitochondrial isolate [110,111]. Freshly isolated cortical mitochondria were assayed at 0.5mg protein/ml MiR05 respiration buffer to a final volume of 2mL per chamber in an O2K Oxygraph (Oroboros, Innsbruck, Austria). Baseline oxygen uptake was established over 10 minutes and oxygen flux was measured and normalized to protein concentration under State 4 respiration in the presence of glutamate (10mM), and malate (2mM) and State 3 respiration was determined by the addition of ADP (2mM) [110–112] over a 10-minute time-span for each. We calculated a "pseudo" respiratory control ratio by normalizing oxygen consumed by isolated mitochondria after the addition of ADP (State 3) to the oxygen consumed at baseline in the absence of substrate or inhibitor (State 4). While some variation is normal and can reflect quality of isolate preparation, the averages per group were not significantly different by ANOVA ($p= 0.1576$), though the $SVCT2^{+/-}$ had a lower overall mean ratio than the other groups. Where indicated (**Fig. 3**), 100μM ASC (Sigma Aldrich, USA) diluted in Miro5 was added directly to a closed respirometer chamber containing mitochondrial isolates after baseline oxygen uptake was determined. The same volume of Miro5 was added to the untreated chamber. Total oxygen consumption due to auto-oxidation of ASC was determined after an hour in the closed chamber, representing the entire duration of a respirometry experiment, and was found to be marginal (~5 pmol/s*ml).

Western Blotting. Isolated mitochondria were obtained as described above and disrupted using RIPA buffer (Sigma Aldrich, USA) with protease inhibitors (*cOmplete* protease inhibitor cocktail, Roche, Switzerland). SVCT2 expression (SVCT2 H-70 (rabbit) sc-30114, dil. 1:1000, Santa Cruz Biotechnology, Inc., USA) was normalized to heat shock protein-60 (HSP-60 (mouse) A302-844A, dil. 1:2000, Bethyl Laboratories, USA). Hippocampal tissue was

collected from APP/PSEN1 and SVCT2$^{+/-}$;APP/PSEN1 mice at 4 and 12 months of age using RIPA buffer with protease inhibitors, as described above and probed for amyloid β (β – amyloid (rabbit) D54D2, dil. 1:1000, Cell Signaling
Technology, USA). Anti-rabbit and anti-mouse IgG-HRP conjugated secondary antibodies were used at 1:5000 (Promega, USA).

Vitamin C (ASC, ascorbic acid) content. ASC was measured in cerebellum by HPLC with electrochemical detection as described previously [62]. Cerebellum levels are a good reflection of levels in cortical and hippocampal areas [113]. Values were calculated per gram tissue wet weight.

Mitochondrial membrane potential and oxidative stress. Isolated mitochondria from cortical tissue were obtained using 230mM mannitol (Sigma Aldrich, USA), 70mM sucrose (Gibco), 20mM HEPES (Sigma Aldrich, USA), and 0.5mM EGTA (Sigma Aldrich, USA) and 0.05mM saponin (Sigma Aldrich, USA) [23,114]. Isolates were resuspended in buffer without saponin to obtain protein concentration. Isolates were divided and 325µg incubated with 100nM tetramethylrhodamine, ethyl ester (TMRE, ThermoFisher Cat.#T669, ex/em:540,575nm) in DMEM for 15 minutes at 37°C, or 20µM dihydrofluorescein diacetate (Sigma Aldrich, USA Aldrich, USA, ex/em:480,520nm) in PBS with fetal bovine serum (FBS, 3%) for 30 minutes at 37°C. Isolates incubated in TMRE were pelleted using centrifugation and pellets were washed in PBS/BSA (TMRE pellets) or PBS/FBS (dihydrofluorescein diacetate) twice before plating 100µg in triplicate on black bottom 96-well plates. TMRE quenching was assessed using serial dilutions of 2,4-dinitrophenol DNP). Fluorescence was measured using Synergy^{TM} H4 Hybrid microplate reader (Biotek Instruments, USA).

ATP/ADP ratio. Measurements of ATP and ADP were conducted using an ADP/ATP Ratio Bioluminescent Assay Kit (ab65313 Abcam, USA) on cortical tissue. Briefly, a single-cell suspension was prepared using one cortical hemisphere from wild type, SVCT2+/- or APP/PSEN1 animals (average age 21 weeks). Tissue was dissociated using 0.5% trypsin-EDTA, followed by DNase 1 treatment. Samples were prepared according to kit instructions and luminescence was measured using Synergy^{TM} H4 Hybrid microplate reader (Biotek Instruments, USA). Protein concentration in each well was measured and used for normalization.

Statistical analyses. Results are presented as mean ± SEM. Differences between SVCT2 and APP/PSEN1 genotypes were assessed by a two-way analysis of variance (ANOVA) with post hoc testing using Bonferroni tests when overall significance was obtained using SPSS. Student's t-tests were made in GraphPad Prism 7.0 when comparisons were made between two groups. Differences in fluorescence percent change between genotypes were assessed by two-way ANOVA with post hoc testing using Bonferroni tests when overall significance was obtained using GraphPad Prism 7.0. Greater variability, and artificially elevated ASC in SVCT2-Tg would mask the more subtle differences in SVCT2$^{+/-}$ versus WT, which represents the human-relevant condition of mild chronic deficiency versus replete status, respectively. Therefore, we made the *a priori* decision to perform individual analyses between WT and SVCT2$^{+/-}$, or WT and SVCT2^{Tg}. These were performed by individual Student's t-tests.

Methods for Chapter 5

Table 3. Total number of mice used in gene expression studies

	4 months		12 months	
	APP/PSEN1-	**APP/PSEN1+**	**APP/PSEN1-**	**APP/PSEN1+**
SVCT2+/-	**"SVCT2$^{+/-}$"** (n=4)	**"SVCT2$^{+/-}$;APP/PSEN1^{+}"** (n=4)	**"SVCT2$^{+/-}$"** (n=4)	**"SVCT2$^{+/-}$;APP/PSEN1^{+}"** (n=4)
SVCT2+/+	**"Wild type"** (n=4)	**"APP/PSEN1^{+}"** (n=4)	**"Wild type"** (n=4)	**"APP/PSEN1^{+}"** (n=4)

mRNA Isolation. All reagents used to isolate mRNA were included in RNeasy Mini Kit (Qiagen, USA). Briefly, hippocampal tissue was dissected from 4- and 12- month old mice and mechanically homogenized in *RLT* buffer with β-mercaptoethanol. Debris were pelleted by centrifugation. Supernatants were mixed with 1 volume of ethanol (70%) before adding to spin column. Genomic DNA was removed using RNase-free DNase kit (Qiagen, USA, cat. 79254). Column-bound mRNA was washed with *RW1*(1x) and *RPE* (2x) and eluted using RNase-free water.

Gene Expression. We assessed gene expression changes in 84 genes implicated in AD pathogenesis and progression on hippocampal tissue collected from wild-type and APP/PSEN1 mice with differential SVCT2 expression at 4 and 12 months of age using Alzheimer's Disease RT^2 Profiler PCR Array (Qiagen, PAMM-057Z). Isolated mRNA was reverse transcribed to cDNA using the RT^2 First Strand kit (Qiagen) and loaded on to pre-prepared 96-well plates with RT^2 SYBR Green qPCR mastermix (Qiagen).

Hippocampal 5-hmC Quantification. We measured 5-hmC levels in DNA isolated from hippocampal tissue collected from wild-type and APP/PSEN1 mice with differential SVCT2 expression at 4 and 12 months of age using an ELISA-based Global 5-hmC Quantification assay (Active Motif, cat. #55108). Absorbance was measured at 450nm using Synergy^{TM} H4 Hybrid microplate reader (Biotek Instruments, USA).

Statistical analyses. Data were analyzed using GeneGlobe RT^2 Data Analysis Center (Qiagen). Results are represented as fold-change (2^ (-ΔΔC$_T$)) defined as the normalized gene expression (2^(- ΔC$_T$)) in the Test Sample (SVCT2+/-, APP/PSEN1, SVCT2+/-;APP/PSEN1) divided the normalized gene expression (2^ (- ΔC$_T$)) in the Control Sample (wild-type). APP/PSEN1 serves as the Control Sample in comparisons made between APP/PSEN1 and SVCT2+/-;APP/PSEN1. The p values are calculated based on a Student's t-test of the replicate 2^ (- ΔC$_T$) values for each gene in the control group and test groups (*p<0.05, **p<0.001, ***p<0.0001). Differences in 5-hmC quantification were analyzed using a 2x2x2 ANOVA (Age, SVCT2 status, APP/PSEN1 status) with Bonferroni post hoc testing w

Chapter III: Ascorbate Deficiency in the Brain Impairs Cognition, Increases Amyloid Accumulation and Deposition, and Oxidative Stress in APP/ PSEN1 and Normally Aging Mice

A large portion of the Western world may be deficient in vitamin C, and in several studies lower blood vitamin C correlated with cognitive impairment [66,115–117]. Under normal circumstances ASC is maintained at high concentrations in brain tissue where it is critical for maintenance of oxidative balance [118]. 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 [119,120] and ASC deficiency is likely to be a major contributing factor to pathology. Several studies have thus sought to define the role for ASC in Alzheimer's disease, but population studies have yielded mixed results as to the effectiveness of dietary supplements in older adults [67,121–123]. It is therefore critical to determine how prolonged sub-clinical ASC 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 ASC deficiency in Alzheimer's disease by crossing SVCT2 heterozygous knockout mice [59] with a bigenic mouse carrying two mutations known to cause early-onset Alzheimer's disease ($SVCT2^{+/-}$;$APP/PSEN1^{+}$). These mice have intracellular ASC deficiency, but normal circulating levels, since they can synthesize the vitamin in liver. We hypothesized that low ASC in the brain 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 ASC on normal aging in the non-transgenic mice.

Results and Discussion

We predicted that lower brain ASC 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 testing was conducted in 5-month old mice. Data for this test were not normally distributed and therefore a $\log_{10}$ transformation was used on the data. Baseline exploration levels did not vary among the genotypes for either the water or familiar odor trials ($Fs<3.68$, $ps>0.06$). 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 4A, (SVCT2$^{+/-}$ $t(14)=2.176$ $p=0.047$; wild-type $t(16)=4.15$ $p<0.001$, **Fig. 4A** (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. 4A** (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 performance [124] and may predict the likelihood of further cognitive decline [125].

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. 4B**). Although the effect appeared larger in SVCT2$^{+/-}$APP/PSEN1^{+} mice, there were no significant effects of SVCT2 genotype (Fs<0.87, ps>0.36). At 12 months, there were no differences according to APP/PSEN1 genotype (Fs<0.58, ps>0.45), however, mice with low vitamin C made fewer alternations ($F_{1, 49}$=4.92, p=0.031, **Fig. 4C**). 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.

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 (Fs<0.77, ps>0.39, **Fig. 4D**). 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. 4E**). There was no effect of SVCT2 genotype (Fs<0.19, ps>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.

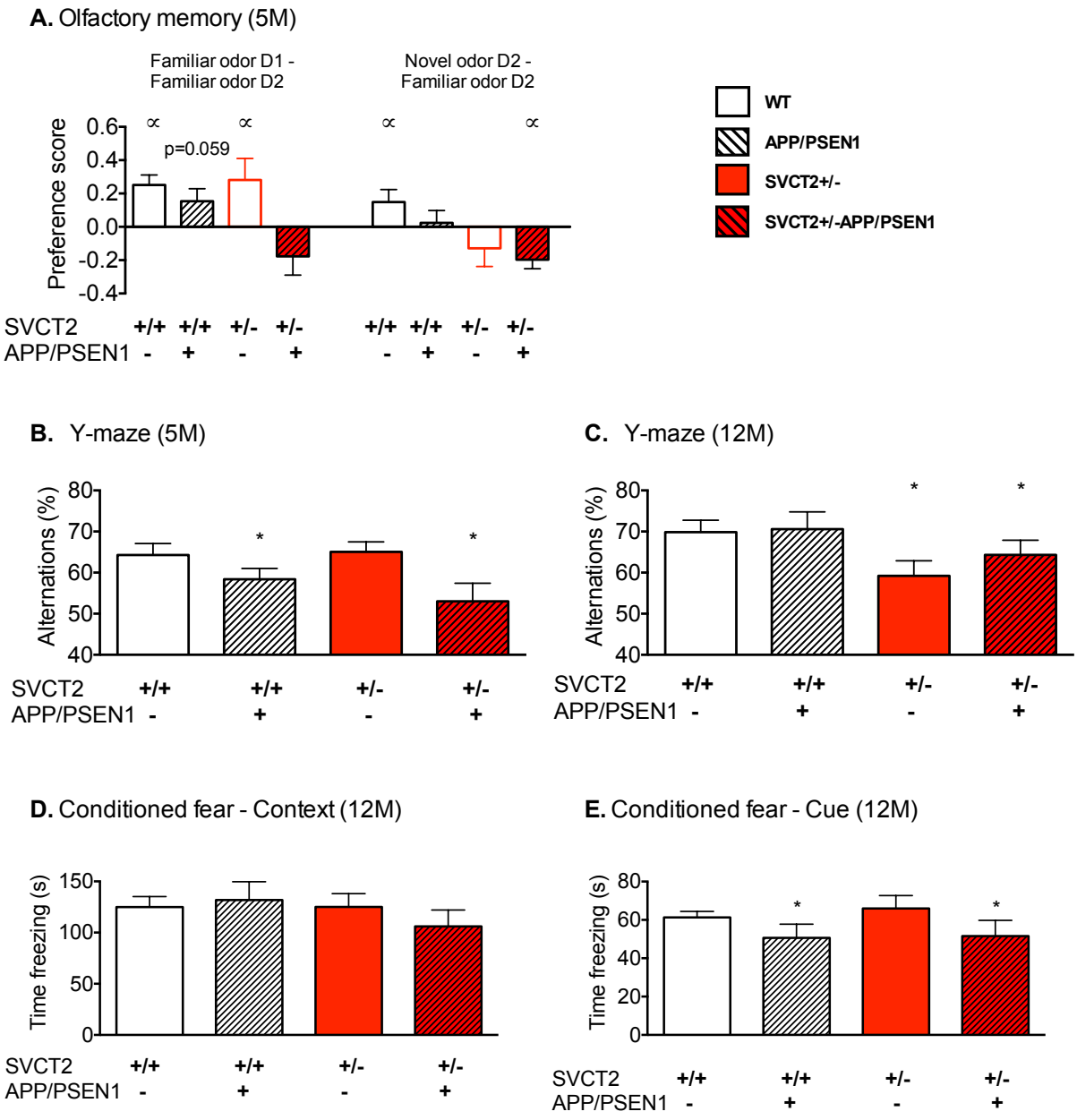


Figure 4 Learning and memory tasks. At 5 months of age (5M), SVCT2+/–APP/PSEN1+ mice were the only group to show no decrease in the interest of the familiar odor on the second day of testing (24 h recall) (A, left). Only wild-type mice showed evidence of a significant preference for the novel over the 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 min 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 +SEM $\propto p < 0.05$ 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–.

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. 5A**; 12M $F_{7, 336}$=34.46, p<0.001, **Fig. 5E**). 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 (Fs<3.41, ps>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 (Fs<2.61, ps>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. 5I**). At both ages all groups showed a significant preference for the platform quadrants (Fs>4.90, ps<0.05, **Figs. 5B,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 (Fs<0.58, ps>0.45; **Fig. 5C**). At 12 months of age performance was similar across the groups (Fs<0.93, ps>0.34, **Fig. 5G**). Platform crossings did not differ among the groups at 5 months (Fs<1.14, ps>0.71, **Fig. 5D**). 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. 5H**). There was no main effect of APP/PSEN1 genotype and no interaction (Fs<0.72, ps>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 (Fs<2.10, ps>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 tasks [126], although other reports have failed to show deficits in the Y-maze at 7 months [127]. The APP/PSEN1 mouse line was originally developed on a hybrid background [128], 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 [129,130]).

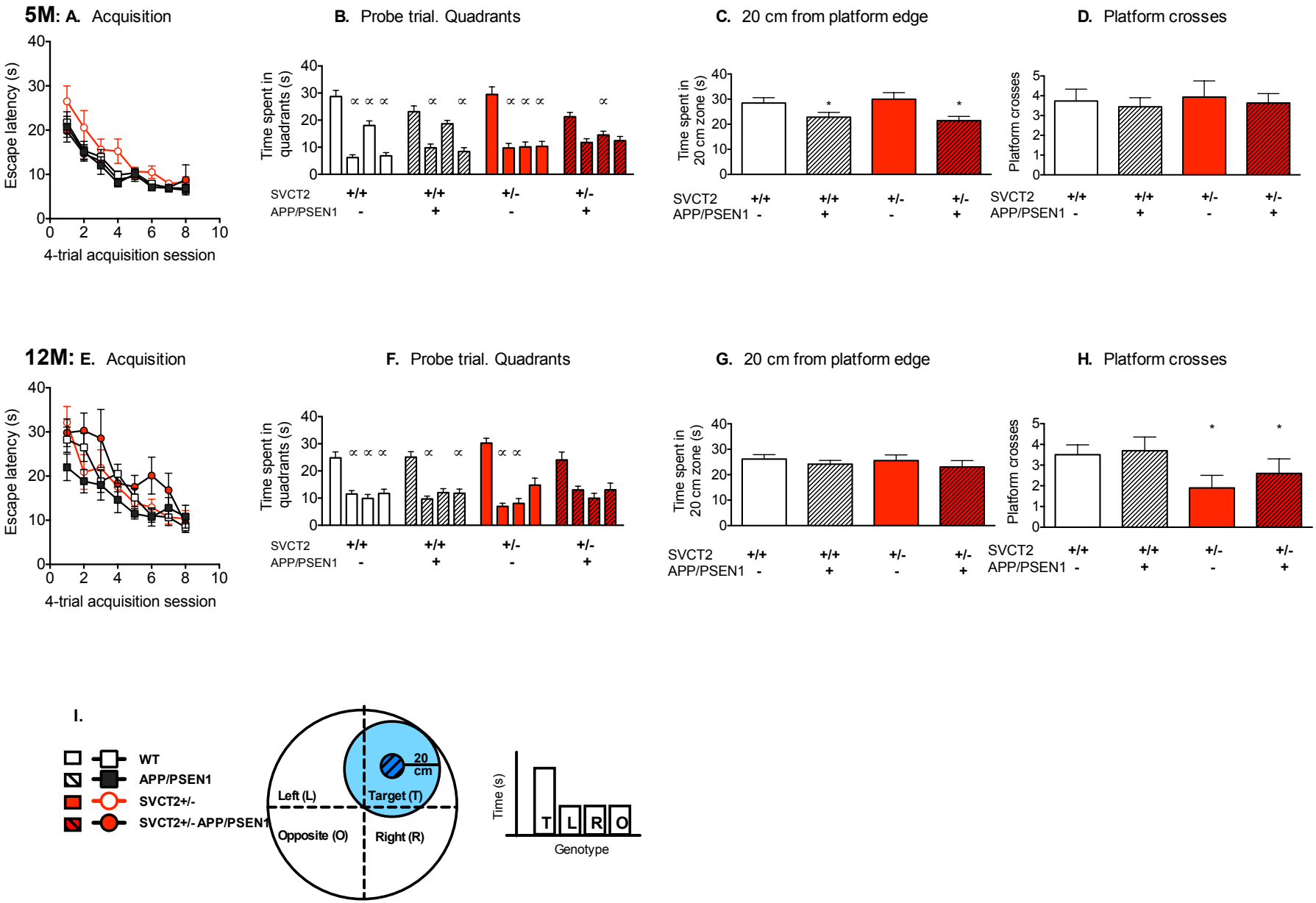

5M: A. Acquisition
Escape latency (s)
4-trial acquisition session
B. Probe trial. Quadrants
Time spent in quadrants (s)
C. 20 cm from platform edge
Time spent in 20 cm zone (s)
D. Platform crosses
Platform crosses
SVCT2 +/+ +/+ +/- +/-
APP/PSEN1 - + - +
12M: E. Acquisition
F. Probe trial. Quadrants
G. 20 cm from platform edge
H. Platform crosses
I.
WT
APP/PSEN1
SVCT2+/-
SVCT2+/- APP/PSEN1
20 cm
Target (T)
Left (L)
Opposite (O)
Right (R)
Time (s)
T L R O
Genotype

Figure 5 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 +SEM ∝ $p < 0.05$ 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–.

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. 6A; 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).

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 (Fs>10.68, ps<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. 6E**; 12 M: $F_{1,44}$=10.096, p=0.003 **Fig. 6F**). The SVCT2 is expressed [131] 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 mice [60], 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 junction [132]. 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 [62,133] These mice are not known to have major cognitive deficits, although testing has mostly been limited to mice younger than 5 months of age [62,133]. The deficits observed in strength and co-ordination 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.

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 (Fs<1.96, ps>0.17, *data not shown).* At 5 months there were no differences in exploration of the elevated zero maze (Fs<2.47, ps>0.12, **Fig. 6C**), 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. 6D**). There were no differences on the time spent in the closed zones at either age (Fs<0.72, ps>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 [134,135], 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 task [69]. Extreme vitamin C deficiency leads to severe lethargy and low activity [49], but agitation and motor disturbances are features of Alzheimer's disease that may be being modeled by the hyperlocomotion in mouse models [134,136,137] and it is therefore interesting that the modest decrease in vitamin C contributed to this increased activity in the older cohort of mice.

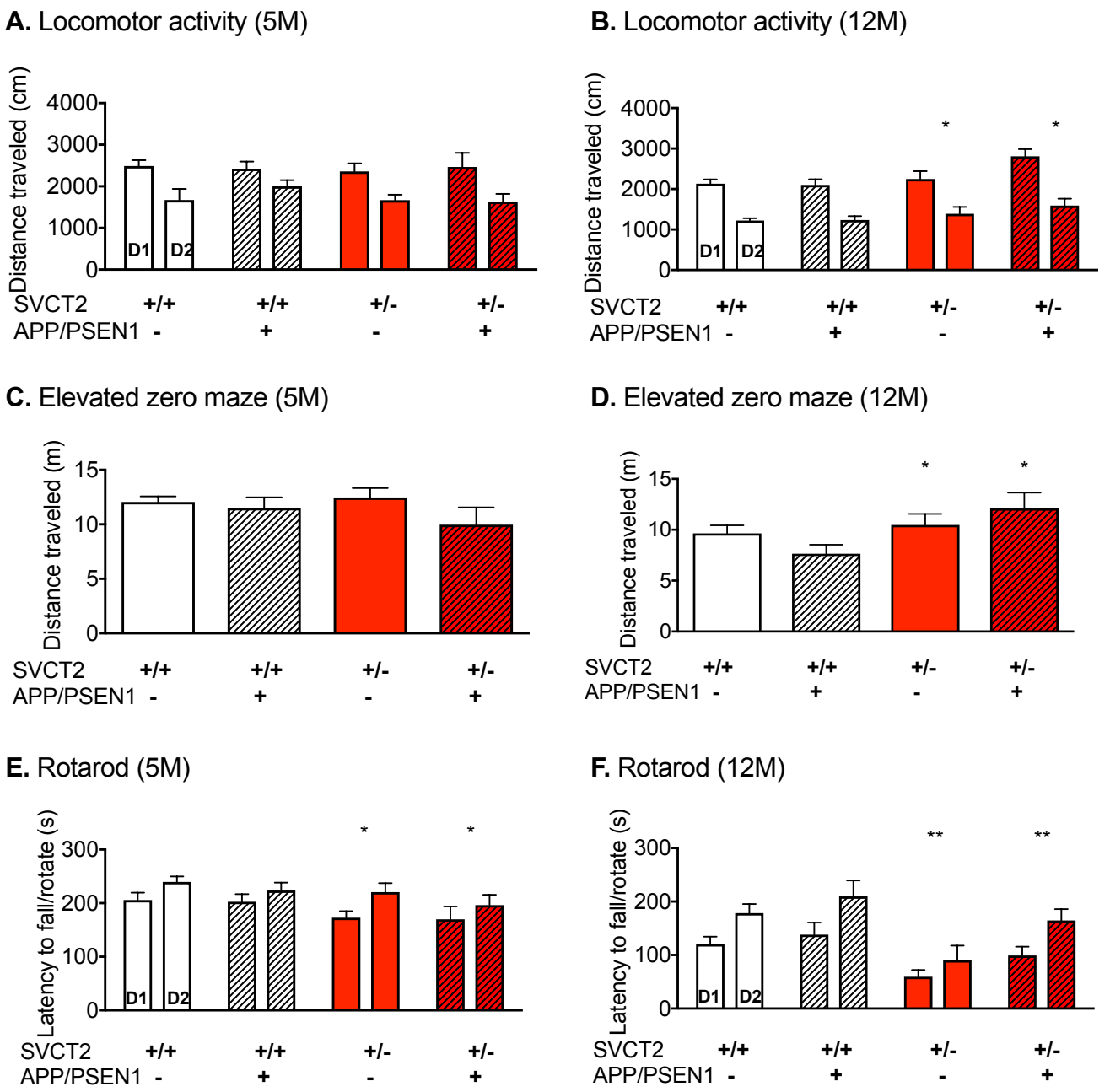


Figure 6 Activity, anxiety and neuromuscular coordination. Locomotor activity was tested in two 15 min sessions on 2 consecutive days (D1, D2). At 5 months of age (5M), there were no differences according to genotype (A), but at 12 months (12M), SVCT2+/– mice were slightly hyperactive compared to SVCT2+/+ 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 +SEM * $p < 0.05$, **$p < 0.01$, main effect of SVCT2+/– compared to SVCT2+/+.

