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Mitochondrial dysfunction in the APP/PSEN1 mouse model of Alzheimer's disease and a novel protective role for ascorbate

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Abstract

Mitochondrial dysfunction is elevated in very early stages of Alzheimer's disease and exacerbates oxidative stress, which contributes to disease pathology. Mitochondria were isolated from 4-month-old wild-type mice, transgenic mice carrying the APP_{SWE} and $PSEN1_{dE9}$ mutations, mice with decreased brain and mitochondrial ascorbate (vitamin C) via heterozygous knockout of the sodium dependent vitamin C transporter (SVCT2+/–) and transgenic APP/PSEN1 mice with heterozygous SVCT2 expression. Mitochondrial isolates from SVCT2+/– mice were observed to consume less oxygen using high-resolution respirometry, and also exhibited decreased mitochondrial membrane potential compared to wild type isolates. Conversely, isolates from young (4 months) APP/PSEN1 mice consumed more oxygen, and exhibited an increase in mitochondrial membrane potential, but had a significantly lower ATP/ADP ratio compared to wild type isolates. Greater levels of reactive oxygen species were also produced in mitochondria isolated from both APP/PSEN1 and SVCT2+/– mice compared to wild type isolates. Acute administration of ascorbate to mitochondria isolated from wild-type mice increased oxygen consumption compared with untreated mitochondria suggesting ascorbate may support energy production. This study suggests that both presence of amyloid and ascorbate deficiency can contribute to mitochondrial dysfunction, even at an early, prodromal stage of Alzheimer's disease, although occurring via different pathways. Ascorbate may, therefore, provide a useful preventative strategy against neurodegenerative disease, particularly in populations most at risk for Alzheimer's disease in which stores are often depleted through mitochondrial dysfunction and elevated oxidative stress.

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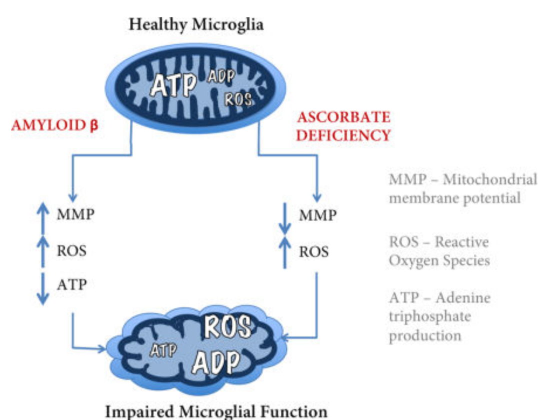
Graphical abstract



Keywords

Alzheimer's disease; mitochondria; oxidative stress; ascorbate; energy production

I. Introduction

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 [1–3], emerging before the appearance of the classical pathological features of Alzheimer's disease, such as amyloid plaques and neurofibrillary tangles [4–6]. Damage to both mitochondrial DNA and nuclear DNA have been identified in patients with mild cognitive impairment and early-stage Alzheimer's disease [7–9]. Mitochondrial dysfunction, in turn, increases production of reactive oxygen species (ROS), contributing further to oxidative stress [10–12] and damage to lipids [13,14], proteins [15,16] and nucleic acids [17–19]. 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 [20,21]. Furthermore, ROS generated from mitochondrial dysfunction can drive amyloidogenesis and tau phosphorylation [20,22,23], thus creating a cycle that decreases the energy available for proper cellular function, and drives the pathology and progression of cell death [24–26].

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 vitamin C (ascorbate, ASC), particularly to correct states of deficiency, confers some protection against the cognitive decline associated with Alzheimer's disease [27–30]. 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 the sodium-dependent vitamin C transporter, type 2 (SVCT2) and efficient storage and recycling mechanisms, suggesting an important role of ASC in

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neuronal health [31]. 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 [32]. Animal and cell culture studies offer additional evidence that supplementation with ASC not only ameliorates oxidative stress, but also mitigates the production of Aβ [33–37]. Previously published data from our group show a significant increase in soluble $A\beta_{1-42}$ and $A\beta_{1-40}$ peptides in cortical tissue from $SVCT2^{+/-}$; APP/PSEN1 mice at 6 months compared with APP/PSEN1 mice that have normal SVCT2 transporter expression, indicating that compromised ASC capacity increases the momentum of Alzheimer's disease pathological processes [38]. Consistent with this data, other groups showed chronic ascorbate (ASC) supplementation resulted in a decrease in total soluble $A\beta_{1-42}$ and a reduction in overall amyloid plaque burden in Alzheimer's disease mouse models [33,34].

