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Combined Vitamin C & E deficiency induces motor defects in gulo$^{-/-}$/SVCT2$^{+/-}$ mice

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Abstract

Objectives—Key antioxidants, vitamins C and E, are necessary for normal brain development and neuronal function. In this study, we depleted both of these vitamins in two mouse models to determine if oxidative stress due to combined vitamin C and E dietary deficiency altered their neurological phenotype. The first model lacked both alleles for the Gulonolactone oxidase gene (Gulo$^{-/-}$) and therefore was unable synthesize vitamin C. To obtain an additional cellular deficiency of vitamin C, the second model also lacked one allele for the cellular vitamin C transporter gene (Gulo$^{-/-}$/SVCT2$^{+/-}$).

Methods—The experimental treatment was 16 weeks of vitamin E deprivation followed by 3 weeks of vitamin C deprivation. Mice were assessed for motor coordination deficits, vitamin levels, and oxidative stress biomarkers.

Results—In the first model, defects in motor performance were more apparent in both vitamin C-deficient groups (VE+VC−, VE−VC−) compared to vitamin C-supplemented groups (VE+VC+, VE−VC+) regardless of vitamin E level. Analysis of brain cortex and liver confirmed decreases of at least 80% for each vitamin in mice on deficient diets. Vitamin E deficiency doubled oxidative stress biomarkers (F_2-isoprostanes and malondialdehyde). In the second model, Gulo$^{-/-}$/ SVCT2$^{+/-}$ mice on the doubly deficient diets showed deficits in locomotor activity, Rota-rod performance, and other motor tasks, with no concomitant change in anxiety or spatial memory.

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Discussion—Vitamin E deficiency alone caused a modest oxidative stress in brain that did not affect motor performance. Adding a cellular deficit in vitamin C to dietary deprivation of both vitamins significantly impaired motor performance.

Keywords

Ascorbic acid; Brain; Mouse behavior; Oxidative stress; SVCT2; Vitamin C deficiency; Vitamin E deficiency

INTRODUCTION

Oxidative stress is a key feature of normal aging, and is markedly increased in stroke and certain neurodegenerative conditions. Neurons are especially prone to oxidative stress due to their high metabolic activity.[1] Chief among the central nervous system's defenses against oxidative stress are low-molecular-weight antioxidants such as vitamins E and C. Vitamin E (mostly α-tocopherol) is lipid-soluble and provides protection in cellular membranes. Vitamin C is water-soluble and is transported from blood into the cerebrospinal fluid (CSF) and from the CSF in ventricles into neurons by the sodium-dependent vitamin C transporter – type 2 (SVCT2).[2] This transporter is responsible for generating vitamin C concentrations up to 2–6 mM in neurons.[3] Mice that are homozygous for the SVCT2 gene deletion die shortly after birth with undetectable vitamin C levels in the brain.[4] This demonstrates the importance both of vitamin C content and regulation by SVCT2 in brain.

In vitro[5] and in vivo[6] studies have demonstrated a synergistic relationship between vitamin C and vitamin E that helps to preserve neuronal function and survival.[7] Guinea pigs, like humans, are unable to synthesize vitamin C and have long served as a model for study of both vitamin E and C deficiencies. Using this model, it was demonstrated that first, high dietary fat and cholesterol exacerbated chronic vitamin C deficiency by both decreasing vitamin C levels and increasing its oxidation ratio.[8] Second, a diet deficient in both vitamins had greater oxidative stress than animals deficient in either vitamin alone. Lastly, the doubly deficient animals developed a phenotype of progressive paralysis and death due to neuronal and fiber loss in the long tracks of the spinal cord.[9] This severe phenotype precluded further studies with guinea pigs.