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. 7A**). APP/PSEN1 genotype had no effect on brain vitamin C level at either age (Fs<3.78, ps>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 health [138]. Clinical and population studies of plasma vitamin C routinely report levels in the depleted and deficient range (<28 μM) in 10-15% of subjects, with clinical scurvy reported in some populations [68,139].

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. 7B**). 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. 7C**). There were no main effects of either SVCT2 or APP/PSEN1 mutation alone at 6 months ($Fs_{1, 37}$<2.49, ps>0.12) and there were no

differences among groups at 14 months ($Fs_{1, 29}$<0.88, ps>0.36). At 6 months, F_2-isoprostanes were highest in APP/PSEN1 mutant mice ($F_{1, 37}$=4.56, p=0.039, **Fig. 7D**) with no further effect of SVCT2 genotype (Fs<0.12, ps>0.73). At 14 months, there were no differences among the groups (Fs<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 $Fs_{1, 35}$<0.85, ps>0.36, **Fig. 7E**). There were no differences among groups in 14-month old mice ($Fs_{1, 26}$<2.28, ps>0.14). The ratio between GSH:GSSG did not differ among the groups at either age (Fs<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 ($Fs_{1, 14}$=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. 7F**), but there were no further effects of SVCT2 genotype (Fs<0.40, ps>0.54). Similarly, Murakami et al. [69] reported decreased carbonyls following vitamin C supplementation to normal mice, and increased GSH, but no change in GFAP expression.

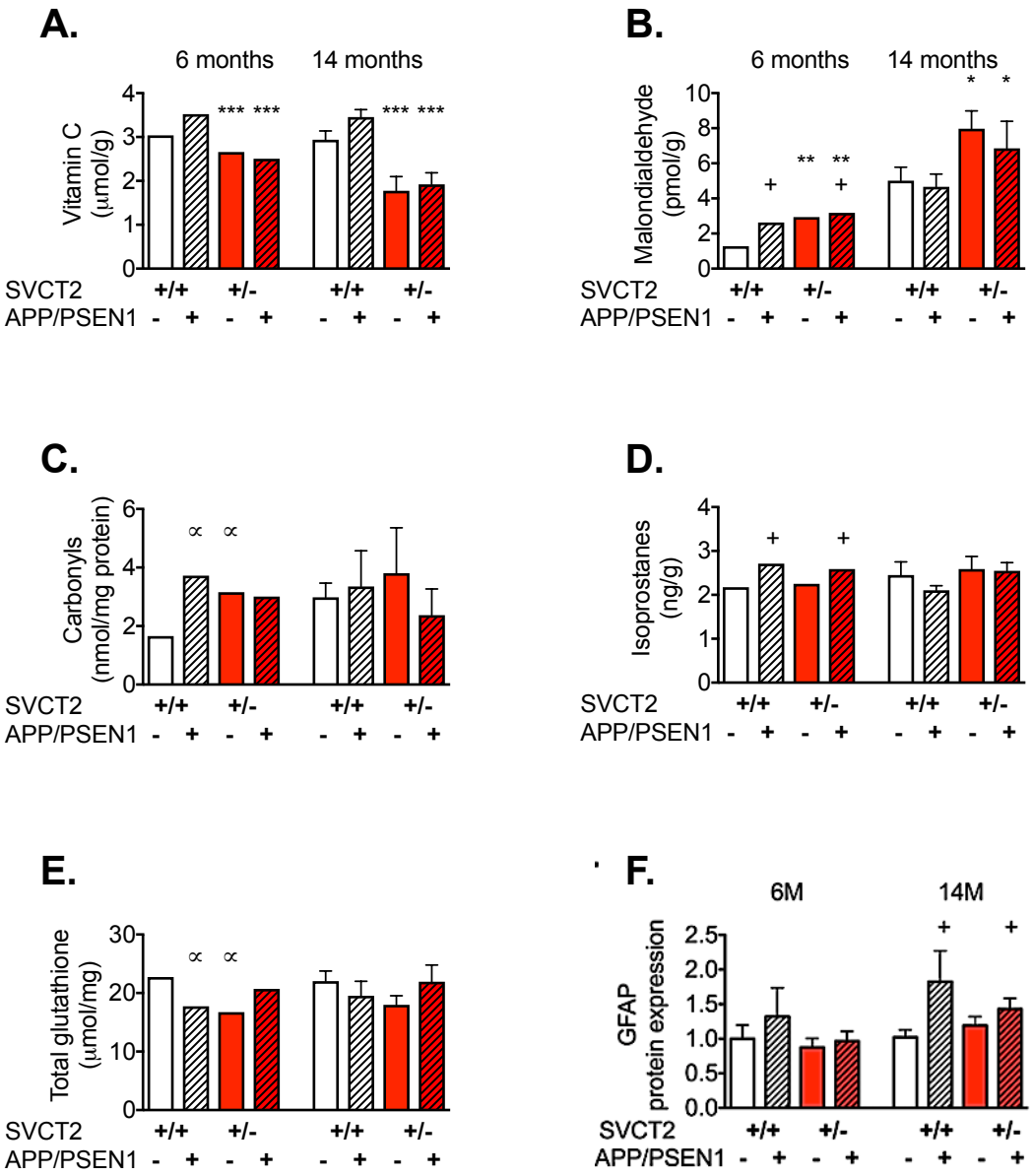
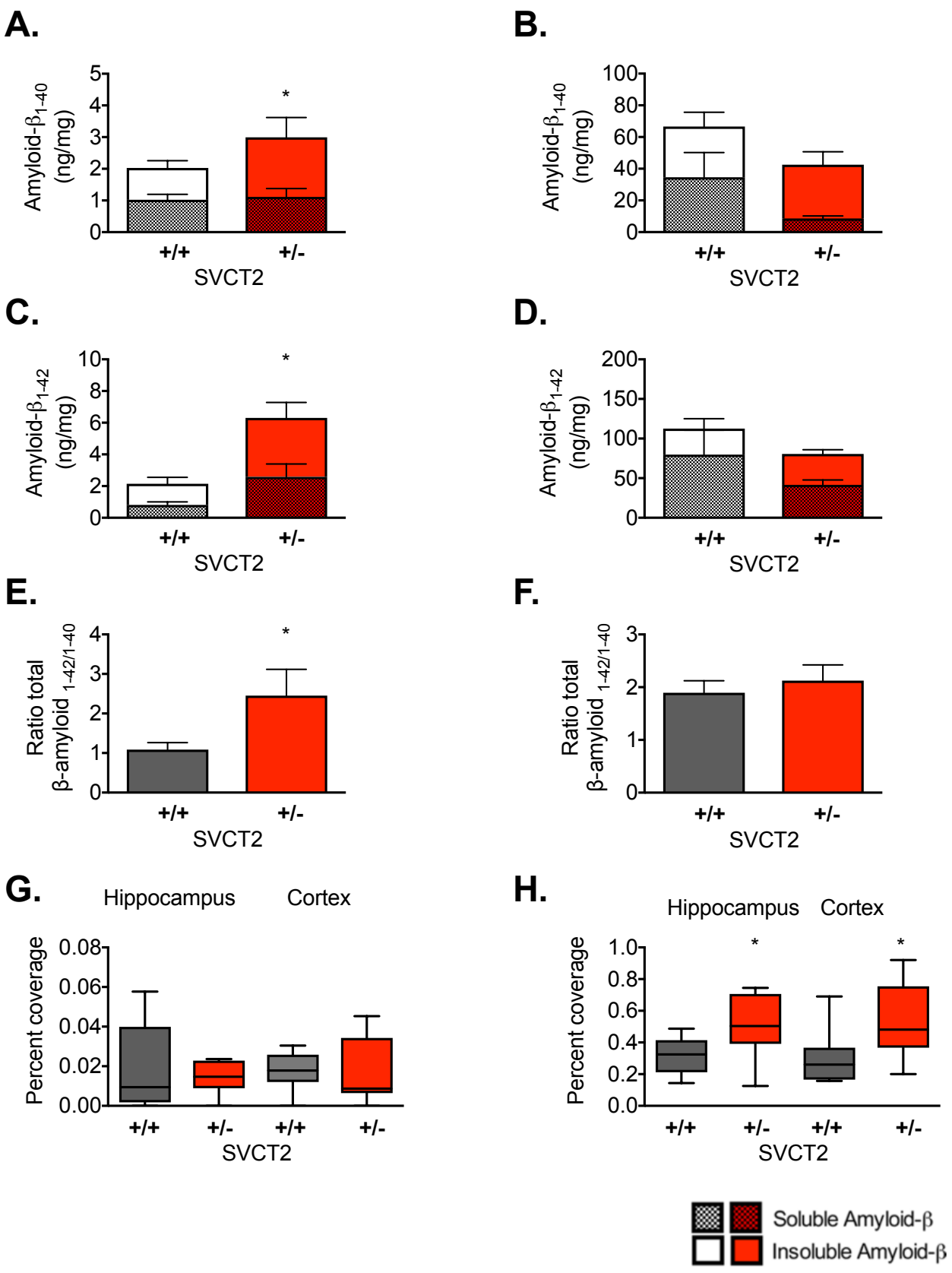


Figure 7 Measures of antioxidant status, oxidative stress and neuroinflammation. Mice that had undergone behavioral testing were sacrificed at 6 months (6M: "5 month" group) and 14 months of age (14M: "12 month" group). Lacking one copy of the SVCT2 successfully lowered vitamin C in the brains of SVCT2+/– 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 +SEM *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, 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).

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 $A\beta_{1-40}$ and $A\beta_{1-42}$ 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 $A\beta_{1-40}$ did not vary solely according to SVCT2 genotype (ps>0.077, **Fig. 8A**), but soluble and insoluble $A\beta_{1-42}$ 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. 8C**). This increase in $A\beta_{1-42}$ 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. 8E**). 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 (ps>0.87, **Fig. 8G, 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 (ps>0.53, **Fig. 8B, 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, ps=0.025, **Fig. 8H, I**) indicating that the earlier increase in $A\beta_{1-42}$ may have contributed to more robust amyloid seeding.


A.
Amyloid-β1-40 (ng/mg)
5
4
3
2
1
0
*
+/+
+/-
SVCT2
B.
Amyloid-β1-40 (ng/mg)
100
80
60
40
20
0
+/+
+/-
SVCT2
C.
Amyloid-β1-42 (ng/mg)
10
8
6
4
2
0
*
+/+
+/-
SVCT2
D.
Amyloid-β1-42 (ng/mg)
200
150
100
50
0
+/+
+/-
SVCT2
E.
Ratio total β-amyloid 1-42/1-40
4
3
2
1
0
*
+/+
+/-
SVCT2
F.
Ratio total β-amyloid 1-42/1-40
3
2
1
0
+/+
+/-
SVCT2
G.
Hippocampus
Cortex
Percent coverage
0.08
0.06
0.04
0.02
0.00
+/+
+/-
+/+
+/-
SVCT2
H.
Hippocampus
Cortex
Percent coverage
1.0
0.8
0.6
0.4
0.2
0.0
*
*
+/+
+/-
+/+
+/-
SVCT2
Soluble Amyloid-β
Insoluble Amyloid-β

I.

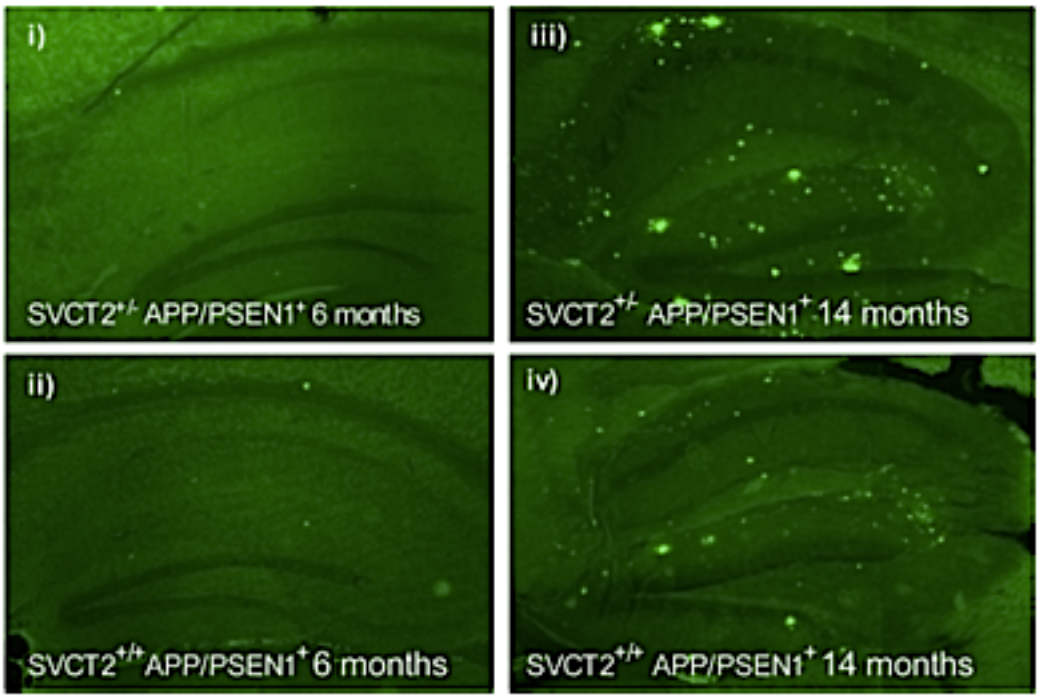


Figure 8 Measurement of amyloid β. Amyloid-β levels are only reported in mice that carry APP/PSEN1 mutations. At 6 months of age (6M), soluble and insoluble amyloid-β1–40 and amyloid-β1–42 were very low, and insoluble amyloid proteins of both lengths were more numerous in mice with low vitamin C (A, 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 +SEM. 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.

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 oligomerization [69]. 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 Aβ oligomers [140]. Less specific changes in oxidative stress can also influence factors in the pathways for over-production of amyloid-β (e.g. BACE1 enzymatic function) [141]. 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 survival [61]. 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) [70]. 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 junctions [142], 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.

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.

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 changes in energy homeostasis and genetic integrity that can contribute to

impaired synaptogenesis and neurotransmitter function and cell death. ASC is a critical co-factor in the biosynthesis and maintenance of collagen, which is linked to the formation of myelin in cell culture studies, as well as in the synthesis of catecholamines and in modulating receptor binding of neurotransmitters [reviewed in 96]. The specific role of vitamin C deficiency in each of these areas has yet to be shown but they can be attributed to a lack of available ASC due to the imbalance in the redox state.

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 age represents the stage of disease pathogenesis during which significant amyloid production has been triggered and progression is inevitable, but what remains unclear is how ASC deficiency contributes during prodromal stages of the disease, at which point intervention may be successful. 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.

Chapter IV: Mitochondrial dysfunction in the APP/PSEN1 mouse model and a novel protective role for ascorbate

Increased mitochondrial dysfunction and oxidative stress are well-established features of the normal aging processes, and are accompanied by decreases in the activity of endogenous and nutrient-based antioxidants. Alterations in mitochondrial function are elevated at early clinical stages of sporadic Alzheimer's disease compared with age-matched controls [144–146], emerging before the appearance of the classical pathological features of Alzheimer's disease, such as amyloid plaques and neurofibrillary tangles [16,22,147]. Damage to both mitochondrial DNA and nuclear DNA have been identified in patients with mild cognitive impairment and early-stage Alzheimer's disease [33,36,148]. Mitochondrial dysfunction, in turn, increases production of reactive oxygen species (ROS), contributing further to oxidative stress [10,15,149] and damage to lipids [26,38], proteins [27,46] and nucleic acids [150–152]. The accumulation of amyloid beta (Aβ) peptides in the Arctic APP-over expressing mouse model decreased ATP production, impaired mitochondrial membrane potential, and inhibited complex IV activity, in addition to exacerbating oxidative stress [11,23]. Furthermore, ROS generated from mitochondrial dysfunction can drive amyloidogenesis and tau phosphorylation [11,32,153], thus creating a cycle that decreases the energy available for proper cellular function, and drives the pathology and progression of cell death [14,41,42].

Preventative strategies that slow pathological development by attenuating oxidative stress are projected to delay age of onset of cognitive decline. Clinical studies suggest antioxidant supplementation with ASC, particularly to correct states of deficiency, confers

some protection against the cognitive decline associated with Alzheimer's disease [65–68]. ASC is a powerful antioxidant that protects against oxidative damage throughout the entire body. The highest concentrations of ASC are found in the brain, due to expression of SVCT2 and efficient storage and recycling mechanisms, suggesting an important role of ASC in neuronal health [154]. A meta-analysis evaluating oxidative stress biomarkers confirmed decreased antioxidant capacity in the plasma of patients with Alzheimer's disease, suggesting that antioxidant defenses are consumed more rapidly in these individuals [39]. Animal and cell culture studies offer additional evidence that supplementation with ASC not only ameliorates oxidative stress, but also mitigates the production of Aβ [69–73]. In the previous chapter, we reported that compromised ASC capacity increases the momentum of Alzheimer's disease pathological processes with regard to soluble $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$ peptides in cortical tissue from $SVCT2^{+/-}$; APP/PSEN1 mice at 6 months compared with APP/PSEN1 mice that have normal SVCT2 transporter expression [155]. Consistent with this data, other groups showed chronic ascorbate (ASC) supplementation resulted in a decrease in total soluble $A\beta_{1\text{-}42}$ and a reduction in overall amyloid plaque burden in Alzheimer's disease mouse models [69,70].

It was originally believed that mitochondria took up the twice-oxidized form of ASC, dehydroascorbate, and reduced the molecule for use [85,156], but SVCT2 expression has been demonstrated on mitochondria in cell lines, indicating direct transport of ASC for a specific and localized requirement in that organelle [83,87]. Low ASC supplementation led to morphological changes in mitochondria of $gulo^{-/-}$;5XFAD mice unable to synthesize their own ASC, compared with the high ASC supplementation group suggesting ASC is necessary for maintaining mitochondrial integrity by mitigating oxidative damage to the organelle [70]. It is

still unknown how ASC deficiency can compromise cellular function at the subcellular level *in vivo*, particularly in regard to aging and disease. In the present study we tested the hypothesis that decreased neuronal ASC contributes to impaired mitochondrial function and oxidative stress generation, and thus, contributes to the subcellular damage and cell death associated with Alzheimer's disease pathogenesis. The onset of Alzheimer's disease in the human population is diagnosed by progressive cognitive decline indicating that significant damage has already accumulated in the brain, at which point supplementation is unlikely to reverse damage.

The goal of the present study was to assess the effect of ASC deficiency on mitochondrial facets of early disease progression. This could potentially introduce an easy and inexpensive lifestyle modification to slow the progression of sporadic Alzheimer's disease. We measured mitochondrial respiration, membrane potential, ROS generation and energy production in cortical mitochondrial isolates from the APP/PSEN1 mouse model for Alzheimer's disease at 4 months of age, before the emergence of cognitive deficits and significant amyloid deposition [155,157]. We also investigated the extent to which ASC affects mitochondrial function in mice with heterozygous expression of the highly selective SVCT2 transporter ($SVCT2^{+/-}$). This mutation results in commensurate decreases in cellular and mitochondrial ASC concentrations. Additionally, we measured respiration after acute ASC administration in wild type cortical mitochondria, and membrane potential and ROS generation in a transgenic mouse model that over-expresses SVCT2 ($SVCT2^{Tg}$), which results in life-long increases in intracellular ASC concentrations, in order to elucidate the potential contributions of supra-adequate ASC levels to mitochondrial function.

Results and Discussion

Disrupted aspects of mitochondrial function during early or prodromal stages of Alzheimer's disease contribute to disease progression by augmenting oxidative stress and cell death. We hypothesized that these detrimental aspects of the disease are accelerated by deficiency of intracellular ASC.

ASC status and APP/PSEN1 drive changes in oxygen consumption during increased mitochondrial activity. We have previously shown that cognitive deficits and amyloidogenic pathologies emerge at 5-6 months in SVCT2$^{+/-}$;APP/PSEN1 mice, and these features of the disease are more pronounced compared to APP/PSEN1 mice, suggesting that ASC deficiency speeds the onset and progression of the disease [155]. Expression of SVCT2 protein in mitochondria was decreased in mice heterozygous for the SVCT2 gene by approximately 20% (SVCT2: $F(1,14) = 5.624$, $p = 0.03$; **Fig. 9A**). The magnitude of the effect is consistent with decreased ASC content in mitochondria from SVCT2 heterozygous mice ($t(8) = 2.766$, $p = .024$, **Fig. 9B**). These data confirm that SVCT2 is not preferentially conserved in mitochondria and ASC concentration within mitochondria is primarily determined by expression of the transporter. Aβ peptides can interact with mitochondrial membrane proteins leading to structural and functional alterations, which may then result in inefficient ATP production and an increase in mitochondrial activity [17,18,158]. While there is little amyloid accumulation prior to 6 months in the APP/PSEN1 mouse model [157,159], the Aβ peptide is present and measureable at 4 months in the APP/PSEN1 mouse model, as demonstrated qualitatively by western blot (**Fig. 9C**). There is thus the potential for Aβ to interact with and compromise mitochondrial function.

Using high-resolution respirometry, we measured oxygen consumption by mitochondria isolates from cortical tissue collected from wild-type, SVCT2$^{+/-}$, APP/PSEN1 and

SVCT2$^{+/-}$;APP/PSEN1 mice at 4 months of age. Three substrates (glutamate, malate and ADP) were added sequentially in order to maximize the amount of additional ATP that can be produced by oxidative phosphorylation [160], thus driving mitochondria to their full, or reserve, respiratory capacity. Mitochondrial isolates from SVCT2$^{+/-}$ mice consumed less oxygen compared to mice with SVCT2$^{+/+}$ expression at full respiratory capacity (SVCT2: $F(1, 18) = 4.892$, $p=0.04$; **Fig. 9D**), with a trend toward less oxygen consumption emerging after the addition of glutamate and malate ($p=0.054$), suggesting that adequate ASC in the mitochondria is necessary for efficient energy production. ASC deficiency may, therefore, contribute to an energy deficit both within and outside of the context of disease. In contrast, mitochondrial isolates from APP/PSEN1 mice consumed significantly more oxygen compared to isolates from WT and SVCT2$^{+/-}$ mice that did not carry the transgenes at full respiratory capacity (APP/PSEN1: $F(1, 18) = 10.495$, $p=0.005$; **Fig. 9D**). These data provide strong evidence that mitochondrial function is altered in both SVCT2$^{+/-}$ and APP/PSEN1 isolates because oxygen consumption in these groups is different than that observed in the control group. Interestingly, oxygen consumption in the combined SVCT2$^{+/-}$;APP/PSEN1 appeared to normalize back to rates similar to those seen in the wild-type control group, presumably due to competing direction of effects seen in SVCT2$^{+/-}$ and APP/PSEN1 groups separately. Nevertheless, it is important to note that while respirometry measures how much oxygen is consumed, this design does not describe the fate of that oxygen, and whether it contributes to ATP or ROS production. We therefore assessed the efficiency of the electron transport chain in mitochondrial isolates from the SVCT2$^{+/-}$ and the APP/PSEN1 mice by measuring mitochondrial membrane potential and ATP/ADP ratios to identify individual contributions of each mutation [23,112,161]. We used dihydrofluorescein to measure production of ROS production given that disruption of the electron transport chain leads to oxidative stress.