It was originally believed that mitochondria took up the twice-oxidized form of ASC, dehydroascorbate, and reduced the molecule for use [39,40], 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 [41,42]. 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 [34]. 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 contribute 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 our 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 [38,43]. We also investigated the extent to which ASC affects these functions 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.

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II. Materials and methods

Animals

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. APP/PSEN1 mice develop appreciable amyloid beta (Aβ) accumulation and oxidative stress from 5–6 months of age [38]. Additionally, APP/PSEN1 mice exhibit structural abnormalities in mitochondria at 6 months [44–46]. $SVCT2^{+/-}$ mice have decreased expression of the SVCT2 transporter and 20–30% decreased brain ASC levels although they retain the ability to synthesize ASC, and peripheral SVCT2-dependent tissue contents are within 50–90% of $SVCT2^{+/+}$ littermates [47]. They were backcrossed at least 10 generations to the C57Bl/6J strain and maintained on that same background. SVCT2 transgenic mice ($SVCT2^{Tg}$), developed for our research group, exhibit a global increase in SVCT2 expression and concomitant increases in ASC concentration [48]. The term "wild-type" (WT) is used to denote mice that do not carry the mutations APP and PSEN1, and are also wild-type for SVCT2 expression. All animals were housed in a temperature and humidity controlled vivarium and were kept on a 12:12 h light/dark cycle. All procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee.

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 [49,50]. 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) [49–51] 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,

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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 [52]. Cerebellum levels are a good reflection of levels in cortical and hippocampal areas [53]. Briefly, tissue samples were weighed and 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. 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) [21,54]. 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™ 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™ 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

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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.

III. 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 [38]. 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. 1A). The magnitude of the effect is consistent with decreased ASC content in mitochondria from SVCT2 heterozygous mice ($t(8) = 2.766$, $p = .024$, Fig. 1B). 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 [45,55,56]. While there is little amyloid accumulation prior to 6 months in the APP/PSEN1 mouse model [43,57], the Aβ peptide is present and measureable at 4 months in the APP/PSEN1 mouse model, as demonstrated qualitatively by western blot (Fig. 1C). 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 [58], 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. 1D), with a trend toward less oxygen consumption emerging after the addition of glutamate and malate ($p=0.054$), suggesting that adequate ASC in the

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mitochondria is necessary for efficient energy production. ASC deficiency may, therefore, contribute to an energy deficit during disease but also in normal function. 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. 1D). 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, although the effects observed were in opposite directions. 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 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 [21,51,59]. We used dihydrofluorescein to measure of ROS production given that disruption of the electron transport chain leads to oxidative stress.

ASC status and APP/PSEN1 genotype affects mitochondrial membrane potential and ATP/ADP ratios and increases oxidative stress

Mitochondrial membrane potential was determined through TMRE accumulation in mitochondrial isolates from wild-type, SVCT2$^{+/-}$, 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. 2A). 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. 2B). These results are consistent with the pattern of oxygen consumption observed in SVCT2$^{+/-}$ and APP/PSEN1 isolates (Fig. 1D). 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 determined the ATP/ADP ratio in mitochondrial isolates from WT, SVCT2$^{+/-}$, 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. 2D) and no significant difference in ATP/ADP ratio

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in the SVCT2$^{+/-}$ isolates compared to wild type (Fig. 2C). 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 [60–62]. Ascorbate 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 [63]. 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 [39]. 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 [64]. 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. 2E) and in mitochondrial isolates from APP/PSEN1 cortex ($t(16) = 3.447$, $p = 0.0033$; Fig. 2F), suggesting that the oxygen being consumed is not being used efficiently but is rather contributing to oxidative stress. 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 [65] 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.

ASC increases oxygen consumption and decreases ROS generation

Having observed that ASC deficiency resulted in reduced mitochondrial respiration and increased ROS generation, we considered whether the acute supplementation of ASC to isolated mitochondria would increase respiration. Mitochondria were isolated from WT cortex 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

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consumption significantly over its own baseline level and the untreated group ($F(1,40)= 11.31$, $p= 0.0017$; Fig. 3A). Both ASC+ and ASC-groups significantly increased respiration with the sequential addition of substrates (glutamate, malate and ADP) until reaching full respiratory capacity ($F(4,40)= 26.95$, $p<0.0001$; Fig. 3).