To develop a more tractable model that could also be genetically manipulated, we have adapted the double-vitamin deficiency model to gulo$^{-/-}$ mice. These mice lack functional gulonolactone oxidase (gulo),[10] which is required for vitamin C synthesis. Vitamin C tissue levels in these mice can be readily manipulated by dietary means.[10,11] Vitamin E can also be removed from their diets, thus yielding a combined vitamin C and vitamin E-deficient model for whole-body oxidative stress. In addition to decreasing dietary antioxidants, we also increased fat and sucrose components, as both of these dietary factors have also been shown to increase oxidative stress.[12,13] This model is physiologically relevant because humans deficient in one vitamin are often marginal in the other. Furthermore, those most likely to have vitamin C deficiency tend to eat a diet low in fruits and vegetables[14,15] and high in triglycerides and cholesterol.

The purpose of this study was to determine the effect of oxidative stress due to combined vitamin C and E dietary deficiency on the neurological phenotype of mice. The first experiment induced both single and double vitamin deficiencies then assessed the subsequent impact on motor and cognitive skills in gulo$^{-/-}$ mice. The second experiment, designed to establish a more severe oxidative stress and specifically deplete intracellular vitamin C, induced double vitamin deficiency in gulo mice that also lacked one allele for the SVCT2 and included further behavioral testing.

METHODS

Animals and diets

Experiment 1—Heterozygous gulo$^{+/-}$ mice were originally obtained from Mutant Mouse Regional Resource Centers (http://www.mmrrc.org, #000015-UCD) and maintained as gulo$^{-/-}$ on a C57BL/6J background. Gulo$^{-/-}$ mice are unable to synthesize vitamin C and were supplied with 0.33 g/l vitamin C in their drinking water except during experimental manipulations. This supplement level provides adult (non- pregnant) gulo$^{-/-}$ mice with approximately wild-type levels of vitamin C in tissues.[10,11] To minimize oxidation of vitamin C, deionized water was supplemented with 0.01 mmol/l EDTA.

Male and female gulo$^{-/-}$ mice were maintained on a normal rodent chow that contained essentially no vitamin C and 42 IU/kg of vitamin E (total tocopherols) (Lab Diet, #5001). At 4–5 weeks of age, the mice were randomly divided into four groups on a Westerndiet (16% lard, 34% sucrose, 0.2 cholesterol). This was to increase oxidative stress and to provide a physiological condition relevant to humans, as those deficient in these vitamins are likely to also consume a diet high in saturated fats and sucrose. Vitamin E-deficient and supplemented diets were custom made by Harlan Teklad (TD.07310 and TD.07311, respectively).

The four treatment groups each consisted of 16 weeks of vitamin E treatment via diet followed by 3 weeks of vitamin C treatment via water supplementation: (1) control group – vitamin E treatment as 150 IU/kg with continued supplementation of vitamin C at 0.33 g/l; VE+VC+ (N = 9), (2) vitamin C deficient group – vitamin E treatment as 150 IU/ kg and a final vitamin C treatment of 0g/l; VE+VC− (N = 8), (3) vitamin E-deficient group – vitamin E treatment as 0IU/kg with continued vitamin C supplementation of 0.33 g/l; VE−VC+ (N = 6), and (4) doubly deficient group – vitamin E treatment as 0 IU/kg with a final vitamin C treatment 0 g/L; VE−VC− (N = 8). The four groups, containing approximately equal males and females, are shown in Experimental Scheme 1 (Fig. 1).