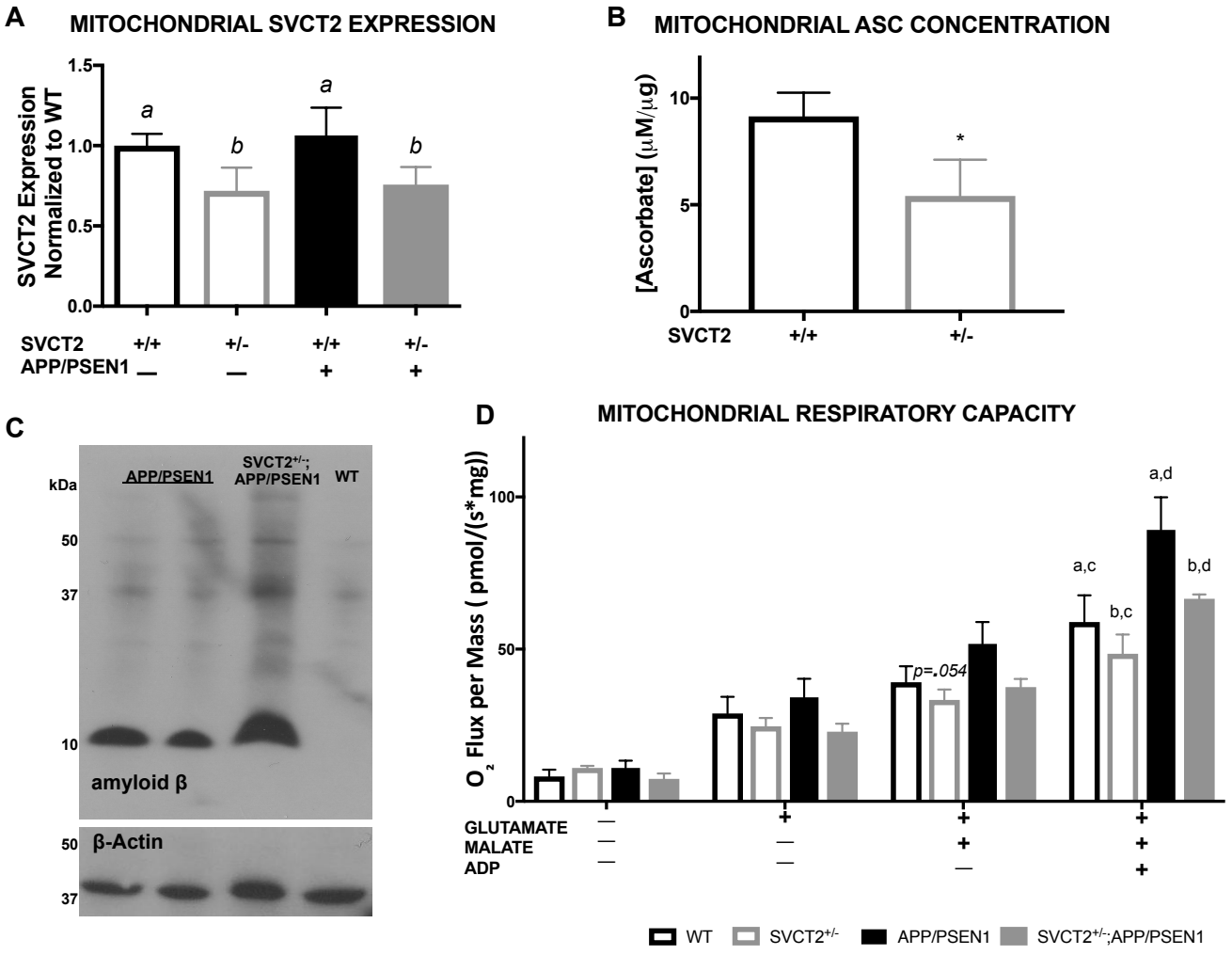


Figure 9 ASC deficiency and APP/PSEN1 affect oxygen consumption in mitochondria. A) SVCT2 expression is decreased in mitochondrial isolates from mice heterozygous for SVCT2 compared to isolates with wild-type SVCT2 expression ($p = 0.03$, n = 5-6 per group). B) ASC content is significantly lower in $SVCT2^{+/-}$ mitochondria at 4 months ($p = 0.024$, n = 5 per group). C) Qualitative western blot analysis demonstrates detectable amyloid β expression in APP/PSEN1 and $SVCT2^{+/-}$;APP/PSEN1 mice at 4 months of age, that already appears more severe in the compound mutant mice. D) $SVCT2^{+/-}$ mitochondrial isolates consume less oxygen ($p=0.04$), while mitochondrial isolates from APP/PSEN1 transgenic mice consume more oxygen when at full respiratory capacity ($p = 0.005$). WT n = 6, $SVCT2^{+/-}$ n = 5, APP/PSEN1, $SVCT2^{+/-}$;APP/PSEN1 n = 6. Letters indicate a main effect of SVCT2 (*a,b*) and APP/PSEN1 (*c,d*).

ASC status and APP/PSEN1 genotype affects mitochondrial membrane potential and ATP/ADP ratios and increases oxidative stress. We found that oxygen consumption in isolated mitochondria from the combined SVCT2+/-;APP/PSEN1 group appeared to normalize in the high-resolution respirometry experiment due to opposing effects of the SVCT2 and APP/PSEN1 mutations, rather than because the combination genotype actually reflected an improvement in function. We therefore made the decision to investigate the individual effects of APP/PSEN1 and ASC deficiency. Mitochondrial membrane potential was determined through TMRE accumulation in mitochondrial isolates from wild-type, $SVCT2^{+/-}$, and APP/PSEN1 mice, which reflects the extent to which oxygen consumption results in the generation of an effective proton gradient. As predicted, mitochondria isolated from $SVCT2^{+/-}$ mice showed a decreased TMRE fluorescence signal compared to WT mitochondria indicating loss of membrane polarization ($t(9)= 2.278$, $p = 0.048$, **Fig. 10A**). A greater TMRE fluorescence signal was observed in mitochondria isolated from APP/PSEN1 cortex indicating increased polarization across the APP/PSEN1 mitochondrial membrane compared to WT isolates ($t(13) = 2.435$, $p = 0.03$; **Fig. 10B**). These results are consistent with the pattern of oxygen consumption observed in $SVCT2^{+/-}$ and APP/PSEN1 isolates (**Fig. 10D**). The accumulation of TMRE at 100nM can cause aggregation of the fluorescent probe within the mitochondrial matrix, resulting in fluorescence self-quenching. To determine the directionality of the fluorescence differences observed in this study, the proton ionophore 2,4-dinitrophenol (DNP) was added in decreasing concentrations (1.0 μM, 0.5 μM, 0.1 μM) after TMRE pre-loading to dissipate mitochondrial membrane potential. We observed no effect of genotype and no effect of DNP concentration in the percent change in

fluorescence from TMRE baseline between groups (*data not shown*). This suggests that fluorescence quenching occurred in mitochondrial isolates across all genotypes, but to the same degree, thus quenching did not mask the overall effect.

Mitochondrial membrane potential can affect overall energy production. We next determined the ATP/ADP ratio in mitochondrial isolates from WT, SVCT2$^{+/-}$, and APP/PSEN1 mice. We observed a significant decrease in the ATP/ADP ratio in APP/PSEN1 isolates compared to WT isolates ($t(11)= 2.459$, $p= 0.03$, **Fig. 10D**) and no significant difference in ATP/ADP ratio in the SVCT2$^{+/-}$ isolates compared to wild type (**Fig. 10C**). Based on the increase in oxygen consumption and mitochondrial membrane potential, an increase in the ATP/ADP ratio would have been expected; however the decrease supports the hypothesis that the oxygen being consumed is not being used efficiently toward energy production. It is suggested that ASC can contribute to the electron transport chain by aiding in the reduction of cytochrome c [162–164]. ASC acts as an enzymatic co-factor with the 2-oxoglutarate-dependent dioxygenases and is believed to play a role in iron reduction in these reactions [52]. Given that the heme-bound iron atoms in cytochrome c are reduced and oxidized with electron transfer, it is likely that ASC can assist in this redox reaction due to its low reduction potential; however, it is important to note that canonical cytochrome c reduction requires ubiquinol oxidation, which occurs in the absence of ASC. The twice-oxidized form of ASC, dehydroascorbate, appears to a have specific role in maintaining the redox balance within mitochondria by readily accepting electrons, thereby preventing leakage to the formation of ROS [156]. This is further evidenced by the observation that ASC concentrations increase when mitochondrial isolate preparations are stimulated by the addition of substrates, primarily due to reduction of dehydroascorbate [142]. Additionally, the similar ratios observed between WT and SVCT2$^{+/-}$

isolates suggest that the effects of ASC deficiency on mitochondria are distinct from mechanisms or processes specific to the disease. Although these mechanisms have not yet been clearly defined, either pathway could be magnified in the increased oxidative stress environment and presence of amyloid associated with Alzheimer's disease progression.

In concurrent experiments, the mitochondria isolated from WT, SVCT2$^{+/-}$ and APP/PSEN1 cortices were incubated with dihydrofluorescein to measure the generation of ROS. We observed a significant increase in fluorescence in SVCT2$^{+/-}$ mitochondrial isolates compared with WT isolates ($t(23)= 2.312$, $p= 0.03$, **Fig. 10E**) and in mitochondrial isolates from APP/PSEN1 cortex ($t(16)= 3.447$, $p = 0.0033$; **Fig. 10F**), suggesting that the oxygen being consumed is not being used efficiently but is rather contributing to oxidative stress in both genotypes. Superoxide, the primary reactive oxygen species produced through oxidative phosphorylation, is reduced to hydrogen peroxide by superoxide dismutase. The brain has lower enzymatic antioxidant activity compared to peripheral tissues [165] and ASC can therefore scavenge ROS, including hydrogen peroxide and hydroxyl radical, providing additional support to the endogenous antioxidant barrier. These observations together indicate that within the context of Alzheimer's disease, which impacts energy production, ASC deficiency can exacerbate dysfunctional mitochondrial respiration and contribute to a redox imbalance within mitochondria.

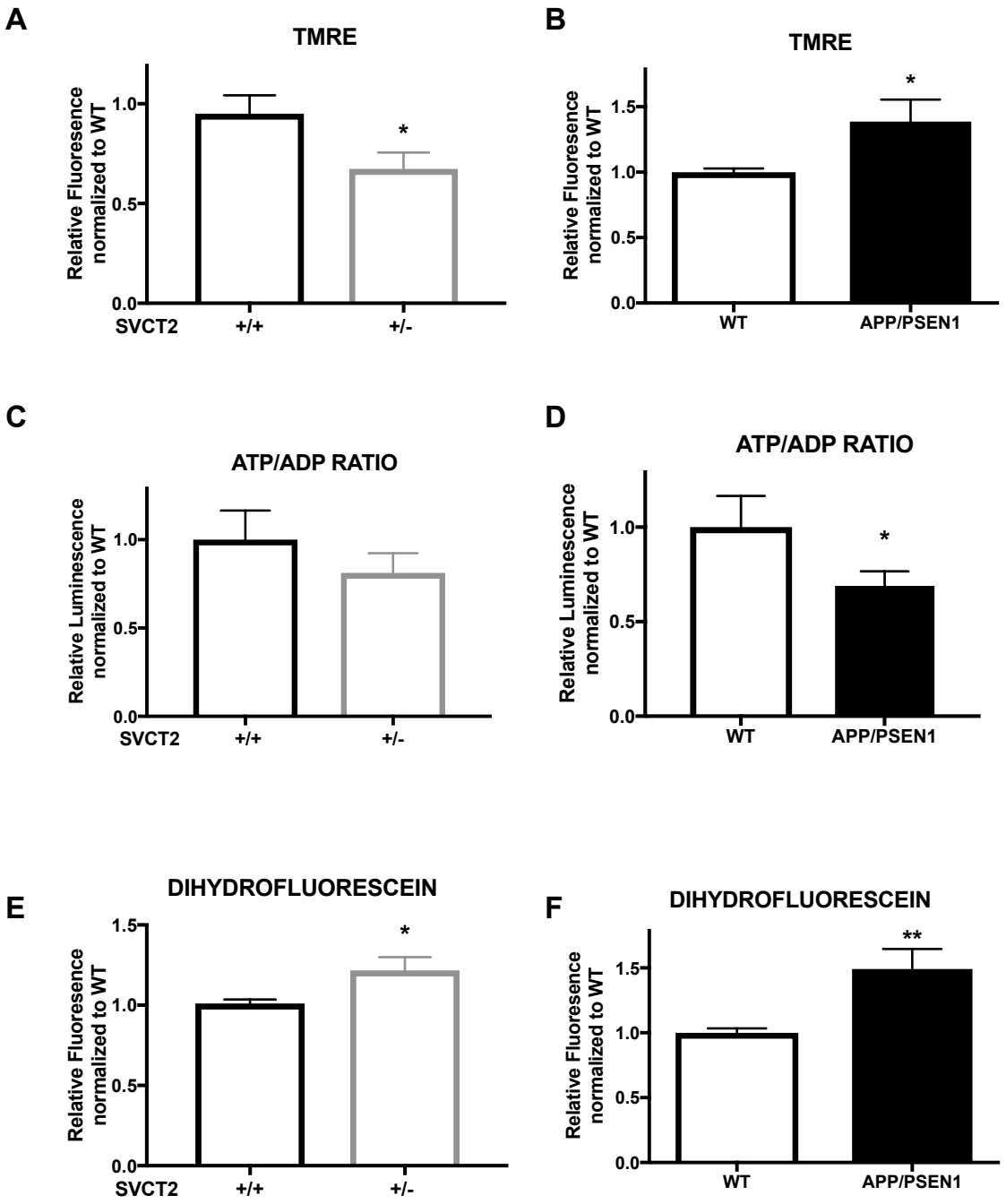


Figure 10 APP/PSEN1 genotype affects mitochondrial membrane potential and increases oxidative stress. A) Mitochondrial isolates from SVCT2$^{+/-}$ mice show decreased mitochondrial membrane potential measured by relative fluorescence from accumulated TMRE ($p = 0.049$, $n = 5$-6 per group). B) Mitochondrial isolates from APP/PSEN1 transgenic mice show increased membrane potential measured by relative fluorescence from accumulated TMRE ($p = 0.0085$, $n = 7$-8 per group). C) The ATP/ADP ratio in SVCT2$^{+/-}$ isolates were not significantly different from WT ($p= 0.195$). D) The ATP/ADP ratio in APP/PSEN1 transgenic isolates was significantly lower than in WT isolates ($p= 0.0299$). E) Mitochondrial isolates from SVCT2$^{+/-}$ mice had significantly greater relative fluorescence from oxidized dihydrofluorescein ($p = 0.03$) than did mice that were wild-type for SVCT2. F) Mitochondrial isolates from APP/PSEN1 transgenic mice also show increased levels of ROS as measured by relative fluorescence from oxidized dihydrofluorescein ($p = 0.038$).

ASC increases oxygen consumption and decreases ROS generation. ASC deficiency resulted in decreased mitochondrial respiration and increased ROS generation, thus we considered whether the acute supplementation of ASC to isolated mitochondria would increase respiration. Mitochondria were isolated from WT cortices as above, and equal concentrations from a single isolation were used for both ASC-supplemented and control conditions in each experimental replication. Once baseline oxygen consumption was established, either vehicle or 100μM ASC was added to mitochondria in a closed respirometer chamber. As expected, the addition of ASC increased oxygen consumption significantly over its own baseline level and the untreated group ($F(1,40)= 11.31$, $p= 0.0017$; **Fig. 11A**). Both ASC+ and ASC- groups significantly increased respiration with the sequential addition of substrates until reaching full respiratory capacity ($F(4,40)= 26.95$, $p<0.0001$; **Fig. 11A**).

Using the $SVCT2^{Tg}$ mouse model, in which SVCT2 is globally over-expressed, we measured mitochondrial membrane potential and ROS generation as above. SVCT2 expression was increased in mitochondrial isolates in the $SVCT2^{Tg}$ model by 17% ($t(6) = 0.625$, $p = 0.55$) and although this difference was not significant it was accompanied by an increase in mitochondrial ASC concentration ($t(12) = 2.166$, $p = 0.05$) (**Fig. 11B, C**). The variability of SVCT2 expression level within the mitochondria of the $SVCT2^{Tg}$ mice is consistent with previously published data from our lab of expression and ASC level in multiple organs, including brain [103]. Unlike $SVCT2^{+/-}$ isolates, we observed no significant change in TMRE fluorescence in $SVCT2^{Tg}$ mice, indicating that a chronic increase in mitochondrial ASC did not directly affect membrane potential. Additionally, we observed a significant decrease in dihydrofluorescein fluorescence in $SVCT2^{Tg}$ mitochondrial isolates compared to WT isolates ($t(12) = 4.414$, $p =$

0.0008) indicating lower ROS production in those mice.

While we observe no significant difference Aβ 42/40 ratio (ELISA, Abcam, USA) between APP/PSEN1 and SVCT2-Tg; APP/PSEN1 mice in hippocampal tissue at 5 months of age (**Fig 12A**), ASC levels in $SVCT2^{Tg}$; APP/PSEN1 mice cortex are ~16% lower at 5 months (p=0.14) and ~29% lower at 12 months (p= 0.002) compared to $SVCT2^{Tg}$. Wild-type and $SVCT2^{Tg}$ data were collected at the same time, but wild-type Aβ ELISA and cortical ASC data only were previously presented in Chapter 3. This observation suggests that the ASC is being consumed more rapidly in $SVCT2^{Tg}$;APP/PSEN1 mice when there is over-abundance, as this change in cortical concentration is not observed between WT and APP/PSEN1 mice at either 5 or 12 months. ASC levels increase slightly between WT and APP/PSEN1, but it is important to note that mice biosynthesize ASC and can increase systemic levels that are limited by transporter expression. Interestingly, ASC levels are clearly not limited by the ability to regulate biosynthesis, in that mice carrying $SVCT2^{Tg}$ exhibit substantially greater concentrations, thus supporting a critical role for ASC. Together, these data suggest that the role of ASC in mitochondria is not directly related to driving Aβ pathology, but rather its effects may contribute to overall mitochondrial function and maintaining redox homeostasis, as demonstrated above.

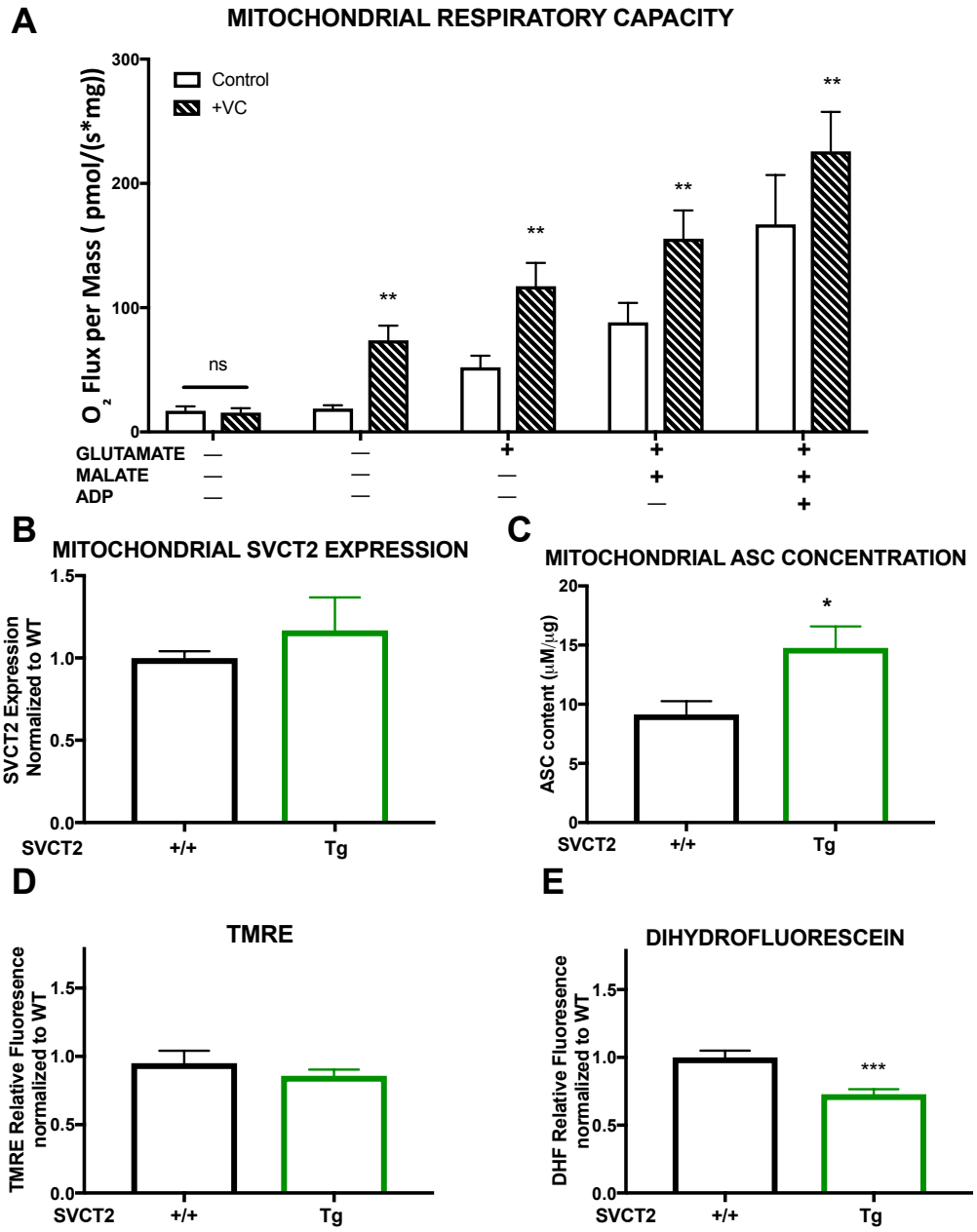


Figure 11 ASC increases oxygen consumption in isolated mitochondria and decreases ROS production. A) The oxygen consumed by mitochondrial isolated from WT cortex was significantly increased with the addition of 100μM ASC ($p = 0.0017$) compared to vehicle following establishment of baseline. . Oxygen consumption within groups increased with the addition of each new substrate compared to the prior condition ($p < 0.0001$). Asterices (*) indicate a main effect of ASC following addition of ASC and of each substrate B) SVCT2 xpression is only slightly increased, (not significant), in mitochondria isolated from $SVCT2^{Tg}$ mice compared to WT mitochondria ($p = 0.55$, n= 5 per group). C) ASC content is significantly elevated,, in $SVCT2^{Tg}$ mitochondria at 4 months ($p = 0.05$, n = 5-9 per group). D) Mitochondrial isolates from $SVCT2^{Tg}$ mice show no difference in mitochondrial membrane potential measured by relative fluorescence from accumulated TMRE ($p = 0.3409$, n = 5-7 per group). E) Mitochondrial isolates from $SVCT2^{Tg}$ mice show a significant decrease in levels of ROS as measured by relative fluorescence from oxidized dihydrofluorescein ($p = 0.0008$, n = 6-8).

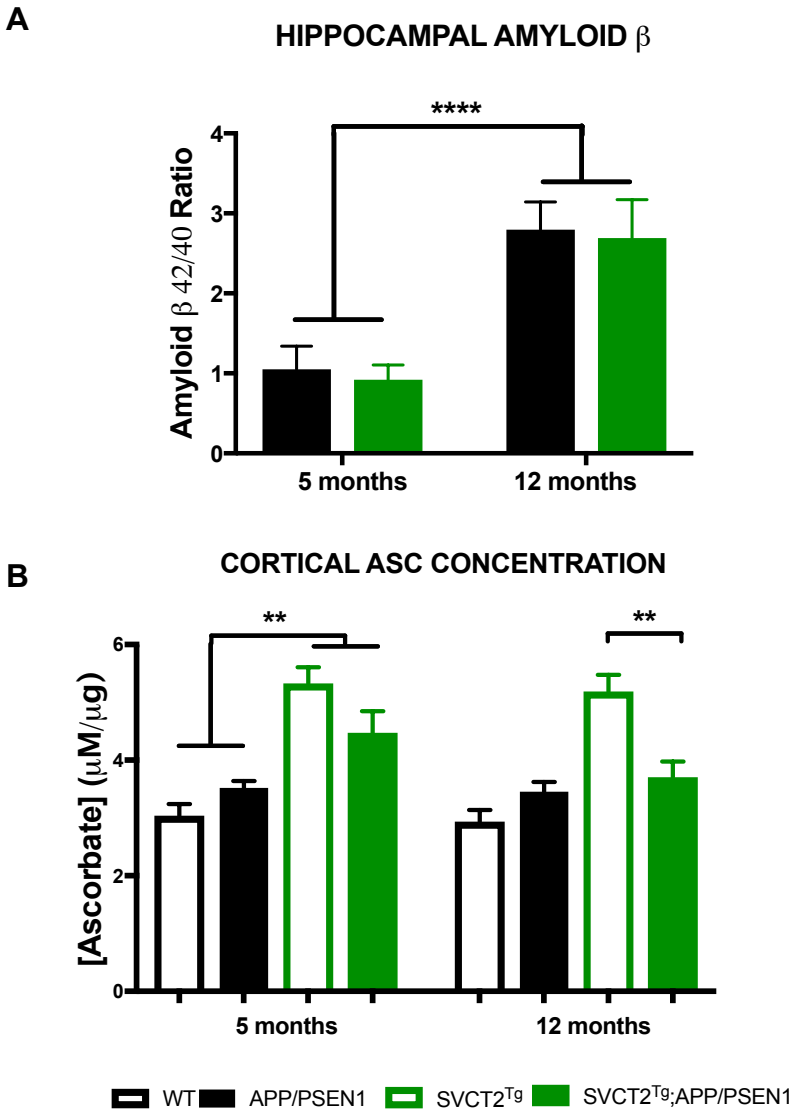


Figure 12 Intracellular ASC concentration is altered in SVCT2^{Tg} ;APP/PSEN1 mice. A) Amyloid β 42/40 ratio is significantly greater at 12 months compared with 5 months in APP/PSEN1 and SVCT2^{Tg};APP/PSEN1 mice ($p< 0.0001$) but does not differ according to genotype with each age group. B) Cortical ASC concentration is significantly elevated in SVCT2^{Tg} mice compared to mice with normal SVCT2 expression ($p=0.005$) at 5 months, but at 12 months a significant difference is observed between SVCT2^{Tg} and SVCT2^{Tg};APP/PSEN1 only ($p=0.0002$).

Summary and Conclusions

ASC is critical for mitochondrial health and ASC deficiency is a major cause of oxidative stress that contributes to Alzheimer's disease risk. The data presented in the previous chapter demonstrated a relationship between ASC deficiency, oxidative stress and amyloid pathology. Here we report that ASC deficiency and the APP/PSEN1 genotype alter mitochondrial respiration, membrane potential, energy production and increase ROS generation at much earlier stages in disease progression. While optimal levels of ASC are unknown [154], adequate ASC supports both the redox balance within mitochondria, and oxygen consumption. The key features of oxidative stress and mitochondrial dysfunction that are elevated at prodromal stages are believed to drive disease pathology, and may provide potential biomarkers before the manifestation of clinical symptoms. Elderly individuals are at even greater risk as the activity of endogenous antioxidant enzymes decreases with age, a phenomenon that is exaggerated in Alzheimer's disease suggesting that the antioxidant barrier is being overwhelmed by increasing oxidative stress [11,166]. However, given that biological changes are observed in humans long before cognitive symptoms manifest [24,167], ASC deficiency in mid-life may have an impact on the risk for developing AD. We next sought to determine whether ASC deficiency and oxidative stress influence the age of onset for disease-driven gene expression changes associated with the pathogenesis of AD.