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. 3B, 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 [48]. Unlike $SVCT2^{+/-}$ isolates, we observed no significant change in TMRE fluorescence in $SVCT2^{Tg}$ mice, indicating that a chronic increase in mitochondrial ASC does 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 4A), 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 $SVCT2^{Tg}$. These data were collected prior to this study and some (WT: ELISA and cortical ASC) has appeared in a previous publication [38]. This observation suggests that the ASC is being consumed more rapidly 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.

IV. Conclusion

ASC is critical for mitochondrial health and ASC deficiency is a major cause of oxidative stress that contributes to Alzheimer's disease risk. We have shown that ASC deficiency and the APP/PSEN1 genotype alter mitochondrial respiration, membrane potential, energy production and increase ROS generation. While optimal levels of ASC are unknown [31], adequate ASC supports both the redox balance within mitochondria, and oxygen consumption. The key features of oxidative stress and mitochondrial dysfunction are elevated in very early stages of the disease, and may provide potential biomarkers before the manifestation of clinical symptoms, and are believed to drive disease pathology. 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

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that the antioxidant barrier is being overwhelmed by increasing oxidative stress [20,66]. 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 than are currently typically addressed.

Acknowledgments

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Abbreviations

SVCT	sodium-dependent vitamin C transporter
APP	amyloid precursor protein
PSEN1	presenilin 1
ASC	ascorbate
ROS	reactive oxygen species
Aβ	amyloid beta peptide
TMRE	tetramethylrhodamine, ethyl ester

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Highlights

- APP/PSEN1 genotype increases mitochondrial oxygen consumption and generation of reactive oxygen species
- Ascorbate deficiency exacerbates mitochondrial oxidative stress
- Ascorbate supplementation mitigates generation of reactive oxygen species and supports mitochondrial health

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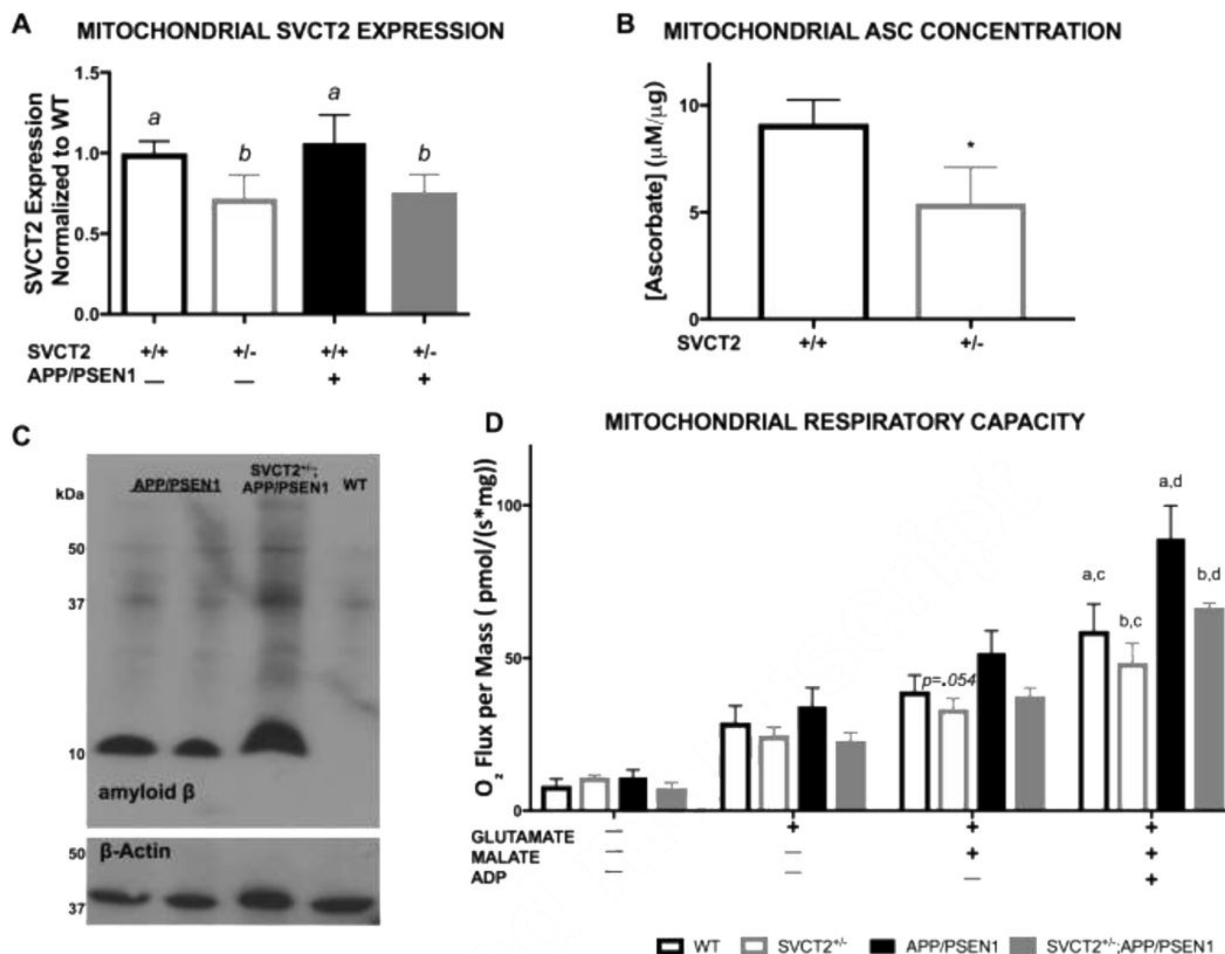