Experiment 2—To enable both systemic and intracellular vitamin C depletion, gulo$^{-/-}$ mice were crossed with mice lacking one allele of the SVCT2 (SVCT2$^{+/-}$)[4] to generate gulo$^{-/-}$/SVCT2$^{+/-}$ mice. SVCT2$^{+/-}$ mice were originally provided by Dr Robert Nussbaum and were placed on the C57BL/6J background as described.[16] Additional groups assessed included two single mutant genotypes (gulo$^{-/-}$ and SVCT2$^{+/-}$ mice), as well as wild-type C57BL/6J mice (gulo$^{+/+}$/ SVCT2$^{+/+}$). At 4–5 weeks of age, all mice were placed on the vitamin E-deficient, Western diet (TD.07310) and were provided with 0.33 g/l vitamin C in their drinking water for 16 weeks. The mice were then all deprived of vitamin C for 3 weeks before sacrifice. Thus, the four groups containing approximately equal males and females were wild- type control; gulo$^{+/+}$/SVCT2$^{+/+}$ (N = 10), Gulo; gulo$^{-/-}$/SVCT2$^{+/+}$ (N = 5), SVCT2; gulo$^{+/+}$/ SVCT2$^{+/-}$ (N = 9) and gulo/SVCT2; gulo$^{-/-}$/ SVCT2$^{+/-}$ (N = 10) as shown in Experiment Scheme 2 (Fig. 2).

All animals were housed in tub cages in a temperature- and humidity-controlled vivarium on a 12:12-h light:dark cycle with lights on at 6 AM. All procedures conformed to Institutional IACUC guide- lines. Mice had free access to food and water for the duration of experiment.

Weight

Scurvy in mice typically manifests after 5–6 weeks of vitamin C deprivation10 and early signs include weight loss. Mice were weighed on a standard gram scale twice per week and observed for signs of overt scurvy.

Behavior assessment schedule

Experiment 1—To determine the effect of vitamin E deprivation only, an initial behavior assessment (Test 1) was conducted after 16 weeks of the respective vitamin E treatments. A second assessment (Test 2) was performed after 3 weeks of the respective vitamin C treatments to determine the effect of the combined vitamin deficiency, as shown in Experimental Scheme 1. Each assessment spanned two consecutive days. Day 1 included a Rota-rod training session to acclimate the mice to the task. Day 2 included gait testing, locomotor activity, inverted screen, and the Rota-rod test session.

Experiment 2—This experiment was designed to confirm the findings from experiment 1 and expand the results to include three additional genotypes. The first assessment (Test 1) was done 16 weeks after the vitamin E deficient diet followed by two separate assessments at weeks 2 and 3 of the vitamin C treatment phase which corresponds to 18 and 19 weeks of vitamin E deficiency, (Tests 2 and 3, respectively), as shown in Experimental Scheme 2. Each assessment spanned two consecutive days with the more energy demanding tasks on day 2. Day one included one session of locomotor activity, zero-maze, inverted screen, and Rota-rod training, sequentially. Day 2 included one session of gait, Y-maze, horizontal beam, wire-hang, and Rota-rod test, sequentially.

All tests were performed under similar lighting. Repeated testing of cohorts was performed at the same time of day (morning or afternoon). Experiments and data analysis were performed in part through the use of the Murine Neurobehavior Core Laboratory at the Vanderbilt University Medical Center.

Motor and strength assessments

Gait analysis—Shorter stride length can be indicative of muscle weakness or ataxia. To assess this, hind-foot printing was performed to monitor changes in stride length. Average stride length was calculated as the distance between two consecutive footprints, averaged from three strides as described.[11]

Locomotor activity—In each 10 minutes session, the mice were allowed to individually explore an open 27.9 cm × 27.9 cm chamber (Med Associates, OFA-510). Locomotor activity (distance traveled and velocity) was automatically recorded by the breaking of infrared beams as described.[11]

Inverted screen—Mice were assessed for general muscle grip strength in the inverted screen test. In each 5 minutes trial, mice were placed on a 1-cm wire mesh and slowly inverted completely over a collection box. The latency to fall was recorded for each mouse.

Rota-rod activity—All groups were tested for motor coordination on the Rota-rod. Mice with severe coordination problems will have difficulty remaining on the Rota-rod even at low speeds. Performance generally improves across subsequent trials; therefore a training session was conducted. In each 5 minutes trial, mice were placed on a ridged, rotating beam that slowly accelerated from 4–40 rpm. The time it took the mouse to fall was recorded. If the mice simply clung to the beam and rotated along with it without falling this was recorded as a ‘rotation’.