Chapter V: Early gene changes in Alzheimer's disease are exacerbated by ascorbate deficiency

Gene changes that occur early in Alzheimer's disease may be crucial in dictating the rate of decline in later life. Many important pathways such as energy metabolism, protein degradation and antioxidant defense become less efficient with age. However, the appearance of deficiencies in each pathway may occur more rapidly in those individuals at risk for developing AD, and in turn contribute to accelerated overall decline [11,12,168].

Early gene changes also likely represent pathways that are more amenable to pharmacological or lifestyle interventions. Increased oxidative stress has been associated with upregulation of genes related to AD. For this reason, maintaining high levels of nutrient-based antioxidants may be an essential adjuvant therapy. ASC is a vital molecule for its antioxidant and enzymatic co-factor capabilities. ASC has a low reduction potential, which allows it to reduce most radicals and oxidants it interacts with in the aqueous phase [47,64], and has the ability to recycle other antioxidants such as the lipophilic antioxidant vitamin E [48]. In clinical studies, ascorbate supplementation decreased the prevalence of DNA adducts and DNA strand breaks after mutagen or carcinogen exposure, specifically when plasma concentrations exceeded 50μmol/L [53]. ASC deficiency is often studied in guinea pigs because they are incapable of ascorbate biosynthesis, similar to the human condition. Lykkesfeldt et al. (2007) reported increased DNA oxidation and DNA incision repair in neonatal guinea pigs with ASC deficiency [169]. We have previously reported that ASC deficiency in mice, either through heterozygous expression of the SVCT2, or through an inability to biosynthesize ASC (gulo-/-), is correlated with increased levels of oxidative

damage in the brain [59,62,113]. ASC deficient mice crossed with APP/PSEN1, show an accelerated pattern of both cognitive and pathological deficits compared with ASC-replete control, as discussed in previous chapters. SVCT2$^{+/-}$ mice have a life-long, 30% decrease in global ASC tissue concentration compared to wild-type mice [59,60], but SVCT2$^{+/-}$;APP/PSEN1 mice have significantly lower cortical ASC concentration compared with SVCT2$^{+/-}$ mice at 14 month, suggesting more rapid consumption of ASC in the brains of these animals due to the presence of AD pathology.

In addition to the role of ASC as an antioxidant to directly scavenge ROS, it also functions as an enzymatic cofactor in 2-oxoglutarate-dependent dioxygenases (2-ODDs) reactions, such as TET hydroxylases [55,56,58,170], AlkB DNA repair proteins [52,171]. These observations indicate that compromised ASC capacity increases the momentum of AD pathological processes and may hasten AD-related epigenetic modification and oxidative DNA damage because of a diminished capacity to maintain the cellular redox balance and diminished availability as an enzymatic cofactor. To investigate the effect of ASC deficiency on AD-driven gene expression, we used a pre-designed array with 84 genes implicated in AD pathogenesis and progression to determine alterations in gene expression in hippocampi collected from wild-type and APP/PSEN1 mice with differential SVCT2 expression at 4 and 12 months of age. Given the previous findings of increases in both oxidative stress and amyloid pathology at 6 months, along with the emergence of cognitive deficits at 5 months in the SVCT2$^{+/-}$;APP/PSEN1 mice discussed in the previous chapters, we were specifically interested in expression changes in genes related to amyloid generation and clearance, as well as neuronal health and apoptosis in order to elucidate the direct contribution of ASC deficiency in disease pathogenesis.

Results and Discussion

Significant oxidative damage precedes the classical pathological features of AD, contributing to the progression of the disease [36,172]. Once established, the relationship between oxidative stress and AD becomes cyclical. As an antioxidant, ASC combats the deleterious effects oxidative stress has on DNA integrity and epigenetic regulation, and contributes to DNA repair mechanisms as an enzymatic cofactor, thereby maintaining genome integrity. We proposed that low ASC is particularly damaging in young mice with a greater risk for disease, and thus our primary focus was on genes known to directly govern disease pathology and progression at very early stages of the disease. These we divided into four general classifications according to functional contribution to disease pathology: (1) amyloid and tau pathology, (2) amyloid clearance, (3) apoptosis, and (4) neuronal health. A complete table of fold-regulation and p-values are reported in Table 4 and Table 5.

The same genes were evaluated at 4 and 12 months to determine expression changes as a result of age and disease progression, allowing us to directly test the hypothesis that disease-driven genetic changes occur earlier in the context of ASC deficiency, thus protracting the length of abnormal disease pathology rather than exaggerating the ultimate level of damage. At 4 months, gene expression associated with (1) amyloidogenesis, (2) amyloid clearance, and (3) apoptosis are up-regulated in both of the APP/PSEN1+ groups compared to the wild-type group, *but the magnitude of change in gene expression in the combined SVCT2$^{+/-}$;APP/PSEN1 mice were elevated to a greater degree at this age*. The same genes were down-regulated in young SVCT2$^{+/-}$ mice. These observations were effectively reversed by 12 months when compared to wild-type expression, presumably due to age-related gene expression changes in the wild type at 12 months. Specific genes and

their potential role in AD neuropathology are discussed in detail below.

ASC status and APP/PSEN1 genotype drive gene expression changes related to Aβ generation and microtubule dynamics in young mice. We observed increased expression of the APP gene in both APP/PSEN1 and SVCT2$^{+/-}$;APP/PSEN1 hippocampi at 4 months over wild-type expression (**Fig. 13A**), as expected due to the expression of the human APP transgene in this mouse model. While the SVCT2$^{+/-}$ alone group also showed decreased APP gene expression compared to wild-type animals, the combined SVCT2$^{+/-}$;APP/PSEN1 exhibited a more than 4-fold increase in expression over wild type. This observation is consistent with our hypothesis that ASC deficiency exacerbates AD pathology, presumably through redox imbalance. It is are also consistent with data discussed previously in Chapter 3 in which soluble $A\beta_{1-42}$ and $A\beta_{1-40}$ peptides were increased in cortical tissue from SVCT2$^{+/-}$;APP/PSEN1 mice at 6 months, and Aβ plaque deposition was greater in the hippocampus and cortex at 14 months, compared with APP/PSEN1 mice that have normal ASC transporter expression.

We also observed an increase in gene expression related to tau proteins (microtubule-associated proteins: Mapt, Map2) in the APP/PSEN1 and SVCT2$^{+/-}$;APP/PSEN1, but not in the SVCT2$^{+/-}$ group (**Fig 13A**). Hyperphosphorylated tau proteins cause the aberrant microtubule organization associated with the formation of neurofibrillary tangles characteristic to AD pathogenesis, but the overexpression of tau alone has been shown to cause neurodegeneration in several animal models [173]. As expected Bace1, Bace2 and Psen1 (presenilin 1) were also up-regulated as they make up the γ-secretase complex. Interestingly, the expression of Psen2 (presenilin 2) appears to be affected by ASC deficiency as indicated by the increased expression observed in the SVCT2$^{+/-}$ and SVCT2$^{+/-}$;APP/PSEN1 groups, although not in the APP/PSEN1 group (**Fig 13A**). Psen1 and Psen2 are both catalytically active

in the γ-secretase complex. Although, Aβ cleavage rates are higher with Psen1 than Psen2 [174,175], the combination of increased expression of both could contribute to the elevated Aβ generation observed in earlier studies. Also of note, expression of the two homologs differs among tissue and in development, as indicated by distinctly different phenotypes between Psen1 and Psen2 knock-out models [174,175].

ASC status and APP/PSEN1 drive gene expression changes related to Aβ clearance in young mice. The low-density lipoprotein receptor-related proteins 1 and 8 (Lrp1, Lrp8) are specialized receptors for the endocytosis of lipoproteins and have been implicated in Aβ clearance. Specifically, AD patients have lower Lrp density compared with age-matched controls. Additionally, a single nucleotide polymorphism in Lrp C776T has been associated with increased risk of developing AD [176], indicating that Lrp plays a crucial role in Aβ clearance and degradation. Both Lrp1 and Lrp8 were upregulated in APP/PSEN1 and SVCT2$^{+/-}$;APP/PSEN1 groups (**Fig. 13B**), which is consistent with an increase in amyloid production. Lrp1 expression in the SVCT2$^{+/-}$ group was also increased over wild-type (**Fig. 13B**). Lrp1 and Lrp8 are also known as Alpha-2-macroglobulin (A2m) and Apolipoprotein E (Apoe) receptors. A2m is implicated in Aβ degradation by directly binding to Aβ while retaining its ability to bind to several proteases. This induces a conformational change that exposes a binding site for Lrps, thereby creating a direct pathway for degradation and preventing aggregation [177]. A2m appears to be down-regulated more than 2.5 fold in the SVCT2$^{+/-}$ and APP/PSEN1 groups, while the magnitude of the down-regulation is not as dramatic in the SVCT2$^{+/-}$;APP/PSEN1 group at a 1.5-fold decrease (**Fig. 13B**). Concentrations of A2m are generally low and increased concentrations are associated with injury and inflammation [176]. In clinical studies, elevated

plasma concentrations of A2m positively correlate with increased concentrations of AD markers in the CSF at preclinical stages of the disease, indicating greater risk of disease progression [178]. The difference in fold-regulation magnitude in the SVCT2$^{+/-}$;APP/PSEN1 group compared to the APP/PSEN1 group may indicate that A2m expression is regulated in part by ASC availability and progression of AD pathology.

Apoe is involved in lipid transport and has been associated with extracellular Aβ clearance. Mice that overexpress APP but are null for Apoe show no Aβ plaque formation, while overall levels of Aβ remain unchanged. When human apoE3 and apoE4 genes are expressed in mice, overexpressing APP appears to affect APP processing and Aβ deposition [179]. Humans carrying the apoE ε4 allele have a greater risk of developing AD [180], perhaps due to an inherent inefficiency in the resulting protein. Interestingly, all groups show an increase in Apoe expression over wild-type expression (**Fig. 13B**); however, the role of Apoe may be two-fold, which would account for the apparent additive effect seen in the SVCT2$^{+/-}$;APP/PSEN1 group. Firstly, both ASC deficiency and increased levels of Aβ result in increased levels of oxidative damage to membrane lipids and proteins. Apoe plays a crucial role in membrane repair and remodeling through transport of lipids such as cholesterol and phospholipids; therefore, the increased expression observed in the SVCT2$^{+/-}$ group may be in response to increased levels of lipid peroxidation, which has been reported previously by our group [60,155]. Secondly, increased Aβ production observed in the APP/PSEN1 and the SVCT2$^{+/-}$;APP/PSEN1 group may deplete or overwhelm available Apoe, requiring increased expression of the lipoprotein.

Cathepsin B and D (Ctsb, Ctsd) are lysosomal proteases necessary for endocytosed or autophagized protein degradation, such as those facilitated by Lrps, and may play a role in Aβ

clearance and degradation. Ctsb appears to have a dualistic role in the literature in that it has been shown to degrade Aβ via the cathepsin B-cystain C axis [181]; however, studies also show that inhibition of Ctsb in mice expressing human wild-type APP reduces Aβ burden and improves memory. This is not the case for mice expressing human APP mutations associated with familial AD [reviewed in 35]. We observed a decrease in Cstb expression in both SVCT2$^{+/-}$ and APP/PSEN1 groups over wild-type, but an increase in the SVCT2$^{+/-}$;APP/PSEN1 group alone (**Fig. 13B**). This data is consistent with the increase Aβ burden discussed previously. Age-related Ctsd translocation to the cytosol through lysosomal permeabilization may play a significant role in tau degradation and APP metabolism as well as ApoE processing [182,183]. Lysosomal permeabilization is also associated with apoptosis due to translocation of proteases. In fact, we observed an increase in microtubule associated proteins (Mapt, Map2) in both the APP/PSEN1 and SVCT2$^{+/-}$;APP/PSEN1 groups compared to wild-type expression (**Fig. 13A**). Ctsd protein localizes with Aβ plaques and is up-regulated in AD and mild cognitive impairment [183]. Consistent with this data, we observed an up-regulation in Ctsd in both the APP/PSEN1 and SVCT2$^{+/-}$;APP/PSEN1 groups, but decreased expression in the SVCT2$^{+/-}$ group compared to wild-type.

ASC status and APP/PSEN1 genotype drive gene expression changes related to oxidative stress response and apoptosis in young mice. Hydroxysteroid (17-beta) dehydrogenase 10 is an oxioreductase localized to the mitochondria, expression of which is elevated in the brains of AD patients, supporting an etiological link between mitochondrial dysfunction to AD pathology. Hydroxysteroid (17-beta) dehydrogenase (Hsd17b10) has been shown to bind to intracellular Aβ oligomers, which ultimately inhibits the enzymatic efficiency of Hsd17b10

[184,185], resulting in alterations to neuroprotective steroid metabolism. Increased Hsd17b10 was only observed in the SVCT2$^{+/-}$;APP/PSEN1 group, presumably due to the increase in AD pathology hastened by ASC deficiency (**Fig. 13C**). Increased ROS is observed when Hsd17b10 binds Aβ in mitochondria, which has been shown to contribute to Aβ production and oxidative stress, leading to mitochondrial dysfunction and eventually cell death via mitochondrial membrane permeability [185]. In fact, we observed increased caspase 3 (Casp3) expression in the SVCT2$^{+/-}$;APP/PSEN1 group alone as well, indicating increased cell death in the ASC deficient model of AD (**Fig. 13C**). This is supports the hypothesis that ASC deficiency hastens AD pathology through amyloid-independent pathways. Interestingly, we also observed an increase in caspase 4 (Casp4) expression in both the APP/PSEN1 and SVCT2$^{+/-}$;APP/PSEN1 group (**Fig. 13C**). Casp4 is implicated in activation of the inflammasome and release of the inflammatory cytokine interleukin 1 alpha (Il1a), which is also increased in both the APP/PSEN1 and SVCT2$^{+/-}$;APP/PSEN1 group. Neuroinflammation is elevated in AD and contributes to the redox imbalance that leads to oxidative damage, cellular dysfunction and cell death associated with disease progression [9,30].

Gene expression of cell cycle proteins cyclin-dependent kinases 1 and 5 (Cdk1, Cdk5) were increased in all groups compared to wild type (**Fig. 13C**). Neurons are considered post-mitotic cells and therefore do not re-enter the cell cycle; however, cell cycle proteins are elevated in early stages of AD [186]. As discussed previously, ROS can attack and oxidatively damage DNA leading to strand breaks, mutation and altered gene expression [37]. Cell cycle proteins are implicated in neuroplasticity, highlighting the ability of neurons to adapt; however, cell cycle phosphorylation and dephosphorylation also serve as checkpoints during mitosis and can trigger apoptosis based on compromised integrity of different cellular

components. Mitotic cells can repair DNA damage by re-entering the cell cycle [187], thus the up-regulation of cell cycle proteins Cdk1 and Cdk5 may be a conserved response to accumulating DNA damage and a pathway to programmed cell death. Cdk5 has also been implicated in aberrant tau phosphorylation [188,189].

ASC status and APP/PSEN1 drive gene expression changes related to synaptic plasticity in young mice. Disruptions in the cholinergic system have long been reported in AD. Choline acetyltranferase (Chat) activity, the enzyme necessary for biosynthesis of acetylcholine, is decreased in patients in late stages of AD, while an increase in Chat activity is observed in patients with mild cognitive impairment and early AD [190]. Several pharmacological interventions target the cholinergic system to preserve cognitive function, specifically inhibition of acetylcholinesterase-mediated (Ache) degradation of acetylcholine [159,191]. Ache expression was increased for both SVCT2$^{+/-}$ and SVCT2$^{+/-}$;APP/PSEN1 while expression was decreased in the APP/PSEN1 group, suggesting a direct effect of ASC on the expression of this gene (**Fig. 13D**). Our group has demonstrated that acute administration of ASC appears to exert an inhibitory effect on Ache and facilitates neurotransmitter release from vesicles [48,192] resulting in improved performance in Y-maze alternation and Morris water maze in 12- and 24-month APP/PSEN1 mice [77]. In the present study, Chat expression increased across all groups compared to wild type, suggesting the up-regulation may be a compensatory response to insult (**Fig. 13D**).

Neurotrophic factors support synaptic development and neuronal survival to promote learning and memory, and are neuroprotective against brain injury or insult; however, neurotrophic activity declines with age resulting in a decline in cognitive performance [28].

Acute administration of insulin-like growth factor 2 (Igf2) via hippocampal injection or overexpression has been shown to encourage dendritic spine formation and neural stem cell proliferation, thereby improving memory, while decreasing Aβ accumulation in mouse models of AD [191,193]. We observed a substantial decrease in Igf2 expression (~3-fold) in SVCT2$^{+/-}$ and APP/PSEN1 groups compared to wild-type expression, but a less pronounced decrease in the SVCT2$^{+/-}$;APP/PSEN1 group (**Fig. 13D**). Changes in the expression of brain-derived neurotrophic factor (Bdnf) are associated with different stages of AD. Some studies suggest that Bdnf increases in early AD and mild cognitive impairment while other studies report brain and serum levels are much lower in all stages of AD, significantly lower in advanced stages of AD compared to age-matched controls [194]. Bdnf expression was increased in SVCT2$^{+/-}$ and APP/PSEN1 over wild type, and an additive effect was observed in SVCT2$^{+/-}$;APP/PSEN1, suggesting an up-regulation in response to insult (**Fig. 13D**). Additionally, only the SVCT2$^{+/-}$;APP/PSEN1 group showed an increase in Lrp6, which is closely associated with Wnt/β-catenin signaling [195], and which promotes neurogenesis and synaptic plasticity through transcriptional activation [195,196]. Taken together, these data indicate that at 4 months of age mechanisms to repair or restore neuronal integrity in response to the detrimental effects of both ASC deficiency and AD pathology are activated through independent pathways resulting in an additive effect in the combined SVCT2$^{+/-}$;APP/PSEN1 group.

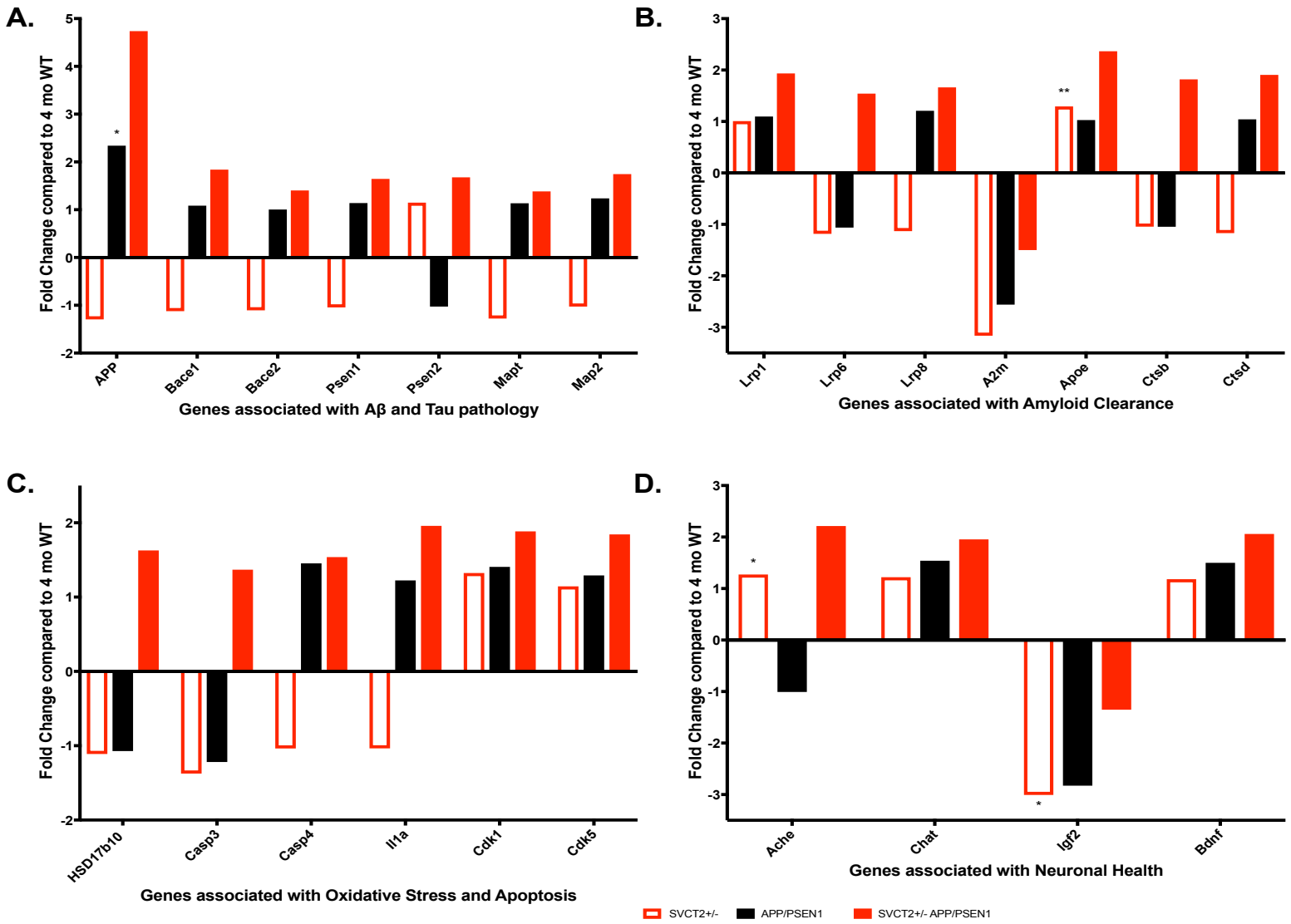


Figure 13 Changes in gene expression compared to wild type at 4 months. A) Genes associated with Aβ and Tau pathology are up-regulated to a greater degree in ASC-deficient APP/PSEN1 mice (App: p=0.047) B) Genes associated with Aβ protein degradation are up-regulated to a greater degree ASC-deficient APP/PSEN1 mice (Apoe: p=0.003) C) Genes associated with apoptosis are up-regulated to a greater degree ASC-deficient APP/PSEN1 mice. D. Genes associated with synaptic maintenance and neuronal health are up-regulated to a greater degree ASC-deficient APP/PSEN1 mice (Ache: p=0.012; Igf2: p=0.03).

Gene expression patterns differ between middle-aged and young mice. We observed a reversal in the expression patterns of several genes in 12-month-old mice compared to expression patterns in 4-month-old mice. Expression of the APP gene in the APP/PSEN1 and SVCT2$^{+/-}$;APP/PSEN1 groups remained higher than wild type at 12 months, but not to the same magnitude observed in the 4-month group. Bace1, Bace2, Psen1, Psen2 and genes related to tau pathology (Mapt, Map2) were decreased in the APP/PSEN1 and SVCT2$^{+/-}$;APP/PSEN1 groups compared to wild type in 12-month-old mice (**Fig. 14A**). Interestingly, expression of App, Bace1, Psen1 and Mapt appear to be up-regulated SVCT2$^{+/-}$ at 12 months, supporting our hypothesis that ASC deficiency can drive AD-relevant pathology in the absence of the two specific mutations carried by the mice, particularly when age is also a factor. Lrp1, Lrp6, Lrp8, and Apoe were down-regulated, while A2m was up-regulated in APP/PSEN1 and SVCT2$^{+/-}$;APP/PSEN1 groups compared to wild type in 12-month-old mice, consistent with clinical data regarding LRP expression in patients with AD (**Fig. 14B**). 176]. Ctsb expression was down regulated in APP/PSEN1 and SVCT2$^{+/-}$;APP/PSEN1 in 12-month-old mice, whereas expression was up-regulated in the SVCT2$^{+/-}$ group compared to wild-type at 12 months. Although expression of Ctsb shows a minimal change in expression at this age, it is reported to remain stable across age in animal studies [182], suggesting that the observed changes are typical. Ctsd was significantly increased in APP/PSEN1 and SVCT2$^{+/-}$;APP/PSEN1 groups compared to wild type in 12-month-old mice, consistent with clinical data (**Fig. 14B**). [183]. Ache, Chat, Hsd17b10 and Caps3 were down regulated in SVCT2$^{+/-}$;APP/PSEN1 mice at 12 months, whereas expression for these genes were increased in this group alone at 4 months. Cdk5 expression was decreased in all groups at 12 months (**Fig. 14C**). Finally, Bdnf was decreased for all groups at 12 months, which is consistent with the literature regarding age-related decreases in

neurotrophic activity (**Fig. 14D**). [189,194].

The observed reversal in expression of several genes in the APP/PSEN1 and SVCT2$^{+/-}$;APP/PSEN1 at 12 months that had been up-regulated at 4 months may be the result of a compensatory mechanism. It is important to note that age-related gene expression changes in wild type may have contributed to the observed changes in expression patterns, such as magnitude of fold-change, because all data are reported relative to age-matched wild type expression, and we did not explicitly compare aged and young groups. However, several genes in the SVCT2$^{+/-}$ group also exhibited a reversal in gene expression directionality compared to wild type at 12 months (App, Bace1, Psen1, Lrp6, etc.), suggesting that within the context of an aging brain, ASC deficiency contributes to pathological changes when the disease is not present.