Figure 1. ASC deficiency and APP/PSEN1 affect oxygen consumption in mitochondria

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*).

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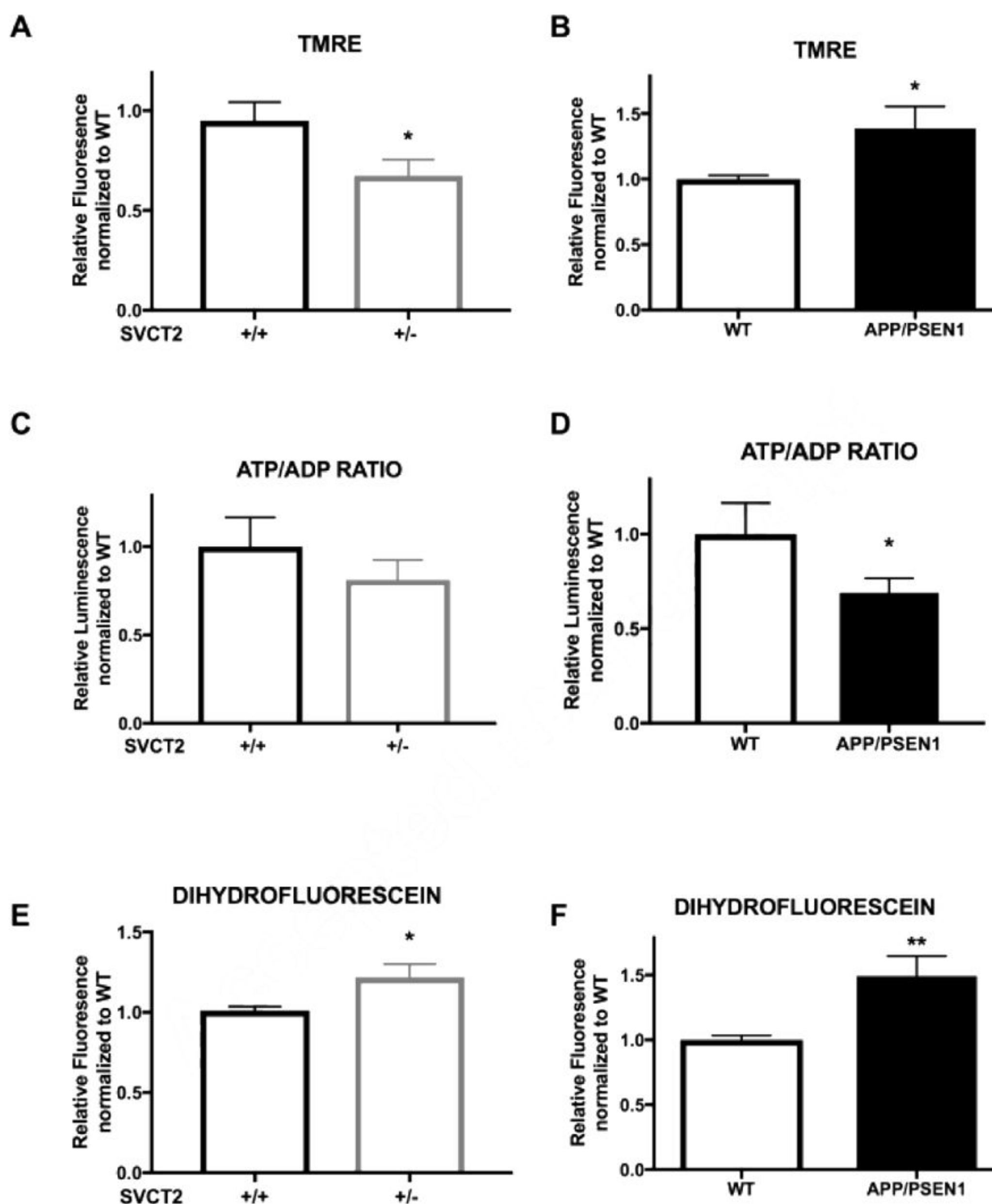