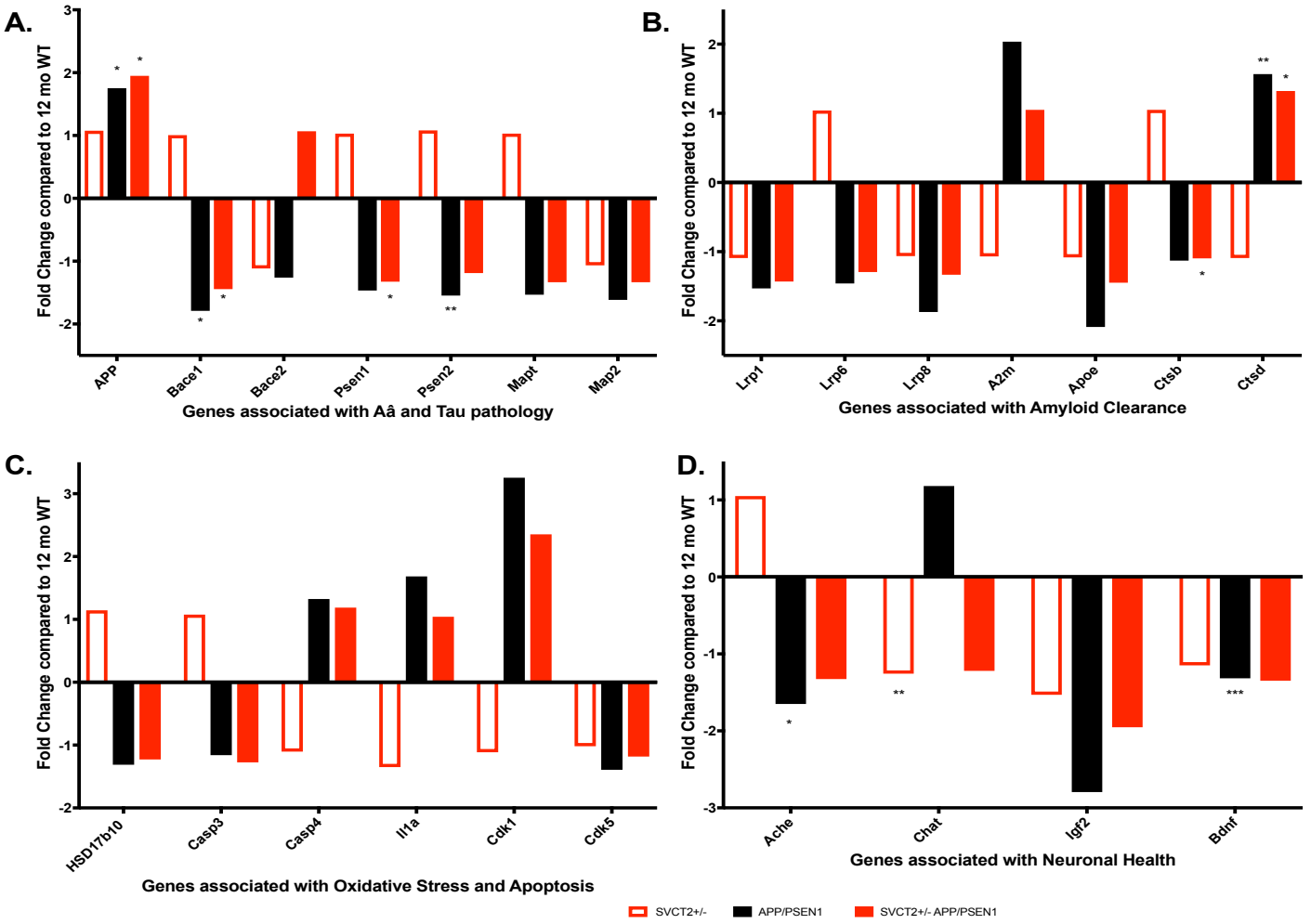


Figure 14 Changes in gene expression compared to wild type at 12 months. A) Genes associated with Aβ and Tau pathology are up-regulated to a greater degree in ASC-deficient mice, but down-regulated in APP/PSEN1 mice (App: p= 0.036, p= 0.018; Bace1: p= 0.026, p= 0.043 ; Psen1: p= 0.038; Psen2: p= 0.004, respectively) B) Genes associated with Aβ protein degradation are down-regulated in ASC-deficient and APP/PSEN1 mice (Ctsb: p= 0.028; Ctsd: p= 0.002, p= 0.035,resp.) C) Genes associated with apoptosis are down-regulated to a greater degree in aged APP/PSEN1 mice. D. Genes associated with synaptic maintenance and neuronal health are down-regulated to a greater degree in aged ASC-deficient and APP/PSEN1 mice (Ache: p= 0.039 ; Chat p = 0.007; Bdnf: p= 0.001, resp.).

Global DNA methylation patterns differ between middle-aged and young mice. One mechanism by which gene transcription is regulated is through addition of methyl groups to DNA. Decreases in methylation occur naturally with age throughout the entire genome; however, epigenetic studies have identified differences in methylation patterns between AD patients and the typically aging population. DNA methylation (5-methylcytosine, 5-mC) effectively suppresses gene expression by inhibiting transcription factor binding at CG islands within gene promoter regions [197,198]. Such differences indicate that this form of gene regulation may contribute to the development or progression of AD [199]. A concurrent hypomethylation of these same genes, including BACE1 and presenilin 1 (amyloid beta production), as well as TNFa, NFkB, IL-6 and IL- 8 (inflammatory markers) was also detected under conditions of oxidative stress [37,166,189]. Similarly, a study by Chen *et al.* reported that treatment of cerebral epithelial cells with amyloid β(1-40) peptide caused global hypomethylation [200]. Paradoxically, the promoter region of neprilysin, an important protein in amyloid degradation, is shown to be hypermethylated in AD patients resulting in decreased expression, suggesting gene-specific regulation [200].

Until recently, 5-hydroxymethylcytosine (5-hmC) was believed to be a DNA oxidation product of 5-mC, but is now believed to be an intermediate in cytosine demethylation [201]. 5-hmC levels are particular abundant in the brain [202], perhaps reflective of the genetic plasticity in the brain, in which case the presence of the stable 5-hmC intermediate effectively primes an inactive gene for activation [203]. Using an ELISA-based assay for global 5-hydroxymethylcytosine (5-hmC) in hippocampus, we observed an overall increase 12 month mice compared to 4 month-old mice (**Fig 15** $F(1,22)=35.86$, $p<0.0001$). Based on the hypothesis that aging is accelerated in the context of disease or ASC deficiency, we

compared genotypes within each age. Interestingly, we observed a main effect of APP/PSEN1 in global 5-hmC levels at 4 months compared to wild type (**Fig 15** $F(1,22)=12.17$, $p<0.002$). This effect was not apparent at 12 months, presumably due to the increased magnitude of 5-hmC observed in the older wild-type mice compared to younger animals. There were no significant differences between groups at 12 months although the SVCT2$^{+/-}$ and SVCT2$^{+/-}$;APP/PSEN1 showed markedly lower levels compared to the wild type and APP/PSEN1 groups, which have wild-type SVCT2 expression. 5-mC is oxidized to 5-hmC by TET enzymes [170], which utilize ASC as an enzymatic co-factor. The decrease in 5-hmC at 12 months may be the result of decreased availability of ASC due to age- and disease-related redox imbalance. Combined, these data suggest that oxidative stress-driven epigenetic modification may contribute to the pathogenesis and progression of AD.

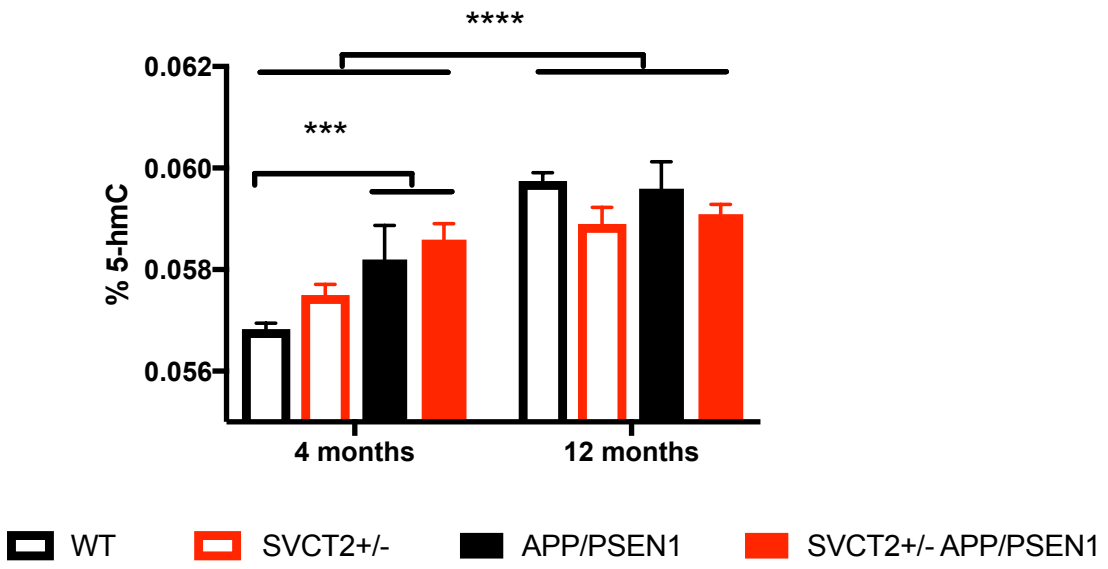


Figure 15 Hippocampal 5-hmC at 4 and 12 months. 5-hmC levels increase significantly with age ($p < 0.0001$). At 4 months, APP/PSEN1 and SVCT2+/-;APP/PSEN1 5-hmC levels were significantly greater than wild type ($p = 0.002$).

Summary and Conclusions

Gene expression changes that occur in response to age, injury and disease reflect the brain's ability to adapt, repair and restore function in a rapidly changing environment. This ability is hindered by the accumulation of oxidative stress and nutrient deficiency. We report here that ASC deficiency in the APP/PSEN1 mouse model of Alzheimer's disease lead to a greater up-regulation of genes associated with AD pathology than in APP/PSEN1 mice with adequate ASC. In response, the expression of genes responsible for repairing and protecting the brain were up-regulated in young mice, as were genes associated with apoptosis. This adaptive response was lost at later stages of the disease in both groups in middle-aged mice due to and contributing to the detrimental cycle of oxidative stress and disease progression that eventually leads to cognitive dysfunction. We also observed up-regulation in genes associated with Aβ generation and apoptosis in middle-aged ASC deficient mice. These observations suggest that in familial AD, where the developing the disease is inevitable in those that inherit the relevant mutations, adequate ASC may delay disease pathology and support the protective adaptive response at very early stages of the disease. With regard to the sporadic form of the disease, the data presented in these chapters support the role of ASC deficiency in accelerating age-related and AD neuropathology, specifically that driven by oxidative stress. ASC deficiency is cheaply and effectively addressed through diet or supplementation, which provides a potential strategy to slow the progression of Alzheimer's disease by attenuating the damaging effects of oxidative stress and proves to be a valuable addition to current Alzheimer's disease therapies. The current data support implementation of antioxidant strategies at far earlier stages of disease development in order to protect against age-related changes that may increase the risk for developing AD.

Table 4 Fold-regulation and p-values for all genes across groups in 4-month-old mice.

		Up-Down Regulation (comparing to control group)					
		SVCT2+/-		APP/PSEN1		SVCT2+/-; APP/PSEN1	
Position	Symbol	Fold Regulation	p-value	Fold Regulation	p-value	Fold Regulation	p-value
A01	A2m	-3.165	0.092	-2.559	0.081	-1.499	0.761
A02	Abca1	-1.101	0.980	1.091	0.540	1.547	0.460
A03	Ache	1.273	0.012	-1.008	0.918	2.213	0.382
A04	Adam9	-1.124	0.633	1.110	0.229	1.619	0.436
A05	Apba1	1.017	0.809	1.099	0.350	2.051	0.407
A06	Apba3	1.221	0.523	1.539	0.375	1.954	0.392
A07	Apbb1	1.520	0.032	1.477	0.470	2.716	0.340
A08	Apbb2	-1.104	0.759	1.047	0.810	1.783	0.436
A09	Aph1a	-1.116	0.443	-1.046	0.798	1.875	0.403
A10	Aplp1	1.093	0.629	1.159	0.467	1.944	0.414
A11	Aplp2	-1.042	0.888	1.019	0.834	1.605	0.445
A12	Apoa1	1.389	0.186	1.016	0.872	2.176	0.379
B01	Apoe	1.293	0.003	1.028	0.755	2.366	0.310
B02	App	-1.294	0.640	2.340	0.047	4.738	0.328
B03	Bace1	-1.122	0.835	1.084	0.545	1.838	0.425
B04	Bace2	-1.106	0.577	1.004	0.896	1.403	0.476
B05	Bche	1.108	0.773	1.371	0.297	1.777	0.441
B06	Bdnf	1.182	0.503	1.500	0.216	2.060	0.403
B07	Casp3	-1.375	0.287	-1.219	0.189	1.368	0.482
B08	Casp4	-1.037	0.859	1.454	0.361	1.538	0.458
B09	Cdk1	1.324	0.445	1.406	0.426	1.884	0.398
B10	Cdk5	1.144	0.558	1.291	0.395	1.844	0.416
B11	Cdkl1	-1.111	0.648	1.044	0.995	1.433	0.494
B12	Chat	1.221	0.523	1.539	0.375	1.954	0.392
C01	Clu	-1.122	0.345	-1.134	0.095	1.557	0.462
C02	Ctsb	-1.044	0.889	-1.047	0.740	1.820	0.431
C03	Ctsc	-1.319	0.542	-1.372	0.187	1.334	0.475
C04	Ctsd	-1.169	0.415	1.043	0.750	1.906	0.393
C05	Ctsg	1.221	0.523	1.539	0.375	1.954	0.392
C06	Ctsl	1.078	0.648	1.113	0.415	1.961	0.404
C07	Ep300	-1.213	0.764	1.106	0.348	1.708	0.438
C08	Ern1	-1.331	0.364	-1.069	0.557	1.713	0.438
C09	Gap43	-1.227	0.840	1.110	0.513	1.680	0.457
C10	Gnao1	-1.096	0.778	1.108	0.272	1.552	0.457
C11	Gnaz	-1.018	0.964	1.174	0.250	1.878	0.419
C12	Gnb1	1.012	0.850	1.156	0.352	1.650	0.440
D01	Gnb2	1.232	0.174	1.170	0.452	2.807	0.323
D02	Gnb4	-1.002	0.738	1.027	0.736	1.812	0.428
D03	Gnb5	1.063	0.533	1.077	0.273	1.851	0.418
D04	Gng10	1.472	0.364	1.663	0.129	2.334	0.375
D05	Gng11	1.185	0.079	-1.128	0.660	1.875	0.396
D06	Gng3	1.187	0.120	1.112	0.423	1.714	0.441
D07	Gng4	1.027	0.866	1.049	0.812	1.564	0.465
D08	Gng5	1.212	0.466	1.171	0.391	2.029	0.370
D09	Gng7	-1.076	0.942	1.138	0.567	1.697	0.429
D10	Gng8	1.246	0.379	1.386	0.356	2.165	0.399
D11	Gngt1	1.221	0.523	1.539	0.375	1.954	0.392
D12	Gngt2	1.144	0.517	1.375	0.223	2.057	0.382
E01	Gsk3a	-1.277	0.352	-1.202	0.148	1.508	0.473
E02	Gsk3b	-1.086	0.745	1.147	0.312	1.576	0.458
E03	Hsd17b10	-1.111	0.423	-1.072	0.564	1.628	0.455
E04	Ide	1.229	0.408	1.314	0.455	1.819	0.425
E05	Igf2	-3.008	0.215	-2.828	0.027	-1.352	0.995
E06	Il1a	-1.035	0.737	1.224	0.430	1.958	0.395
E07	Insr	-1.076	0.827	1.064	0.207	1.778	0.424
E08	Lpl	1.098	0.615	1.487	0.277	2.096	0.351
E09	Lrp1	1.008	0.966	1.096	0.536	1.934	0.408
E10	Lrp6	-1.183	0.367	-1.062	0.447	1.542	0.439
E11	Lrp8	-1.132	0.657	1.208	0.287	1.663	0.443
E12	Mapt	-1.277	0.219	1.136	0.525	1.383	0.487
F01	Mpo	1.221	0.523	1.731	0.301	1.954	0.392
F02	Map2	-1.027	0.974	1.238	0.127	1.745	0.433
F03	Nae1	1.396	0.418	1.598	0.157	2.285	0.382
F04	Ncstn	-1.099	0.730	-1.049	0.580	1.570	0.459
F05	Pkp4	1.058	0.726	1.019	0.872	1.753	0.440
F06	Plat	-1.256	0.274	1.015	0.876	1.469	0.465
F07	Plau	-1.009	0.835	-1.004	0.902	1.529	0.477
F08	Plg	1.221	0.523	1.539	0.375	1.954	0.392
F09	Prkca	-1.416	0.075	1.163	0.408	1.464	0.463
F10	Prkcb	-1.164	0.200	1.066	0.630	1.554	0.457
F11	Prkcg	1.001	0.997	1.020	0.859	1.641	0.446
F12	Prkcd	-1.049	0.557	-1.087	0.520	1.880	0.408
G01	Prkce	-1.018	0.869	1.139	0.325	1.754	0.438
G02	Prkci	-1.250	0.214	1.073	0.507	1.925	0.426
G03	Prkcq	-1.704	0.185	-1.868	0.000	-1.044	0.605
G04	Prkcz	1.088	0.350	1.082	0.499	1.789	0.431
G05	Psen1	-1.044	0.793	1.139	0.326	1.644	0.443
G06	Psen2	1.147	0.121	-1.026	0.717	1.677	0.447
G07	Serpina3c	1.190	0.530	1.315	0.448	1.693	0.422
G08	Snca	1.193	0.068	1.402	0.099	2.133	0.362
G09	Sncb	1.314	0.017	1.079	0.443	1.828	0.424
G10	Ubqln1	-1.016	0.959	1.245	0.054	1.742	0.434
G11	Uqcrc1	1.314	0.066	-1.016	0.950	1.357	0.262
G12	Uqcrc2	-1.044	0.734	1.051	0.580	1.368	0.468

Table 5 Fold-regulation and p-values for all genes across groups in 12-month-old mice.

		Up-Down Regulation (comparing to control group)					
		SVCT2+/-		APP/PSEN1		SVCT2+/-; APP/PSEN1	
Position	Symbol	Fold Regulation	p-value	Fold Regulation	p-value	Fold Regulation	p-value
A01	A2m	-1.068	0.900	2.035	0.087	1.050	0.545
A02	Abca1	-1.009	0.876	-1.540	0.252	-1.238	0.383
A03	Ache	1.052	0.669	-1.650	0.039	-1.326	0.126
A04	Adam9	1.076	0.850	-1.257	0.514	-1.183	0.450
A05	Apba1	1.014	0.980	-1.741	0.058	-1.402	0.075
A06	Apba3	-1.257	0.007	1.092	0.505	-1.106	0.678
A07	Apbb1	1.175	0.727	-1.909	0.328	-1.534	0.553
A08	Apbb2	1.019	0.862	-1.597	0.087	-1.354	0.052
A09	Aph1a	-1.002	0.995	-1.508	0.068	-1.352	0.287
A10	Aplp1	1.021	0.957	-1.378	0.071	-1.242	0.126
A11	Aplp2	1.091	0.405	-1.392	0.059	-1.242	0.038
A12	Apoa1	1.397	0.208	-1.268	0.373	1.133	0.609
B01	Apoe	-1.083	0.819	-2.089	0.261	-1.449	0.329
B02	App	1.076	0.689	1.753	0.036	1.949	0.018
B03	Bace1	1.005	0.947	-1.790	0.026	-1.444	0.043
B04	Bace2	-1.115	0.830	-1.261	0.280	1.068	0.717
B05	Bche	1.019	0.947	-1.368	0.452	-1.212	0.438
B06	Bdnf	-1.149	0.317	-1.317	0.116	-1.347	0.001
B07	Casp3	1.076	0.802	-1.161	0.955	-1.275	0.326
B08	Casp4	-1.104	0.160	1.324	0.153	1.187	0.109
B09	Cdk1	-1.113	0.480	3.255	0.307	2.354	0.210
B10	Cdk5	-1.018	0.893	-1.392	0.130	-1.183	0.329
B11	Cdkl1	-1.055	0.713	-1.427	0.353	-1.145	0.432
B12	Chat	-1.257	0.007	1.181	0.457	-1.221	0.074
C01	Clu	1.121	0.628	-1.310	0.264	-1.204	0.356
C02	Ctsb	1.050	0.354	-1.129	0.148	-1.096	0.028
C03	Ctsc	-1.021	0.781	1.308	0.060	1.133	0.182
C04	Ctsd	-1.092	0.202	1.567	0.002	1.322	0.035
C05	Ctsg	-1.257	0.007	1.112	0.493	-1.221	0.074
C06	Ctsl	-1.007	0.989	1.091	0.112	1.052	0.283
C07	Ep300	1.119	0.671	-1.567	0.141	-1.290	0.252
C08	Ern1	-1.091	0.709	-1.315	0.492	-1.427	0.180
C09	Gap43	1.110	0.641	-2.354	0.102	-1.477	0.068
C10	Gnao1	-1.012	0.952	-1.633	0.131	-1.400	0.158
C11	Gnaz	1.057	0.655	-1.545	0.023	-1.248	0.067
C12	Gnb1	-1.053	0.848	-9.110	0.047	-1.373	0.147
D01	Gnb2	-1.121	0.949	-1.844	0.411	-1.772	0.530
D02	Gnb4	1.117	0.475	-1.564	0.039	-1.225	0.270
D03	Gnb5	-1.009	0.886	-1.694	0.006	-1.392	0.017
D04	Gng10	-1.141	0.331	-1.422	0.106	-1.313	0.111
D05	Gng11	-1.092	0.515	-1.796	0.008	-1.534	0.051
D06	Gng3	1.108	0.320	-1.441	0.001	-1.283	0.009
D07	Gng4	1.167	0.667	-1.983	0.052	-1.385	0.154
D08	Gng5	-1.098	0.123	-1.214	0.013	-1.195	0.029
D09	Gng7	-1.048	0.422	-1.529	0.052	-1.404	0.003
D10	Gng8	1.021	0.978	1.246	0.432	-1.272	0.292
D11	Gngt1	-1.257	0.007	-1.378	0.094	-1.221	0.074
D12	Gngt2	-1.072	0.688	1.388	0.141	1.189	0.357
E01	Gsk3a	1.268	0.016	-1.622	0.002	-1.299	0.011
E02	Gsk3b	1.023	0.910	-1.495	0.243	-1.366	0.253
E03	Hsd17b10	1.145	0.475	-1.313	0.131	-1.229	0.219
E04	Ide	-1.149	0.399	-1.407	0.096	-1.457	0.075
E05	Igf2	-1.532	0.313	-2.794	0.241	-1.952	0.231
E06	Il1a	-1.350	0.226	1.685	0.176	1.043	0.841
E07	Insr	-1.106	0.641	-1.741	0.167	-1.432	0.231
E08	Lpl	-1.159	0.320	-1.004	0.869	-1.018	0.888
E09	Lrp1	-1.092	0.818	-1.532	0.135	-1.432	0.277
E10	Lrp6	1.041	0.839	-1.459	0.282	-1.295	0.293
E11	Lrp8	-1.064	0.792	-1.873	0.113	-1.333	0.272
E12	Mapt	1.030	0.894	-1.534	0.110	-1.336	0.175
F01	Mpo	-1.193	0.172	-1.198	0.261	-1.179	0.132
F02	Map2	-1.066	0.834	-1.616	0.193	-1.336	0.349
F03	Nae1	-1.087	0.695	-1.419	0.087	-1.261	0.172
F04	Ncstn	1.221	0.317	-1.500	0.198	-1.104	0.438
F05	Pkp4	1.077	0.423	-1.417	0.006	-1.221	0.124
F06	Plat	1.064	0.391	-1.340	0.029	-1.297	0.021
F07	Plau	-1.268	0.487	1.997	0.131	1.759	0.181
F08	Plg	-1.257	0.007	-1.378	0.094	-1.221	0.074
F09	Prkca	-1.076	0.940	-1.588	0.218	-1.368	0.312
F10	Prkcb	-1.016	0.997	-1.599	0.222	-1.412	0.260
F11	Prkcg	1.079	0.556	-1.688	0.013	-1.320	0.051
F12	Prkcd	1.427	0.147	-1.492	0.202	-1.338	0.358
G01	Prkce	-1.167	0.408	-1.945	0.019	-1.682	0.021
G02	Prkci	-1.500	0.340	-1.949	0.204	1.091	0.829
G03	Prkcq	1.125	0.237	-1.402	0.165	-1.257	0.020
G04	Prkcz	1.110	0.175	-1.497	0.000	-1.434	0.000
G05	Psen1	1.030	0.778	-1.467	0.103	-1.326	0.038
G06	Psen2	1.081	0.566	-1.548	0.004	-1.189	0.222
G07	Serpina3c	-1.061	0.827	1.359	0.408	1.125	0.471
G08	Snca	1.005	0.939	-1.495	0.002	-1.283	0.052
G09	Sncb	1.149	0.383	-1.696	0.005	-1.322	0.049
G10	Ubqln1	-1.025	0.868	-1.597	0.146	-1.326	0.232
G11	Uqcrc1	1.011	0.979	-1.561	0.166	-1.414	0.255
G12	Uqcrc2	-1.081	0.520	-1.545	0.073	-1.275	0.148

Chapter VI: Discussion and Future Directions

Maintaining adequate levels of cellular ASC is necessary for proper brain health and even mild deficiency can disrupt cellular function and efficiency. There is a direct connection between ASC deficiency and oxidative stress, which is considered to be a key contributor to the pathogenesis and progression of Alzheimer's disease. Data from human clinical trials of antioxidant supplementation seldom show clear efficacy against the clinical manifestation of Alzheimer's disease (such as $A\beta_{1\text{-}42}$ levels, or cognitive decline [204], although supplements do increase vitamin levels (C and E) and decrease measures of oxidative stress in CSF after only one month of supplementation [205,206]. 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 [62,133], but the effects of prolonged non-scorbutic deficiency of ascorbate (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-thirties [167].

We have shown here that ASC deficiency in conjunction with the APP/PSEN1 mouse model for Alzheimer's disease can hasten disease pathology in aged mice, manifesting in the earlier emergence of amyloidogenesis and aggregation and correlated with a more rapid

cognitive decline than APP/PSEN1 animals with adequate ASC levels. However, the contribution of ASC deficiency may not be directly tied to disease pathology, but rather may increase the overall risk to threshold for developing the disease. ASC deficiency alone can impact neurological function based on the role of ASC in the synthesis and regulation of neurotransmitters [143]. In fact, an identifying characteristic of scurvy is lassitude, which is also observed in neurodegenerative diseases and psychiatric disorders [47,143]. These observations led to investigation into the role of ASC deficiency in early events associated with Alzheimer's in prodromal APP/PSEN1 mice.

Disruptions in energy homeostasis and mitochondrial function are observed in early stages of Alzheimer's disease, but ASC deficiency also appears to impact mitochondrial function. According to the data presented herein, the effects of amyloid beta and the effects of ASC deficiency have seemingly opposing influence on mitochondrial function. Thus the combination of ASC deficiency and the presence of amyloid beta compound mitochondrial dysfunction by acting on different aspects. These observations suggest that different pathways are being affected by each condition. Although under experimental conditions, this appeared to result in a normalization of function, in vivo the result is more likely to result in a compounding effect on dysfunction. Amyloid beta has been shown to directly interact with mitochondria, which may be associated with the changes in mitochondrial morphology and gene expression observed in the APP/PSEN1 mouse model [16,18,19]. It has also been demonstrated that chronic ASC deficiency through inadequate supplementation in the 5XFAD mouse model for Alzheimer's disease increases maladaptive morphology in mitochondria, but high ASC supplementation can protect against these morphological changes and decreases overall amyloid burden [70]. The result of this two-hit insult to energy homeostasis creates an

energy deficit that can profoundly inhibit normal cellular function rendering cells more vulnerable to cell death and neurodegeneration. The mechanism by which ASC supports mitochondrial function is not yet known. The most obvious inference is that ASC supports redox homeostasis within the highly metabolically active organelle, thus protecting against oxidative damage. However, ASC is also a co-factor for several classes of enzymes, including those involved in cell signaling and DNA modification and repair [56,58,169,170]. Further investigation is necessary to determine the specific roles for ASC in mitochondria. Specifically, it would be interesting to investigate the overall stability of mitochondrial DNA within the context of ASC deficiency given changes in gene expression associated with mitochondrial function are observed in young APP/PSEN1 mice [17]. Increased ROS production within the cell has the potential to compromise DNA in the nucleus and in mitochondria, leading to the cell death associated with cognitive dysfunction in AD. Additionally, mitochondrial DNA is not structurally protected by histones, increasing vulnerability to ROS attack and oxidative damage. A common 4977 base-pair deletion in mitochondrial DNA has been shown to increase with age [207,208] and can be used to assess oxidative damage. Additionally, it will be necessary to investigate DNA repair mechanisms and modification in the mitochondria [197]. ASC may also contribute directly to electron transport activity in oxidative phosphorylation [162–164]. A straightforward method to identify a specific site of action would be to measure oxygen consumption by isolating individual oxidative phosphorylation protein complexes using inhibitors and substrates in the presence or absence of ASC. The effects of prolonged ASC deficiency on individual complexes could also be investigated using this method in mitochondrial isolated from SVCT2$^{+/-}$ mice across several ages, as oxidative damage accumulates, and how this damage

from deficiency impacts energy production by directly measuring ATP production. Finally, the data presented herein describe the individual contributions of ASC deficiency (SVCT2$^{+/-}$) and APP/PSEN1 on mitochondria, but it would be valuable to investigate the combined effects in the SVCT2$^{+/-}$;APP/PSEN1 model across age to elucidate the scope of long term deficiency within the context of disease.

Epigenetic studies have identified differences in methylation patterns in individuals with AD, indicating that this form of genetic modification may contribute to disease pathology and progression [37,198–200]. The data presented herein suggest that ASC deficiency may hasten disease-driven epigenetic modification through a diminished capacity in its role as an enzymatic cofactor and in maintaining the cellular redox balance. The most interesting aspect of this study is the observed acceleration in aging with regard to gene expression observed in the APP/PSEN1+ groups and that the magnitude of gene expression changes were impacted by ASC deficiency in the combined group in young, presumably prodromal, mice. This is evidenced by the decrease in magnitude of gene expression changes observed at 12 months when normal age-related expression changes in wild-type mice essentially “caught up” with the APP/PSEN1+ mice.

However, it is important to note that the magnitude of expression changes in some genes was again impacted by ASC deficiency in aged SVCT2+/- mice. The follow-up study regarding global 5-hmC levels further highlighted this observation of accelerated aging in the APP/PSEN1+ groups in that the levels of 5-hmC were significantly elevated at 4 months, but showed minimal change above that at 12 months, at which point the wild type and SVCT2+/- were at similar levels. Given that epigenetic modification in the brain speaks to the adaptability of the central nervous system, these data suggest that there is heightened

response to the presence of disease pathology at early stages, which is then overwhelmed by progression of the disease. It would be interesting to compare 5-mC and 5-hmC status for individual genes associated with Alzheimer's disease and the contribution of ASC deficiency to gather insight into how the brain is compensating or responding to the disease and how the additional insult changes the genetic response. A redox imbalance due to ASC deficiency would increase oxidation of DNA nucleotides leading to mutagenesis, single- and double-stranded DNA breaks, because of or in combination with diminished DNA repair mechanisms has been documented in patients with AD [151]. Guanine is particularly vulnerable to oxidative attack due to its low oxidation potential, making the product 8-hydroxy-2-deoxyguanine (8-OHdG) a substantial biomarker to measure the accumulation of damage [148,151,209]. Modification and removal of methyl groups restores or increases gene expression; however the presence of 8-OHdG in the promoter region of genes can inhibit transcription factor binding and/or methylation, essentially eliminating the regulation of gene transcriptional response to stimuli [37]. It would be interesting to measure 8-OHdG levels in the described groups across age to determine if the neural genetic response is being hindered by oxidative damage. The primary risk factor for developing Alzheimer's disease is age and the phenotypic expression of aging is believed to be the result of accumulated oxidative damage, therefore it stands to reason that any condition that contributes to oxidative stress can accelerate the aging process [210]. Together, information gained from these studies might provide insights into the etiology of the disease in order to develop new and more effective therapeutic strategies and interventions.

One further potential role for ascorbate 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 genotypes [211]. There is a known association between APP mutations and seizures [212–214]. Seizures can independently induce additional β-amyloid production, as well as cognitive deficits, and also significantly increase oxidative stress [215–218]. 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.

Implications

As new insights into this disease emerge, more and more evidence suggests that the etiology lies not in a single event but rather in the culmination of several overlapping events resulting in a maladaptive neural response that ultimately leads to widespread degeneration. However, risk modifiers can combat maladaptive pathologies. While some conditions include unavoidable circumstances such as chronic disease or genetic predisposition, lifestyle choices often have the most profound effects on health outcomes. Nutrition and how the body acquires and uses nutrients have been studied for centuries and yet malnutrition is prevalent in modern societies. Low socioeconomic status and lack of interest and understanding lead to food insecurity and malnutrition in even the most industrialized areas and contribute to health disparities. The economic and public health advantages of promoting and supporting good nutrition and accessibility to food far outweigh the cost-effectiveness programs currently in place. It is the hope of this investigator that new research in the field of nutritional neuroscience continues to uncover the benefits adequate nutrition has not only in the context of disease but on overall health and quality of life.

Bibliography

[1] M.J. Lazarczyk, P.R. Hof, C. Bouras, P. Giannakopoulos, Preclinical Alzheimer disease: identification of cases at risk among cognitively intact older individuals., BMC Med. 10 (2012) 127. doi:10.1186/1741-7015-10-127.

[2] R.H. Swerdlow, Pathogenesis of Alzheimer's disease., Clin. Interv. Aging. 2 (2007) 347–59. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2685260&tool=pmcentrez&rendertype=abstract (accessed May 28, 2014).

[3] A. Prasansuklab, T. Tencomnao, Amyloidosis in Alzheimer's Disease: The Toxicity of Amyloid Beta (A β), Mechanisms of Its Accumulation and Implications of Medicinal Plants for Therapy., Evid. Based. Complement. Alternat. Med. 2013 (2013) 413808. doi:10.1155/2013/413808.

[4] Y. Zhao, B. Zhao, Oxidative stress and the pathogenesis of Alzheimer's disease., Oxid. Med. Cell. Longev. 2013 (2013) 316523. doi:10.1155/2013/316523.

[5] K. Stamer, R. Vogel, E. Thies, E. Mandelkow, E.-M. Mandelkow, Tau blocks traffic of organelles, neurofilaments, and APP vesicles in neurons and enhances oxidative stress., J. Cell Biol. 156 (2002) 1051–63. doi:10.1083/jcb.200108057.

[6] B. Halliwell, Oxidative stress and neurodegeneration: where are we now?, J. Neurochem. 97 (2006) 1634–58. doi:10.1111/j.1471-4159.2006.03907.x.

[7] M. Castello, S. Soriano, On the origin of Alzheimer's disease. Trials and tribulations of the amyloid hypothesis, Ageing Res. Rev. 13 (2014) 10–12.

doi:10.1016/j.arr.2013.10.001.

[8] K. Herrup, The case for rejecting the amyloid cascade hypothesis, Nat Neurosci. 18 (2015) 794–799. doi:10.1038/nn.4017.

[9] G.P. Morris, I. a Clark, B. Vissel, Inconsistencies and controversies surrounding the amyloid hypothesis of Alzheimer's disease., Acta Neuropathol. Commun. 2 (2014) 135. doi:10.1186/s40478-014-0135-5.

[10] M. Lagouge, N.-G. Larsson, The role of mitochondrial DNA mutations and free radicals in disease and ageing., J. Intern. Med. 273 (2013) 529–43. doi:10.1111/joim.12055.

[11] A. Grimm, K. Friedland, A. Eckert, Mitochondrial dysfunction: the missing link between aging and sporadic Alzheimer's disease., Biogerontology. (2015). doi:10.1007/s10522-015-9618-4.

[12] C.D. Kamat, S. Gadal, M. Mhatre, K.S. Williamson, Q.N. Pye, K. Hensley, Antioxidants in central nervous system diseases: preclinical promise and translational challenges., J. Alzheimers. Dis. 15 (2008) 473–93. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2669703&tool=pmcentrez&rendertype=abstract (accessed June 19, 2014).

[13] R.H. Swerdlow, J.M. Burns, S.M. Khan, The Alzheimer's disease mitochondrial cascade hypothesis., J. Alzheimers. Dis. 20 Suppl 2 (2010) S265-79. doi:10.3233/JAD-2010-100339.

[14] C. Cadonic, M.G. Sabbir, B.C. Albensi, Mechanisms of Mitochondrial Dysfunction in Alzheimer's Disease, Mol. Neurobiol. (2015). doi:10.1007/s12035-015-9515-5.

[15] K. Leuner, T. Schütt, C. Kurz, S.H. Eckert, C. Schiller, A. Occhipinti, S. Mai, M. Jendrach, G.P. Eckert, S.E. Kruse, R.D. Palmiter, U. Brandt, S. Dröse, I. Wittig, M. Willem, C. Haass, A.S. Reichert, W.E. Müller, Mitochondrion-Derived Reactive Oxygen Species Lead to Enhanced Amyloid Beta Formation, Antioxid. Redox Signal. 16 (2012) 1421–1433. doi:10.1089/ars.2011.4173.

[16] V. García-Escudero, P. Martín-Maestro, G. Perry, J. Avila, Deconstructing mitochondrial dysfunction in alzheimer disease, Oxid. Med. Cell. Longev. 2013 (2013). doi:10.1155/2013/162152.

[17] I. Pedrós, D. Petrov, M. Allgaier, F. Sureda, E. Barroso, C. Beas-Zarate, C. Auladell, M. Pallàs, M. Vázquez-Carrera, G. Casadesús, J. Folch, A. Camins, Early alterations in energy metabolism in the hippocampus of APPswe/PS1dE9 mouse model of Alzheimer's disease, Biochim. Biophys. Acta - Mol. Basis Dis. 1842 (2014) 1556–1566. doi:10.1016/j.bbadis.2014.05.025.

[18] C.A. Hansson Petersen, N. Alikhani, H. Behbahani, B. Wiehager, P.F. Pavlov, I. Alafuzoff, V. Leinonen, A. Ito, B. Winblad, E. Glaser, M. Ankarcrona, The amyloid beta-peptide is imported into mitochondria via the TOM import machinery and localized to mitochondrial cristae., Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 13145–50. doi:10.1073/pnas.0806192105.

[19] K.J. Choi, M.J. Kim, A.R. Je, S. Jun, C. Lee, E. Lee, M. Jo, Y.H. Huh, H.S. Kweon, Three-dimensional analysis of abnormal ultrastructural alteration in mitochondria of hippocampus of APP/PSEN1 transgenic mouse, J. Biosci. 39 (2014) 97–105. doi:10.1007/s12038-013-9406-8.

[20] M. Manczak, M.J. Calkins, P.H. Reddy, Impaired mitochondrial dynamics and abnormal interaction of amyloid beta with mitochondrial protein Drp1 in neurons from patients with Alzheimer's disease: Implications for neuronal damage, Hum. Mol. Genet. 20 (2011) 2495–2509. doi:10.1093/hmg/ddr139.

[21] M.H. Yan, X. Wang, X. Zhu, Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease., Free Radic. Biol. Med. 62 (2013) 90–101. doi:10.1016/j.freeradbiomed.2012.11.014.

[22] Z. Wu, Y. Zhu, X. Cao, S. Sun, B. Zhao, Mitochondrial Toxic Effects of Aβ Through Mitofusins in the Early Pathogenesis of Alzheimer's Disease, Mol. Neurobiol. (2014) 986–996. doi:10.1007/s12035-014-8675-z.

[23] A. Ronnback, P.F. Pavlov, M. Mansory, P. Gonze, N. Marli??re, B. Winblad, C. Graff, H. Behbahani, Mitochondrial dysfunction in a transgenic mouse model expressing human amyloid precursor protein (APP) with the Arctic mutation, J. Neurochem. 136 (2016) 497–502. doi:10.1111/jnc.13410.

[24] R.J. Bateman, C. Xiong, T.L.S. Benzinger, A.M. Fagan, A. Goate, N.C. Fox, D.S. Marcus, N.J. Cairns, X. Xie, T.M. Blazey, D.M. Holtzman, A. Santacruz, V. Buckles, A. Oliver, K. Moulder, P.S. Aisen, B. Ghetti, W.E. Klunk, E. McDade, R.N. Martins, C.L. Masters, R. Mayeux, J.M. Ringman, M.N. Rossor, P.R. Schofield, R.A. Sperling, S. Salloway, J.C. Morris, Clinical and biomarker changes in dominantly inherited Alzheimer's disease., N. Engl. J. Med. 367 (2012) 795–804. doi:10.1056/NEJMoa1202753.

[25] R. Sultana, M. Perluigi, D. Allan Butterfield, Lipid peroxidation triggers neurodegeneration: a redox proteomics view into the Alzheimer disease brain., Free Radic. Biol. Med. 62 (2013) 157–69. doi:10.1016/j.freeradbiomed.2012.09.027.

[26] E. Niki, Y. Yoshida, Y. Saito, N. Noguchi, Lipid peroxidation: mechanisms, inhibition, and biological effects., Biochem. Biophys. Res. Commun. 338 (2005) 668–76. doi:10.1016/j.bbrc.2005.08.072.

[27] B.S. Berlett, E.R. Stadtman, Protein oxidation in aging, disease, and oxidative stress., J. Biol. Chem. 272 (1997) 20313–6. doi:10.1074/jbc.272.33.20313.

[28] C.K. Lee, R. Weindruch, T. a Prolla, Gene-expression profile of the ageing brain in mice., Nat. Genet. 25 (2000) 294–7. doi:10.1038/77046.

[29] P. Fehlbaum-Beurdeley, A.C. Jarrige-Le Prado, D. Pallares, J. Carrière, C. Guihal, C. Soucaille, F. Rouet, D. Drouin, O. Sol, H. Jordan, D. Wu, L. Lei, R. Einstein, F. Schweighoffer, L. Bracco, Toward an Alzheimer's disease diagnosis via high-resolution blood gene expression., Alzheimers. Dement. 6 (2010) 25–38. doi:10.1016/j.jalz.2009.07.001.

[30] D. Krstic, I. Knuesel, Deciphering the mechanism underlying late-onset Alzheimer disease., Nat. Rev. Neurol. 9 (2013) 25–34. doi:10.1038/nrneurol.2012.236.

[31] E. Tamagno, G. Robino, A. Obbili, P. Bardini, M. Aragno, M. Parola, O. Danni, H2O2 and 4-hydroxynonenal mediate amyloid beta-induced neuronal apoptosis by activating JNKs and p38MAPK., Exp. Neurol. 180 (2003) 144–55. doi:10.1016/S0014-4886(02)00059-6.

[32] Y. Tong, W. Zhou, V. Fung, M. a Christensen, H. Qing, X. Sun, W. Song, Oxidative stress potentiates BACE1 gene expression and Abeta generation., J. Neural Transm. 112 (2005) 455–69. doi:10.1007/s00702-004-0255-3.

[33] G.T. Sutherland, B. Chami, P. Youssef, P.K. Witting, Oxidative stress in Alzheimer's disease: Primary villain or physiological by-product?, Redox Rep. 18 (2013) 134–41. doi:10.1179/1351000213Y.0000000052.

[34] R. Roychaudhuri, M. Yang, M.M. Hoshi, D.B. Teplow, Amyloid beta-protein assembly and Alzheimer disease., J. Biol. Chem. 284 (2009) 4749–53. doi:10.1074/jbc.R800036200.

[35] X.-H. Li, L.-L. Du, X.-S. Cheng, X. Jiang, Y. Zhang, B.-L. Lv, R. Liu, J.-Z. Wang, X.-W. Zhou, Glycation exacerbates the neuronal toxicity of β-amyloid., Cell Death Dis. 4 (2013) e673. doi:10.1038/cddis.2013.180.

[36] R.A. Floyd, Antioxidants, oxidative stress, and degenerative neurological disorders., Proc. Soc. Exp. Biol. Med. 222 (1999) 236–45. http://www.ncbi.nlm.nih.gov/pubmed/10601882 (accessed June 19, 2014).

[37] J.L. Fleming, C.J. Phiel, A.E. Toland, The role for oxidative stress in aberrant DNA methylation in Alzheimer's disease., Curr. Alzheimer Res. 9 (2012) 1077–96. http://www.ncbi.nlm.nih.gov/pubmed/21605062 (accessed June 19, 2014).

[38] W.R. Markesbery, R.J. Kryscio, M. a Lovell, J.D. Morrow, Lipid peroxidation is an early event in the brain in amnestic mild cognitive impairment., Ann. Neurol. 58 (2005) 730–5. doi:10.1002/ana.20629.

[39] M. Schrag, C. Mueller, M. Zabel, a Crofton, W.M. Kirsch, O. Ghribi, R. Squitti, G. Perry, Oxidative stress in blood in Alzheimer's disease and mild cognitive impairment: a meta-analysis., Neurobiol. Dis. 59 (2013) 100–10. doi:10.1016/j.nbd.2013.07.005.

[40] R. Sultana, M. Baglioni, R. Cecchetti, J. Cai, J.B. Klein, P. Bastiani, C. Ruggiero, P. Mecocci, D.A. Butterfield, Lymphocyte mitochondria: Toward identification of peripheral biomarkers in the progression of Alzheimer disease, Free Radic. Biol. Med. 65 (2013) 595–606. doi:10.1016/j.freeradbiomed.2013.08.001.

[41] W. Liu, M. Kato, a a Akhand, A. Hayakawa, H. Suzuki, T. Miyata, K. Kurokawa, Y. Hotta, N. Ishikawa, I. Nakashima, 4-Hydroxynonenal Induces a Cellular Redox Status-Related Activation of the Caspase Cascade for Apoptotic Cell Death., J. Cell Sci. 113 (2000) 635–41. http://www.ncbi.nlm.nih.gov/pubmed/10652256.

[42] S.O. Abarikwu, A.B. Pant, E.O. Farombi, 4-Hydroxynonenal induces mitochondrial-mediated apoptosis and oxidative stress in SH-SY5Y human neuronal cells., Basic Clin. Pharmacol. Toxicol. 110 (2012) 441–8. doi:10.1111/j.1742-7843.2011.00834.x.

[43] J.N. Keller, Z. Pang, J.W. Geddes, J.G. Begley, A. Germeyer, G. Waeg, M.P. Mattson, Impairment of glucose and glutamate transport and induction of mitochondrial oxidative stress and dysfunction in synaptosomes by amyloid beta-peptide: role of the lipid peroxidation product 4-hydroxynonenal., J. Neurochem. 69 (1997) 273–84. http://www.ncbi.nlm.nih.gov/pubmed/9202320 (accessed June 23, 2014).

[44] C.M. Lauderback, J.M. Hackett, F.F. Huang, J.N. Keller, L.I. Szweda, W.R. Markesbery, D.A. Butterfield, The glial glutamate transporter, GLT-1, is oxidatively

modified by 4-hydroxy-2-nonenal in the Alzheimer's disease brain: the role of Abeta1-42., J. Neurochem. 78 (2001) 413–6. http://www.ncbi.nlm.nih.gov/pubmed/11461977 (accessed June 23, 2014).

[45] W.G. Siems, S.J. Hapner, F.J. van Kuijk, 4-hydroxynonenal inhibits Na(+)-K(+)-ATPase., Free Radic. Biol. Med. 20 (1996) 215–23. http://www.ncbi.nlm.nih.gov/pubmed/8746442 (accessed June 24, 2014).

[46] R. Sultana, M. Perluigi, D.A. Butterfield, Protein oxidation and lipid peroxidation in brain of subjects with Alzheimer's disease: insights into mechanism of neurodegeneration from redox proteomics., Antioxid. Redox Signal. 8 (2006) 2021–37. doi:10.1089/ars.2006.8.2021.

[47] J.M. May, Vitamin C Transport and Its ROle in the Central Nervous System, in: O. Stanger (Ed.), Water Soluble Vitam., Springer Netherlands, Dordrecht, 2012: pp. 85–103. doi:10.1007/978-94-007-2199-9.

[48] F.E. Harrison, J.M. May, Vitamin C function in the brain: vital role of the ascorbate transporter SVCT2., Free Radic. Biol. Med. 46 (2009) 719–30. doi:10.1016/j.freeradbiomed.2008.12.018.

[49] M.S. Ward, J. Lamb, J.M. May, F.E. Harrison, Behavioral and monoamine changes following severe vitamin C deficiency, J. Neurochem. 124 (2013) 363–375. doi:10.1111/jnc.12069.

[50] M.E. Rice, I. Russo-Menna, Differential compartmentalization of brain ascorbate and glutathione between neurons and glia., Neuroscience. 82 (1998) 1213–23.

http://www.ncbi.nlm.nih.gov/pubmed/9466441 (accessed June 19, 2014).

[51] M.C. De Tullio, O. Arrigoni, Hopes, disillusions and more hopes from vitamin C., Cell. Mol. Life Sci. 61 (2004) 209–19. doi:10.1007/s00018-003-3203-8.

[52] M.C. De Tullio, Water Soluble Vitamins, in: O. Stanger (Ed.), Water Soluble Vitam., Springer Netherlands, Dordrecht, 2012: pp. 49–65. doi:10.1007/978-94-007-2199-9.

[53] R.J. Sram, B. Binkova, P. Rossner, Vitamin C for DNA damage prevention, Mutat. Res. - Fundam. Mol. Mech. Mutagen. 733 (2012) 39–49. doi:10.1016/j.mrfmmm.2011.12.001.

[54] M.E. Meredith, F.E. Harrison, J.M. May, Differential regulation of the ascorbic acid transporter SVCT2 during development and in response to ascorbic acid depletion, Biochem. Biophys. Res. Commun. 414 (2011) 737–742. doi:10.1016/j.bbrc.2011.09.146.

[55] J. Chen, L. Guo, L. Zhang, H. Wu, J. Yang, H. Liu, X. Wang, X. Hu, T. Gu, Z. Zhou, J. Liu, J. Liu, H. Wu, S.-Q. Mao, K. Mo, Y. Li, K. Lai, J. Qi, H. Yao, G. Pan, G.-L. Xu, D. Pei, Vitamin C modulates TET1 function during somatic cell reprogramming., Nat. Genet. 45 (2013) 1504–9. doi:10.1038/ng.2807.

[56] R. Yin, S.Q. Mao, B. Zhao, Z. Chong, Y. Yang, C. Zhao, D. Zhang, H. Huang, J. Gao, Z. Li, Y. Jiao, C. Li, S. Liu, D. Wu, W. Gu, Y.G. Yang, G.L. Xu, H. Wang, Ascorbic acid enhances tet-mediated 5-methylcytosine oxidation and promotes DNA demethylation in mammals, J. Am. Chem. Soc. 135 (2013) 10396–10403. doi:10.1021/ja4028346.