Figure 2. APP/PSEN1 genotype affects mitochondrial membrane potential and increases oxidative stress

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 ($p = 0.038$).

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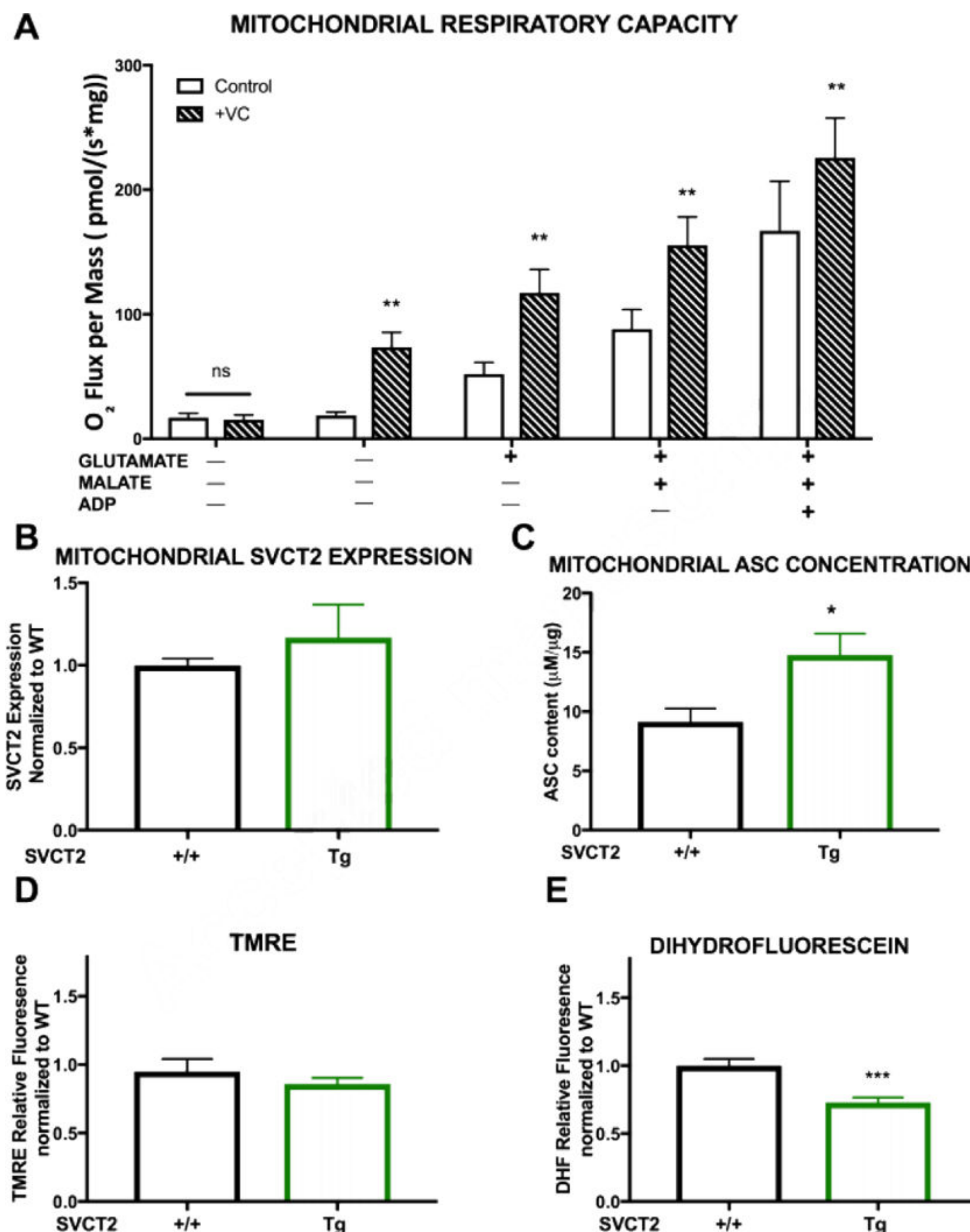

Figure 3. ASC increases oxygen consumption in isolated mitochondria and decreases ROS production

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).