[57] X.-B. He, M. Kim, S.-Y. Kim, S.-H. Yi, Y.-H. Rhee, T. Kim, E.-H. Lee, C.-H. Park, S.

Dixit, F.E. Harrison, S.-H. Lee, Vitamin C Facilitates Dopamine Neuron Differentiation in Fetal Midbrain Through Tet1- aND Jmjd3-Dependent Epigenetic Control Manner, Stem Cells. (2014) 1–18. doi:10.1002/stem.1932.

[58] K. Blaschke, K.T. Ebata, M.M. Karimi, J. a Zepeda-Martínez, P. Goyal, S. Mahapatra, A. Tam, D.J. Laird, M. Hirst, A. Rao, M.C. Lorincz, M. Ramalho-Santos, Vitamin C induces Tet-dependent DNA demethylation and a blastocyst-like state in ES cells., Nature. 500 (2013) 222–6. doi:10.1038/nature12362.

[59] S. Sotiriou, S. Gispert, J. Cheng, Y. Wang, A. Chen, S. Hoogstraten-Miller, G.F. Miller, O. Kwon, M. Levine, S.H. Guttentag, R.L. Nussbaum, Ascorbic-acid transporter Slc23a1 is essential for vitamin C transport into the brain and for perinatal survival., Nat. Med. 8 (2002) 514–7. doi:10.1038/nm0502-514.

[60] F.E. Harrison, S.M. Dawes, M.E. Meredith, V.R. Babaev, L. Li, J.M. May, Low vitamin C and increased oxidative stress and cell death in mice that lack the sodium-dependent vitamin C transporter SVCT2, Free Radic. Biol. Med. 49 (2010) 821–829. doi:10.1016/j.freeradbiomed.2010.06.008.

[61] N. Maeda, H. Hagihara, Y. Nakata, S. Hiller, J. Wilder, R. Reddick, Aortic wall damage in mice unable to synthesize ascorbic acid., Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 841–6. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=15418&tool=pmcentrez&rendertype=abstract.

[62] F.E. Harrison, S.S. Yu, K.L. Van Den Bossche, L. Li, J.M. May, M.P. McDonald,

Elevated oxidative stress and sensorimotor deficits but normal cognition in mice that cannot synthesize ascorbic acid., J. Neurochem. 106 (2008) 1198–208. doi:10.1111/j.1471-4159.2008.05469.x.

[63] M.D. Paidi, J.G. Schjoldager, J. Lykkesfeldt, P. Tveden-Nyborg, Chronic vitamin C deficiency promotes redox imbalance in the brain but does not alter sodium-dependent vitamin C transporter 2 expression., Nutrients. 6 (2014) 1809–22. doi:10.3390/nu6051809.

[64] a C. Carr, B. Frei, Toward a new recommended dietary allowance for vitamin C based on antioxidant and health effects in humans., Am. J. Clin. Nutr. 69 (1999) 1086–107. http://www.ncbi.nlm.nih.gov/pubmed/10357726.

[65] J.S. Goodwin, J.M. Goodwin, P.J. Garry, Association between nutritional status and cognitive functioning in a healthy elderly population., JAMA. 249 (1983) 2917–2921. doi:10.1001/jama.1983.03330450047024.

[66] C.R. Gale, C.N. Martyn, C. Cooper, Cognitive impairment and mortality in a cohort of elderly people., BMJ. 312 (1996) 608–611.

[67] P.P. Zandi, J.C. Anthony, A.S. Khachaturian, S. V Stone, D. Gustafson, J.T. Tschanz, M.C. Norton, K.A. Welsh-Bohmer, J.C.S. Breitner, Reduced risk of Alzheimer disease in users of antioxidant vitamin supplements: the Cache County Study., Arch. Neurol. 61 (2004) 82–88. doi:10.1001/archneur.61.1.82.

[68] G.L. Bowman, H. Dodge, B. Frei, C. Calabrese, B.S. Oken, J.A. Kaye, J.F. Quinn, Ascorbic acid and rates of cognitive decline in Alzheimer's disease., J. Alzheimers. Dis.

16 (2009) 93–8. doi:10.3233/JAD-2009-0923.

[69] K. Murakami, N. Murata, Y. Ozawa, N. Kinoshita, K. Irie, T. Shirasawa, T. Shimizu, Vitamin C restores behavioral deficits and amyloid-β oligomerization without affecting plaque formation in a mouse model of Alzheimer's disease., J. Alzheimers. Dis. 26 (2011) 7–18. doi:10.3233/JAD-2011-101971.

[70] S.-Y. Kook, K.-M. Lee, Y. Kim, M.-Y. Cha, S. Kang, S.H. Baik, H. Lee, R. Park, I. Mook-Jung, High-dose of vitamin C supplementation reduces amyloid plaque burden and ameliorates pathological changes in the brain of 5XFAD mice., Cell Death Dis. 5 (2014) e1083. doi:10.1038/cddis.2014.26.

[71] J. Huang, J.M. May, Ascorbic acid protects SH-SY5Y neuroblastoma cells from apoptosis and death induced by beta-amyloid., Brain Res. 1097 (2006) 52–8. doi:10.1016/j.brainres.2006.04.047.

[72] S. Choi, B.C. Kim, Y. Cho, K. Choi, J. Chang, M. Park, M. Kim, K. Cho, J. Kim, Effects of Flavonoid Compounds on β -amyloid-peptide-induced Neuronal Death in Cultured Mouse Cortical Neurons, Chonnam Med J. 50 (2014) 45–51.

[73] S. Ballaz, I. Morales, M. Rodríguez, J. a Obeso, Ascorbate prevents cell death from prolonged exposure to glutamate in an in vitro model of human dopaminergic neurons., J. Neurosci. Res. 91 (2013) 1609–17. doi:10.1002/jnr.23276.

[74] F.E. Harrison, A.H. Hosseini, M.P. McDonald, J.M. May, Vitamin C reduces spatial learning deficits in middle-aged and very old APP/PSEN1 transgenic and wild-type mice., Pharmacol. Biochem. Behav. 93 (2009) 443–50. doi:10.1016/j.pbb.2009.06.006.

[75] J. a Kennard, F.E. Harrison, Intravenous ascorbate improves spatial memory in middle-aged APP/PSEN1 and wild type mice., Behav. Brain Res. 264 (2014) 34–42. doi:10.1016/j.bbr.2014.01.044.

[76] L. An, T. Zhang, Vitamins C and E reverse melamine-induced deficits in spatial cognition and hippocampal synaptic plasticity in rats., Neurotoxicology. 44 (2014) 132–9. doi:10.1016/j.neuro.2014.06.009.

[77] F.E. Harrison, J. Allard, R. Bixler, C. Usoh, L. Li, J.M. May, M.P. McDonald, Antioxidants and cognitive training interact to affect oxidative stress and memory in APP/PSEN1 mice., Nutr. Neurosci. 12 (2009) 203–18. doi:10.1179/147683009X423364.

[78] K. Fukui, N.-O. Omoi, T. Hayasaka, T. Shinnkai, S. Suzuki, K. Abe, S. Urano, Cognitive impairment of rats caused by oxidative stress and aging, and its prevention by vitamin E., Ann. N. Y. Acad. Sci. 959 (2002) 275–84. http://www.ncbi.nlm.nih.gov/pubmed/11976202.

[79] J.A. Joseph, N.A. Denisova, G. Arendash, M. Gordon, D. Diamond, B. Shukitt-Hale, D. Morgan, Blueberry supplementation enhances signaling and prevents behavioral deficits in an Alzheimer disease model., Nutr. Neurosci. 6 (2003) 153–62. doi:10.1080/1028415031000111282.

[80] S. Sung, Y. Yao, K. Uryu, H. Yang, V.M.-Y. Lee, J.Q. Trojanowski, D. Praticò, 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. 18 (2004)

323–5. doi:10.1096/fj.03-0961fje.

[81] A. Parachikova, K.N. Green, C. Hendrix, F.M. LaFerla, Formulation of a medical food cocktail for Alzheimer's disease: beneficial effects on cognition and neuropathology in a mouse model of the disease., PLoS One. 5 (2010) e14015. doi:10.1371/journal.pone.0014015.

[82] L.M. Broersen, A.A.M. Kuipers, M. Balvers, N. van Wijk, P.J.M. Savelkoul, M.C. de Wilde, E.M. van der Beek, J.W.C. Sijben, R.J.J. Hageman, P.J.G.H. Kamphuis, A.J. Kiliaan, A specific multi-nutrient diet reduces Alzheimer-like pathology in young adult AβPPswe/PS1dE9 mice., J. Alzheimers. Dis. 33 (2013) 177–90. doi:10.3233/JAD-2012-112039.

[83] G. Bánhegyi, A. Benedetti, É. Margittai, P. Marcolongo, R. Fulceri, C.E. Németh, A. Szarka, Subcellular compartmentation of ascorbate and its variation in disease states, Biochim. Biophys. Acta - Mol. Cell Res. 1843 (2014) 1909–1916. doi:10.1016/j.bbamcr.2014.05.016.

[84] Y. Kawai, E. Ono, M. Mizutani, Evolution and diversity of the 2-oxoglutarate-dependent dioxygenase superfamily in plants, Plant J. 78 (2014) 328–343. doi:10.1111/tpj.12479.

[85] O.C. Ingebretsen, P.T. Normann, Transport of ascorbate into guinea pig liver mitochondria., Biochim. Biophys. Acta. 684 (1982) 21–6. http://www.ncbi.nlm.nih.gov/pubmed/7055553.

[86] M. Fiorani, C. Azzolini, L. Cerioni, M. Scotti, A. Guidarelli, C. Ciacci, O. Cantoni, The mitochondrial transporter of ascorbic acid functions with high affinity in the presence of

low millimolar concentrations of sodium and in the absence of calcium and magnesium, Biochim. Biophys. Acta - Biomembr. 1848 (2015) 1393–1401. doi:10.1016/j.bbamem.2015.03.009.

[87] C. Muñoz-Montesino, F.J. Roa, E. Peña, M. González, K. Sotomayor, E. Inostroza, C. a. Muñoz, I. González, M. Maldonado, C. Soliz, A.M. Reyes, J.C. Vera, C.I. Rivas, Mitochondrial ascorbic acid transport is mediated by a low-affinity form of the sodium-coupled ascorbic acid transporter-2, Free Radic. Biol. Med. 70 (2014) 241–254. doi:10.1016/j.freeradbiomed.2014.02.021.

[88] D. Devanand, J. Lee, J. Luchsinger, J. Manly, K. Marder, R. Mayeux, N. Scarmeas, N. Schupf, Y. Stern, Lessons from epidemiologic research about risk factors, modifiers, and progression of late onset Alzheimer's Disease in New York City at Columbia University Medical Center., J. Alzheimers. Dis. 33 Suppl 1 (2013) S447-55. doi:10.3233/JAD-2012-129041.

[89] I. Ampuero, F. Bermejo-Pareja, G. García Ribas, Environmental and Genetic Risk Factors for Alzheimer's Disease in Spain, Eur. Neurol. Rev. 4 (2009) 125. doi:10.17925/ENR.2009.04.02.125.

[90] N. Scarmeas, Y. Stern, M. Tang, J.A. Luchsinger, Mediterranean Diet and Risk for Alzheimer's Disease Nikolaos, Ann. Neurol. 59 (2011) 912–921. doi:10.1002/ana.20854.Mediterranean.

[91] S. Sabia, H. Nabi, M. Kivimaki, M.J. Shipley, M.G. Marmot, A. Singh-Manoux, Health behaviors from early to late midlife as predictors of cognitive function: The Whitehall II

study., Am. J. Epidemiol. 170 (2009) 428–37. doi:10.1093/aje/kwp161.

[92] K. Rahman, Studies on free radicals, antioxidants, and co-factors., Clin. Interv. Aging. 2 (2007) 219–36. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2684512&tool=pmcentrez&rendertype=abstract (accessed June 19, 2014).

[93] M. Levine, New concepts in the biology and biochemistry of ascorbic acid., N. Engl. J. Med. 314 (1986) 892–902. doi:10.1056/NEJM198604033141407.

[94] O.J. Dolberg, A. Elis, M. Lishner, Scurvy in the 21st century., Isr. Med. Assoc. J. 12 (2010) 183–4. http://www.ncbi.nlm.nih.gov/pubmed/20684188.

[95] J.M. Olmedo, J.A. Yiannias, E.B. Windgassen, M.K. Gornet, Scurvy: a disease almost forgotten., Int. J. Dermatol. 45 (2006) 909–13. doi:10.1111/j.1365-4632.2006.02844.x.

[96] A. Mosdøl, B. Erens, E.J. Brunner, Estimated prevalence and predictors of vitamin C deficiency within UK's low-income population., J. Public Health (Oxf). 30 (2008) 456–60. doi:10.1093/pubmed/fdn076.

[97] C.S. Johnston, L.L. Thompson, Vitamin C status of an outpatient population., J. Am. Coll. Nutr. 17 (1998) 366–370.

[98] W.L. Wrieden, M.K. Hannah, C. Bolton-Smith, R. Tavendale, C. Morrison, H. Tunstall-Pedoe, Plasma vitamin C and food choice in the third Glasgow MONICA population survey., J. Epidemiol. Community Health. 54 (2000) 355–60. doi:10.1136/jech.54.5.355.

[99] F.E. Harrison, G.L. Bowman, M.C. Polidori, Ascorbic acid and the brain: rationale for the use against cognitive decline., Nutrients. 6 (2014) 1752–81. doi:10.3390/nu6041752.

[100] C.P. Corpe, H. Tu, P. Eck, J. Wang, R. Faulhaber-Walter, J. Schnermann, S. Margolis, S. Padayatty, H. Sun, Y. Wang, R.L. Nussbaum, M.G. Espey, M. Levine, Vitamin C transporter Slc23a1 links renal reabsorption, vitamin C tissue accumulation, and perinatal survival in mice., J. Clin. Invest. 120 (2010) 1069–83. doi:10.1172/JCI39191.

[101] P. Eck, H.C. Erichsen, J.G. Taylor, M. Yeager, A.L. Hughes, M. Levine, S.J. Chanock, Comparison of the genomic structure and variation in the two human sodium-dependent Vitamin C transporters, SLC23A1 and SLC23A2, Hum. Genet. 115 (2004) 285–294. doi:10.1007/s00439-004-1167-x.

[102] L.E. Cahill, A. El-Sohemy, Vitamin C transporter gene polymorphisms, dietary vitamin C and serum ascorbic acid., J. Nutrigenet. Nutrigenomics. 2 (2009) 292–301. doi:10.1159/000314597.

[103] F.E. Harrison, J.L. Best, M.E. Meredith, C.R. Gamlin, D.-B. Borza, J.M. May, J.M. May, Increased expression of SVCT2 in a new mouse model raises ascorbic acid in tissues and protects against paraquat-induced oxidative damage in lung., PLoS One. 7 (2012) e35623. doi:10.1371/journal.pone.0035623.

[104] R.M. Witt, M.M. Galligan, J.R. Despinoy, R. Segal, Olfactory behavioral testing in the adult mouse., J. Vis. Exp. (2009). doi:10.3791/949.

[105] F.E. Harrison, A.H. Hosseini, M.P. McDonald, Endogenous anxiety and stress

responses in water maze and Barnes maze spatial memory tasks., Behav. Brain Res. 198 (2009) 247–51. doi:10.1016/j.bbr.2008.10.015.

[106] G.L. Milne, S.C. Sanchez, E.S. Musiek, J.D. Morrow, Quantification of F2-isoprostanes as a biomarker of oxidative stress., Nat. Protoc. 2 (2007) 221–6. doi:10.1038/nprot.2006.375.

[107] C.L. Hawkins, P.E. Morgan, M.J. Davies, Quantification of protein modification by oxidants, Free Radic. Biol. Med. 46 (2009) 965–988. doi:10.1016/j.freeradbiomed.2009.01.007.

[108] I. Rahman, A. Kode, S.K. Biswas, Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method., Nat. Protoc. 1 (2006) 3159–65. doi:10.1038/nprot.2006.378.

[109] A. Bernardo, M. McCord, A.M. Troen, J.D. Allison, M.P. McDonald, Impaired spatial memory in APP-overexpressing mice on a homocysteinemia-inducing diet., Neurobiol. Aging. 28 (2007) 1195–205. doi:10.1016/j.neurobiolaging.2006.05.035.

[110] J.P. Fessel, C.R. Flynn, L.J. Robinson, N.L. Penner, S. Gladson, C.J. Kang, D.H. Wasserman, A.R. Hemnes, J.D. West, Hyperoxia synergizes with mutant bone morphogenic protein receptor 2 to cause metabolic stress, oxidant injury, and pulmonary hypertension, Am. J. Respir. Cell Mol. Biol. 49 (2013) 778–787. doi:10.1165/rcmb.2012-0463OC.

[111] H. Lemieux, S. Semsroth, H. Antretter, D. Höfer, E. Gnaiger, Mitochondrial respiratory control and early defects of oxidative phosphorylation in the failing human heart, Int. J.

Biochem. Cell Biol. 43 (2011) 1729–1738. doi:10.1016/j.biocel.2011.08.008.

[112] S. Lores-Arnaiz, P. Lombardi, A.G. Karadayian, F. Orgambide, D. Cicerchia, J. Bustamante, Brain cortex mitochondrial bioenergetics in synaptosomes and non-synaptic mitochondria during aging., Neurochem. Res. 41 (2016) 353–63. doi:10.1007/s11064-015-1817-5.

[113] F.E. Harrison, M.E. Meredith, S.M. Dawes, J.L. Saskowski, J.M. May, Low ascorbic acid and increased oxidative stress in gulo (-/-) mice during development, Brain Res. 1349 (2010) 143–152. doi:10.1016/j.brainres.2010.06.037.

[114] M.R. Wieckowski, C. Giorgi, M. Lebiedzinska, J. Duszynski, P. Pinton, Isolation of mitochondria-associated membranes and mitochondria from animal tissues and cells., Nat. Protoc. 4 (2009) 1582–90. doi:10.1038/nprot.2009.151.

[115] S. Rivière, I. Birlouez-Aragon, F. Nourhashémi, B. Vellas, Low plasma vitamin C in Alzheimer patients despite an adequate diet., Int. J. Geriatr. Psychiatry. 13 (1998) 749–54. http://www.ncbi.nlm.nih.gov/pubmed/9850871.

[116] K.E. Charlton, T.L. Rabinowitz, L.N. Geffen, M.A. Dhansay, Lowered plasma vitamin C, but not vitamin E, concentrations in dementia patients., J. Nutr. Health Aging. 8 (2004) 99–107. http://www.ncbi.nlm.nih.gov/pubmed/14978605.

[117] M.C. Polidori, P. Mecocci, Plasma susceptibility to free radical-induced antioxidant consumption and lipid peroxidation is increased in very old subjects with Alzheimer disease., J. Alzheimers. Dis. 4 (2002) 517–22. http://www.ncbi.nlm.nih.gov/pubmed/12629261.

[118] B. Frei, L. England, B.N. Ames, Ascorbate is an outstanding antioxidant in human blood plasma., Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 6377–81. doi:10.1073/pnas.86.16.6377.

[119] D. Pratico, C.M. Clark, F. Liun, V.Y.-M. Lee, J.Q. Trojanowski, Increase of Brain Oxidative Stress in Mild Cognitive Impairment, Arch Neurol. 59 (2002) 972–976. doi:10.1001/archneur.59.6.972.

[120] D. Praticò, S. Sung, Lipid peroxidation and oxidative imbalance: early functional events in Alzheimer's disease., J. Alzheimers. Dis. 6 (2004) 171–5. http://www.ncbi.nlm.nih.gov/pubmed/15096701.

[121] M.C. Morris, L.A. Beckett, P.A. Scherr, L.E. Hebert, D.A. Bennett, T.S. Field, D.A. Evans, Vitamin E and vitamin C supplement use and risk of incident Alzheimer disease., Alzheimer Dis. Assoc. Disord. 12 (1998) 121–6. http://www.ncbi.nlm.nih.gov/pubmed/9772012.

[122] M.C. Morris, D. a Evans, J.L. Bienias, C.C. Tangney, D. a Bennett, N. Aggarwal, R.S. Wilson, P. a Scherr, Dietary intake of antioxidant nutrients and the risk of incident Alzheimer disease in a biracial community study., JAMA. 287 (2002) 3230–3237. doi:10.1001/jama.287.24.3230.

[123] K.H. Masaki, K.G. Losonczy, G. Izmirlian, D.J. Foley, G.W. Ross, H. Petrovitch, R. Havlik, L.R. White, Association of vitamin E and C supplement use with cognitive function and dementia in elderly men., Neurology. 54 (2000) 1265–72. http://www.ncbi.nlm.nih.gov/pubmed/10746596.

[124] M. Makizako, H. Makizako, T. Doi, K. Uemura, K. Tsutsumimoto, H. Miyaguchi, H. Shimada, Olfactory identification and cognitive performance in community-dwelling older adults with mild cognitive impairment, Chem. Senses. 39 (2014) 39–46. doi:10.1093/chemse/bjt052.

[125] M.Z. Conti, B. Vicini-Chilovi, M. Riva, M. Zanetti, P. Liberini, A. Padovani, L. Rozzini, Odor identification deficit predicts clinical conversion from mild cognitive impairment to dementia due to alzheimer's disease, Arch. Clin. Neuropsychol. 28 (2013) 391–399. doi:10.1093/arclin/act032.

[126] R. Lalonde, H.D. Kim, J.A. Maxwell, K. Fukuchi, Exploratory activity and spatial learning in 12-month-old APP(695)SWE/co+PS1/DeltaE9 mice with amyloid plaques., Neurosci. Lett. 390 (2005) 87–92. doi:10.1016/j.neulet.2005.08.028.

[127] R.S. Reiserer, F.E. Harrison, D.C. Syverud, M.P. McDonald, Impaired spatial learning in the APPSwe + PSEN1DeltaE9 bigenic mouse model of Alzheimer's disease., Genes. Brain. Behav. 6 (2007) 54–65. doi:10.1111/j.1601-183X.2006.00221.x.

[128] D.R. Borchelt, T. Ratovitski, J. Van Lare, M.K. Lee, V. Gonzales, N.A. Jenkins, N.G. Copeland, D.L. Price, S.S. Sisodia, Accelerated amyloid deposition in the brains of transgenic mice coexpressing mutant presenilin 1 and amyloid precursor proteins, Neuron. 19 (1997) 939–945. doi:10.1016/S0896-6273(00)80974-5.

[129] R. Minkeviciene, P. Banerjee, H. Tanila, Memantine improves spatial learning in a transgenic mouse model of Alzheimer's disease., J. Pharmacol. Exp. Ther. 311 (2004) 677–82. doi:10.1124/jpet.104.071027.

[130] A. Savonenko, G.M. Xu, T. Melnikova, J.L. Morton, V. Gonzales, M.P.F. Wong, D.L. Price, F. Tang, A.L. Markowska, D.R. Borchelt, Episodic-like memory deficits in the APPswe/PS1dE9 mouse model of Alzheimer's disease: relationships to beta-amyloid deposition and neurotransmitter abnormalities., Neurobiol. Dis. 18 (2005) 602–17. doi:10.1016/j.nbd.2004.10.022.

[131] M. Low, D. Sandoval, E. Avilés, F. Pérez, F. Nualart, J.P. Henríquez, The ascorbic acid transporter SVCT2 is expressed in slow-twitch skeletal muscle fibres., Histochem. Cell Biol. 131 (2009) 565–74. doi:10.1007/s00418-008-0552-2.

[132] T.M. Manini, S.L. Hong, B.C. Clark, Aging and muscle: a neuron's perspective., Curr. Opin. Clin. Nutr. Metab. Care. 16 (2013) 21–6. doi:10.1097/MCO.0b013e32835b5880.

[133] Y. Chen, C.P. Curran, D.W. Nebert, K. V Patel, M.T. Williams, C. V Vorhees, Effect of vitamin C deficiency during postnatal development on adult behavior: functional phenotype of Gulo-/- knockout mice., Genes. Brain. Behav. 11 (2012) 269–77. doi:10.1111/j.1601-183X.2011.00762.x.

[134] D. Cheng, W. Logge, J.K. Low, B. Garner, T. Karl, Novel behavioural characteristics of the APP Swe /PS1E9 transgenic mouse model of Alzheimer's disease, Behav. Brain Res. 245 (2013) 120–127. doi:10.1016/j.bbr.2013.02.008.

[135] C.R. Hooijmans, C.E.E.M. Van der Zee, P.J. Dederen, K.M. Brouwer, Y.D. Reijmer, T. van Groen, L.M. Broersen, D. Lütjohann, A. Heerschap, A.J. Kiliaan, DHA and cholesterol containing diets influence Alzheimer-like pathology, cognition and cerebral vasculature in APPswe/PS1dE9 mice., Neurobiol. Dis. 33 (2009) 482–98.

doi:10.1016/j.nbd.2008.12.002.

[136] J.A. Chung, J.L. Cummings, Neurobehavioral and neuropsychiatric symptoms in Alzheimer's disease: characteristics and treatment., Neurol. Clin. 18 (2000) 829–46. http://www.ncbi.nlm.nih.gov/pubmed/11072263.

[137] U. Taemeeyapradit, D. Udomittipong, N. Tepparak, Characteristics of behavioral and psychological symptoms of dementia, severity and levels of distress on caregivers., J. Med. Assoc. Thai. 97 (2014) 423–30. http://www.ncbi.nlm.nih.gov/pubmed/24964685.