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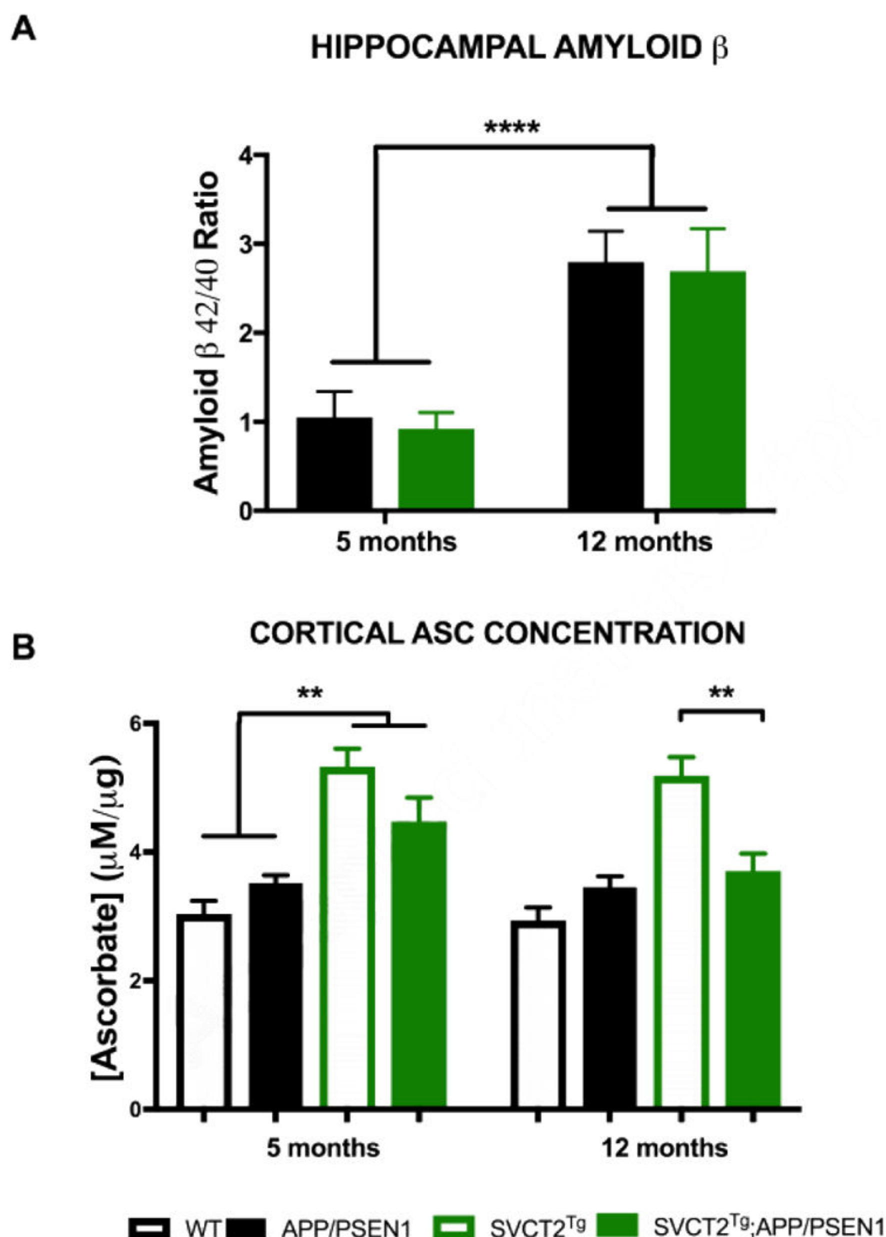

Figure 4. Intracellular ASC concentration is altered in SVCT2^{Tg}; APP/PSEN1 mice

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 ($p=0.0002$).