[138] F.E. Harrison, R.J. Green, S.M. Dawes, J.M. May, Vitamin C distribution and retention in the mouse brain, Brain Res. 1348 (2010) 181–186. doi:10.1016/j.brainres.2010.05.090.

[139] A. Raynaud-Simon, J. Cohen-Bittan, A. Gouronnec, E. Pautas, P. Senet, M. Verny, J. Boddaert, Scurvy in hospitalized elderly patients., J. Nutr. Health Aging. 14 (2010) 407–10. http://www.ncbi.nlm.nih.gov/pubmed/20617280.

[140] F. Cheng, R. Cappai, G.D. Ciccotosto, G. Svensson, G. Multhaup, L.-Å. Fransson, K. Mani, Suppression of amyloid beta A11 antibody immunoreactivity by vitamin C: possible role of heparan sulfate oligosaccharides derived from glypican-1 by ascorbate-induced, nitric oxide (NO)-catalyzed degradation., J. Biol. Chem. 286 (2011) 27559–27572. doi:10.1074/jbc.M111.243345.

[141] M. Guglielmotto, L. Giliberto, E. Tamagno, M. Tabaton, Oxidative stress mediates the pathogenic effect of different Alzheimer's disease risk factors., Front. Aging Neurosci. 2 (2010) 3. doi:10.3389/neuro.24.003.2010.

[142] J.M. May, L. Li, Z. Qu, C.E. Cobb, Mitochondrial recycling of ascorbic acid as a mechanism for regenerating cellular ascorbate., Biofactors. 30 (2007) 35–48. http://www.ncbi.nlm.nih.gov/pubmed/18198400.

[143] J. Kocot, D. Luchowska-kocot, I. Musik, Does Vitamin C Influence Neurodegenerative Diseases and Psychiatric Disorders?, Nutrients. 9 (2017) 659. doi:10.3390/nu9070659.

[144] M. a Bradley, W.R. Markesbery, M. a Lovell, Increased levels of 4-hydroxynonenal and acrolein in the brain in preclinical Alzheimer disease., Free Radic. Biol. Med. 48 (2010) 1570–6. doi:10.1016/j.freeradbiomed.2010.02.016.

[145] P. Podlesniy, J. Figueiro-Silva, A. Llado, A. Antonell, R. Sanchez-Valle, D. Alcolea, A. Lleo, J.L. Molinuevo, N. Serra, R. Trullas, Low cerebrospinal fluid concentration of mitochondrial DNA in preclinical Alzheimer disease., Ann. Neurol. 74 (2013) 655–68. doi:10.1002/ana.23955.

[146] M. Padurariu, A. Ciobica, L. Hritcu, B. Stoica, W. Bild, C. Stefanescu, Changes of some oxidative stress markers in the serum of patients with mild cognitive impairment and Alzheimer's disease, Neurosci. Lett. 469 (2010) 6–10. doi:10.1016/j.neulet.2009.11.033.

[147] P.H. Reddy, R. Tripathi, Q. Troung, K. Tirumala, T.P. Reddy, V. Anekonda, U.P. Shirendeb, M.J. Calkins, A.P. Reddy, P. Mao, M. Manczak, Abnormal mitochondrial dynamics and synaptic degeneration as early events in Alzheimer's disease: implications to mitochondria-targeted antioxidant therapeutics., Biochim. Biophys. Acta. 1822 (2012) 639–49. doi:10.1016/j.bbadis.2011.10.011.

[148] M.S. Cooke, M.D. Evans, M. Dizdaroglu, J. Lunec, Oxidative DNA damage: mechanisms, mutation, and disease., FASEB J. 17 (2003) 1195–214. doi:10.1096/fj.02-0752rev.

[149] M. Valko, D. Leibfritz, J. Moncol, M.T.D. Cronin, M. Mazur, J. Telser, Free radicals and antioxidants in normal physiological functions and human disease., Int. J. Biochem. Cell Biol. 39 (2007) 44–84. doi:10.1016/j.biocel.2006.07.001.

[150] T. Persson, B.O. Popescu, A. Cedazo-Minguez, Oxidative stress in Alzheimer's disease: why did antioxidant therapy fail?, Oxid. Med. Cell. Longev. 2014 (2014) 427318. doi:10.1155/2014/427318.

[151] M. a Lovell, W.R. Markesbery, Oxidative DNA damage in mild cognitive impairment and late-stage Alzheimer's disease., Nucleic Acids Res. 35 (2007) 7497–504. doi:10.1093/nar/gkm821.

[152] A. Barzilai, K.-I. Yamamoto, DNA damage responses to oxidative stress., DNA Repair (Amst). 3 (2004) 1109–15. doi:10.1016/j.dnarep.2004.03.002.

[153] L. Chami, F. Checler, BACE1 is at the crossroad of a toxic vicious cycle involving cellular stress and β-amyloid production in Alzheimer's disease., Mol. Neurodegener. 7 (2012) 52. doi:10.1186/1750-1326-7-52.

[154] A. Monfort, A. Wutz, Breathing-in epigenetic change with vitamin C., EMBO Rep. 14 (2013) 337–46. doi:10.1038/embor.2013.29.

[155] S. Dixit, A. Bernardo, J.M. Walker, J.A. Kennard, G.Y. Kim, E.S. Kessler, F.E. Harrison, Vitamin C deficiency in the brain impairs cognition, increases amyloid accumulation

and deposition, and oxidative stress in APP/PSEN1 and normally aging mice., ACS Chem. Neurosci. 6 (2015) 570–81. doi:10.1021/cn500308h.

[156] J. Mandl, A. Szarka, G. Banhegyi, Vitamin C: Update on physiology and pharmacology, Br. J. Pharmacol. 157 (2009) 1097–1110. doi:10.1111/j.1476-5381.2009.00282.x.

[157] J.L. Jankowsky, D.J. Fadale, J. Anderson, G.M. Xu, V. Gonzales, N.A. Jenkins, N.G. Copeland, M.K. Lee, L.H. Younkin, S.L. Wagner, S.G. Younkin, D.R. Borchelt, Mutant presenilins specifically elevate the levels of the 42 residue ??-amyloid peptide in vivo: Evidence for augmentation of a 42-specific ?? secretase, Hum. Mol. Genet. 13 (2004) 159–170. doi:10.1093/hmg/ddh019.

[158] L. Xu, Y. Shen, X. Wang, L. Wei, P. Wang, H. Yang, C. Wang, Z. Xie, J. Bi, Mitochondrial dynamics changes with age in an APPsw / PS1dE9 mouse model of Alzheimer ' s disease, Neuroreport. (2017) 1–7. doi:10.1097/WNR.0000000000000739.

[159] M. Garcia-Alloza, E.M. Robbins, S.X. Zhang-Nunes, S.M. Purcell, R.A. Betensky, S. Raju, C. Prada, S.M. Greenberg, B.J. Bacskai, M.P. Frosch, Characterization of amyloid deposition in the APPswe/PS1dE9 mouse model of Alzheimer disease, Neurobiol. Dis. 24 (2006) 516–524. doi:10.1016/j.nbd.2006.08.017.

[160] C. Desler, T.L. Hansen, J.B. Frederiksen, M.L. Marcker, K.K. Singh, L. Juel Rasmussen, Is there a link between mitochondrial reserve respiratory capacity and aging?, J. Aging Res. 2012 (2012). doi:10.1155/2012/192503.

[161] E. Trushina, E. Nemutlu, S. Zhang, T. Christensen, J. Camp, J. Mesa, A. Siddiqui, Y. Tamura, H. Sesaki, T.M. Wengenack, P.P. Dzeja, J.F. Poduslo, Defects in

mitochondrial dynamics and metabolomic signatures of evolving energetic stress in mouse models of familial alzheimer's disease, PLoS One. 7 (2012). doi:10.1371/journal.pone.0032737.

[162] I. YAMAZAKI, The reduction of cytochrome c by enzyme-generated ascorbic free radical., J. Biol. Chem. 237 (1962) 224–9. http://www.ncbi.nlm.nih.gov/pubmed/14008924.

[163] T. Ljones, T. Skotland, Evidence from the acceleration of cytochrome c reduction for the formation of ascorbate free radical by dopamine beta-monooxygenase., FEBS Lett. 108 (1979) 25–7. http://www.ncbi.nlm.nih.gov/pubmed/230081.

[164] N.H. Williams, J.K. Yandell, Reduction of oxidized cytochrome c by ascorbate ion., Biochim. Biophys. Acta. 810 (1985) 274–7. http://www.ncbi.nlm.nih.gov/pubmed/2998459.

[165] B. Uttara, A. V Singh, P. Zamboni, R.T. Mahajan, Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options., Curr. Neuropharmacol. 7 (2009) 65–74. doi:10.2174/157015909787602823.

[166] M. Padurariu, A. Ciobica, R. Lefter, I.L. Serban, C. Stefanescu, R. Chirita, The oxidative stress hypothesis in Alzheimer's disease., Psychiatr. Danub. 25 (2013) 401–9. http://www.ncbi.nlm.nih.gov/pubmed/24247053 (accessed June 19, 2014).

[167] K.M. Rodrigue, K.M. Kennedy, M.D. Devous, J.R. Rieck, A.C. Hebrank, R. Diaz-Arrastia, D. Mathews, D.C. Park, β-Amyloid burden in healthy aging: regional

distribution and cognitive consequences., Neurology. 78 (2012) 387–95. doi:10.1212/WNL.0b013e318245d295.

[168] M.P. Mattson, T. Magnus, Ageing and neuronal vulnerability., Nat. Rev. Neurosci. 7 (2006) 278–94. doi:10.1038/nrn1886.

[169] J. Lykkesfeldt, G.P. Trueba, H.E. Poulsen, S. Christen, Vitamin C deficiency in weanling guinea pigs: differential expression of oxidative stress and DNA repair in liver and brain., Br. J. Nutr. 98 (2007) 1116–9. http://www.ncbi.nlm.nih.gov/pubmed/18309548.

[170] X.-B. He, M. Kim, S.-Y. Kim, S.-H. Yi, Y.-H. Rhee, T. Kim, E.-H. Lee, C.-H. Park, S. Dixit, F.E. Harrison, S.-H. Lee, Vitamin C Facilitates Dopamine Neuron Differentiation in Fetal Midbrain Through Tet1- aND Jmjd3-Dependent Epigenetic Control Manner, Stem Cells. (2014) n/a-n/a. doi:10.1002/stem.1932.

[171] A. Monfort, A. Wutz, Breathing-in epigenetic change with vitamin C., EMBO Rep. 14 (2013) 337–46. doi:10.1038/embor.2013.29.

[172] J. Drake, C.D. Link, D.A. Butterfield, Oxidative stress precedes fibrillar deposition of Alzheimer's disease amyloid beta-peptide (1-42) in a transgenic Caenorhabditis elegans model., Neurobiol. Aging. 24 (2003) 415–20. doi:10.1016/S0197-4580(02)00225-7.

[173] W.H. Stoothoff, G.V.W. Johnson, Tau phosphorylation: Physiological and pathological consequences, Biochim. Biophys. Acta - Mol. Basis Dis. 1739 (2005) 280–297. doi:10.1016/j.bbadis.2004.06.017.

[174] M.T. Lai, E. Chen, M.C. Crouthamel, J. DiMuzio-Mower, M. Xu, Q. Huang, E. Price, R.B. Register, X.P. Shi, D.B. Donoviel, A. Bernstein, D. Hazuda, S.J. Gardell, Y.M. Li, Presenilin-1 and presenilin-2 exhibit distinct yet overlapping γ-secretase activities, J. Biol. Chem. 278 (2003) 22475–22481. doi:10.1074/jbc.M300974200.

[175] Y. Yonemura, E. Futai, S. Yagishita, S. Suo, T. Tomita, T. Iwatsubo, S. Ishiura, Comparison of presenilin 1 and presenilin 2 γ-secretase activities using a yeast reconstitution system, J. Biol. Chem. 286 (2011) 44569–44575. doi:10.1074/jbc.M111.270108.

[176] P.G. Ulery, D.K. Strickland, LRP in Alzheimer's disease: friend or foe?, J. Clin. Invest. 106 (2000) 1077–9. doi:10.1172/JCI11455.

[177] A.R. Wyatt, P. Const, H. Ecroyd, C.M. Dobson, M.R. Wilson, J.R. Kumita, J.J. Yerbury, Protease-activated alpha-2-macroglobulin can inhibit amyloid formation via two distinct mechanisms, FEBS Lett. 587 (2013) 398–403. doi:10.1016/j.febslet.2013.01.020.

[178] V.R. Varma, S. Varma, Y. An, T.J. Hohman, S. Seddighi, R. Casanova, A. Beri, E.B. Dammer, N.T. Seyfried, O. Pletnikova, A. Moghekar, M.R. Wilson, J.J. Lah, R.J. O'Brien, A.I. Levey, J.C. Troncoso, M.S. Albert, M. Thambisetty, Alpha-2 macroglobulin in Alzheimer's disease: a marker of neuronal injury through the RCAN1 pathway., Mol. Psychiatry. 22 (2017) 13–23. doi:10.1038/mp.2016.206.

[179] J. Poirier, Apolipoprotein E and Alzheimer's disease. A role in amyloid catabolism., Ann. N. Y. Acad. Sci. 924 (2000) 81–90. http://www.ncbi.nlm.nih.gov/pubmed/11193807.

[180] C. V Zerbinatti, G. Bu, LRP and Alzheimer's disease., Rev. Neurosci. 16 (2005) 123–35. http://www.ncbi.nlm.nih.gov/pubmed/15959937.

[181] T. Yamashima, Reconsider Alzheimer's disease by the "calpain-cathepsin hypothesis"--a perspective review., Prog. Neurobiol. 105 (2013) 1–23. doi:10.1016/j.pneurobio.2013.02.004.

[182] V. Stoka, V. Turk, B. Turk, Lysosomal cathepsins and their regulation in aging and neurodegeneration, Ageing Res. Rev. 32 (2016) 22–37. doi:10.1016/j.arr.2016.04.010.

[183] F. Di Domenico, A. Tramutola, M. Perluigi, Cathepsin D as a therapeutic target in Alzheimer's disease., Expert Opin. Ther. Targets. 20 (2016) 1393–1395. doi:10.1080/14728222.2016.1252334.

[184] L. Aitken, S.D. Quinn, C. Perez-Gonzalez, I.D.W. Samuel, J.C. Penedo, F.J. Gunn-Moore, Morphology-Specific Inhibition of β-Amyloid Aggregates by 17β-Hydroxysteroid Dehydrogenase Type 10., Chembiochem. 17 (2016) 1029–37. doi:10.1002/cbic.201600081.

[185] S.Y. Yang, X.Y. He, C. Isaacs, C. Dobkin, D. Miller, M. Philipp, Roles of 17β-hydroxysteroid dehydrogenase type 10 in neurodegenerative disorders, J. Steroid Biochem. Mol. Biol. 143 (2014) 460–472. doi:10.1016/j.jsbmb.2014.07.001.

[186] L.A.G. van Leeuwen, J.J.M. Hoozemans, Physiological and pathophysiological functions of cell cycle proteins in post-mitotic neurons: implications for Alzheimer???s disease, Acta Neuropathol. 129 (2015) 511–525. doi:10.1007/s00401-015-1382-7.

[187] C. Trovesi, N. Manfrini, M. Falcettoni, M.P. Longhese, Regulation of the DNA damage

response by cyclin-dependent kinases., J. Mol. Biol. 425 (2013) 4756–66. doi:10.1016/j.jmb.2013.04.013.

[188] R.B. Maccioni, J.P. Muñoz, L. Barbeito, The molecular bases of Alzheimer's disease and other neurodegenerative disorders., Arch. Med. Res. 32 (2001) 367–81. doi:10.1016/S0188-4409(01)00316-2.

[189] M.J. Millan, The epigenetic dimension of Alzheimer's disease: Causal, consequence, or curiosity?, Dialogues Clin. Neurosci. 16 (2014) 373–393.

[190] S.T. DeKosky, M.D. Ikonomovic, S.D. Styren, L. Beckett, S. Wisniewski, D.A. Bennett, E.J. Cochran, J.H. Kordower, E.J. Mufson, Upregulation of choline acetyltransferase activity in hippocampus and frontal cortex of elderly subjects with mild cognitive impairment., Ann. Neurol. 51 (2002) 145–55. http://www.ncbi.nlm.nih.gov/pubmed/11835370.

[191] T.J. Mellott, S.M. Pender, R.M. Burke, E.A. Langley, J.K. Blusztajn, IGF2 ameliorates amyloidosis, increases cholinergic marker expression and raises BMP9 and neurotrophin levels in the hippocampus of the APPswePS1dE9 Alzheimer's disease model mice, PLoS One. 9 (2014). doi:10.1371/journal.pone.0094287.

[192] J.-H. Heo, Hyon-Lee, K.-M. Lee, The possible role of antioxidant vitamin C in Alzheimer's disease treatment and prevention., Am. J. Alzheimers. Dis. Other Demen. 28 (2013) 120–5. doi:10.1177/1533317512473193.

[193] M. Pascual-Lucas, S. Viana da Silva, M. Di Scala, C. Garcia-Barroso, G. Gonzalez-Aseguinolaza, C. Mulle, C.M. Alberini, M. Cuadrado-Tejedor, A. Garcia-Osta, Insulin-

like growth factor 2 reverses memory and synaptic deficits in APP transgenic mice, EMBO Mol. Med. 6 (2014) 1246–1262. doi:10.15252/emmm.201404228.

[194] S.E. O'Bryant, V. Hobson, J.R. Hall, S.C. Waring, W. Chan, P. Massman, L. Lacritz, C.M. Cullum, Brain-Derived Nurotrophic Factor Levels in Alzheimer's Disease, J Alzheimers Dis. 17 (2009) 337–341. doi:10.3233/JAD-2009-1051.Brain-Derived.

[195] G. Go, Low-Density Lipoprotein Receptor-Related Protein 6 (LRP6) Is a Novel Nutritional Therapeutic Target for Hyperlipidemia, Non-Alcoholic Fatty Liver Disease, and Atherosclerosis, Nutrients. 7 (2015) 4453–4464. doi:10.3390/nu7064453.

[196] C.-C. Liu, C.-W. Tsai, F. Deak, J. Rogers, M. Penuliar, Y.M. Sung, J.N. Maher, Y. Fu, X. Li, H. Xu, S. Estus, H.-S. Hoe, J.D. Fryer, T. Kanekiyo, G. Bu, Deficiency in LRP6-Mediated Wnt Signaling Contributes to Synaptic Abnormalities and Amyloid Pathology in Alzheimer's Disease, Neuron. 84 (2014) 63–77. doi:10.1016/j.neuron.2014.08.048.

[197] M. Devall, J. Roubroeks, J. Mill, M. Weedon, K. Lunnon, Epigenetic regulation of mitochondrial function in neurodegenerative disease: New insights from advances in genomic technologies, Neurosci. Lett. 625 (2016) 47–55. doi:10.1016/j.neulet.2016.02.013.

[198] D. Mastroeni, A. Grover, E. Delvaux, C. Whiteside, P.D. Coleman, J. Rogers, Epigenetic changes in Alzheimer's disease: decrements in DNA methylation., Neurobiol. Aging. 31 (2010) 2025–37. doi:10.1016/j.neurobiolaging.2008.12.005.

[199] D. Mastroeni, A. McKee, A. Grover, J. Rogers, P.D. Coleman, Epigenetic differences in cortical neurons from a pair of monozygotic twins discordant for Alzheimer's disease.,

PLoS One. 4 (2009) e6617. doi:10.1371/journal.pone.0006617.

[200] K.-L. Chen, S.S.-S. Wang, Y.-Y. Yang, R.-Y. Yuan, R.-M. Chen, C.-J. Hu, The epigenetic effects of amyloid-beta(1-40) on global DNA and neprilysin genes in murine cerebral endothelial cells., Biochem. Biophys. Res. Commun. 378 (2009) 57–61. doi:10.1016/j.bbrc.2008.10.173.

[201] M. Iurlaro, G. Ficz, D. Oxley, E.-A. Raiber, M. Bachman, M.J. Booth, S. Andrews, S. Balasubramanian, W. Reik, A screen for hydroxymethylcytosine and formylcytosine binding proteins suggests functions in transcription and chromatin regulation., Genome Biol. 14 (2013) R119. doi:10.1186/gb-2013-14-10-r119.

[202] S. Kriaucionis, N. Heintz, The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain, Science. 324 (2009) 929–930. doi:10.1126/science.1169786.

[203] D.P. Gavin, K.A. Chase, R.P. Sharma, Active DNA demethylation in post-mitotic neurons: A reason for optimism, Neuropharmacology. 75 (2013) 233–245. doi:10.1016/j.neuropharm.2013.07.036.

[204] D.R. Galasko, E. Peskind, C.M. Clark, J.F. Quinn, J.M. Ringman, G.A. Jicha, C. Cotman, B. Cottrell, T.J. Montine, R.G. Thomas, P. Aisen, Alzheimer's Disease Cooperative Study, Antioxidants for Alzheimer disease: a randomized clinical trial with cerebrospinal fluid biomarker measures., Arch. Neurol. 69 (2012) 836–41. doi:10.1001/archneurol.2012.85.

[205] A. Kontush, U. Mann, S. Arlt, A. Ujeyl, C. Lührs, T. Müller-Thomsen, U. Beisiegel,

Influence of vitamin E and C supplementation on lipoprotein oxidation in patients with Alzheimer's disease., Free Radic. Biol. Med. 31 (2001) 345–54. http://www.ncbi.nlm.nih.gov/pubmed/11461772.

[206] S. Arlt, T. Müller-Thomsen, U. Beisiegel, A. Kontush, Effect of one-year vitamin C- and E-supplementation on cerebrospinal fluid oxidation parameters and clinical course in Alzheimer's disease., Neurochem. Res. 37 (2012) 2706–14. doi:10.1007/s11064-012-0860-8.

[207] N.R. Phillips, J.W. Simpkins, R.K. Roby, Mitochondrial DNA deletions in Alzheimer's brains: A review, Alzheimer's Dement. 10 (2014) 393–400. doi:10.1016/j.jalz.2013.04.508.

[208] F. Mamdani, B. Rollins, L. Morgan, P.A. Sequeira, M.P. Vawter, The somatic common deletion in mitochondrial DNA is decreased in schizophrenia., Schizophr. Res. 159 (2014) 370–5. doi:10.1016/j.schres.2014.08.026.

[209] T.B. Salmon, B. a Evert, B. Song, P.W. Doetsch, Biological consequences of oxidative stress-induced DNA damage in Saccharomyces cerevisiae., Nucleic Acids Res. 32 (2004) 3712–23. doi:10.1093/nar/gkh696.

[210] R. Kohen, A. Nyska, Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification., Toxicol. Pathol. 30 (2002) 620–50. doi:10.1080/0192623029016672.

[211] T.A. Warner, J.-Q. Kang, J.A. Kennard, F.E. Harrison, Low brain ascorbic acid increases susceptibility to seizures in mouse models of decreased brain ascorbic acid

transport and Alzheimer's disease., Epilepsy Res. 110 (2015) 20–5. doi:10.1016/j.eplepsyres.2014.11.017.

[212] J.W. Um, H.B. Nygaard, J.K. Heiss, M.A. Kostylev, M. Stagi, A. Vortmeyer, T. Wisniewski, E.C. Gunther, S.M. Strittmatter, Alzheimer amyloid-β oligomer bound to postsynaptic prion protein activates Fyn to impair neurons., Nat. Neurosci. 15 (2012) 1227–35. doi:10.1038/nn.3178.

[213] J.J. Palop, J. Chin, E.D. Roberson, J. Wang, M.T. Thwin, N. Bien-Ly, J. Yoo, K.O. Ho, G.-Q. Yu, A. Kreitzer, S. Finkbeiner, J.L. Noebels, L. Mucke, Aberrant excitatory neuronal activity and compensatory remodeling of inhibitory hippocampal circuits in mouse models of Alzheimer's disease., Neuron. 55 (2007) 697–711. doi:10.1016/j.neuron.2007.07.025.

[214] R. Minkeviciene, S. Rheims, M.B. Dobszay, M. Zilberter, J. Hartikainen, L. Fülöp, B. Penke, Y. Zilberter, T. Harkany, A. Pitkänen, H. Tanila, Amyloid beta-induced neuronal hyperexcitability triggers progressive epilepsy., J. Neurosci. 29 (2009) 3453–62. doi:10.1523/JNEUROSCI.5215-08.2009.

[215] S. Lesné, C. Ali, C. Gabriel, N. Croci, E.T. MacKenzie, C.G. Glabe, M. Plotkine, C. Marchand-Verrecchia, D. Vivien, A. Buisson, NMDA receptor activation inhibits alpha-secretase and promotes neuronal amyloid-beta production., J. Neurosci. 25 (2005) 9367–77. doi:10.1523/JNEUROSCI.0849-05.2005.

[216] J. Noebels, A perfect storm: Converging paths of epilepsy and Alzheimer's dementia intersect in the hippocampal formation., Epilepsia. 52 Suppl 1 (2011) 39–46.

doi:10.1111/j.1528-1167.2010.02909.x.

[217] J.J. Palop, L. Mucke, Epilepsy and cognitive impairments in Alzheimer disease., Arch. Neurol. 66 (2009) 435–40. doi:10.1001/archneurol.2009.15.

[218] J. Chin, H.E. Scharfman, Shared cognitive and behavioral impairments in epilepsy and Alzheimer's disease and potential underlying mechanisms., Epilepsy Behav. 26 (2013) 343–51. doi:10.1016/j.yebeh.2012.11.040.