Wire hang—In each 60 seconds trial, mice were placed to hang by their front legs in the middle of a plastic wire suspended horizontally between two platforms. A score indicating the activity of the mouse is displayed as: 4 = stabilized itself within 30 seconds; 3.5 = stabilized itself within 60 seconds; 3 = attempts to stabilize itself but fails to do so within 60

seconds; 2=remains hanging from the wire for 60 seconds; 0 = falls from wire within 60 seconds.

Horizontal beam—Similar to the wire hang, in each 60 seconds trial, mice were placed in the middle of a rod suspended horizontally between two platforms. A score indicating the activity of the mouse was recorded with 4=escape within 30 seconds and 0 = fell from rod as described.[11]

Zeromaze—Anxiety was assessed by exploration patterns in the elevated zero maze. In each 5 minutes session, mice were placed on an elevated circular platform with two opposite quadrants enclosed and two open. Mice were allowed to explore the maze and activity was recorded with an overhead camera and analyzed with Any-Maze computer software (Stoelting, USA).

Y-Maze—In each 5 minutes session, mice were placed in one arm of a clear Plexiglas Y-maze consisting of three identical arms void of visual cues. As the mouse explored the maze, the sequence of consecutive individual arm entries was recorded with an overhead camera. An alternation was recorded as a ratio of the number of three non-repeated entries divided by the total number of entries minus two.

Biochemical assessment

Mice were briefly anesthetized with isoflurane and killed quickly by decapitation. Cortex, cerebellum and liver were removed and frozen in dry-ice and stored at −80°C until needed.

Antioxidant analysis

Vitamin C was measured as ascorbic acid in metaphosphoric acid extracts of tissues as described[11] using high-performance liquid chromatography (HPLC) with electrochemical detection. Vitamin E was measured as α-tocopherol in extraction buffer containing reagent alcohol, 10 mg/ml butylated hydroxytoluene, 3% sodium dodecyl sulfate and hexane and subjected to HPLC analysis as described.[17] Reduced glutathione was measured in the vitamin C tissue extracts by an adaptation of the o-phthalaldehyde method as described.[18] Data for all assays were expressed per gram tissue weight (wet weight).

Oxidative stress analysis

To assess lipid peroxidation, malondialdehyde was measured by homogenizing samples in 5% trichloroacetic acid solution and reacted with thiobarbituric acid as described.[18] Tissue contents of esterified F_4- neuroprostanes and F_2-isoprostanes were quantified by stable isotope dilution negative ion chemical ionization gas chromatography/mass spectrometry.[19] These analyses were performed in the Vanderbilt University Eicosanoid Core Laboratory.

Behavioral statistical analysis

In Experiment 1, for all experiments that were conducted at both time points, repeated measures analysis of variance (ANOVA) was conducted with time as the repeated variable (Tests 1 and 2) and vitamin C status (VC+, VC−) and vitamin E status (VE+, VE−) as the between-group variables. For the inverted screen test an univariate ANOVA was conducted with vitamin C status (VC+, VC−) and vitamin E status (VE+, VE−) as the between-group variables. In Experiment 2, repeated measures ANOVA were conducted with time as the repeated variable (Tests 1, 2, and 3) and genotype of the gulonolactone oxidase gene ($gulo^{+/+}$, $gulo^{-/-}$) and vitamin C transporter ($SVCT2^{+/+}$, $SVCT2^{+/-}$) as the between-group variables.

Biochemical and oxidative stress markers statistical analysis

In Experiment 1, univariate ANOVA was conducted for each independent measure with vitamin C status (VC+, VC−) and vitamin E status (VE+, VE−) as the between-group variables. In Experiment 2, univariate ANOVA was conducted with genotype of the gulonolactone oxidase gene ($gulo^{+/+}$, $gulo^{-/-}$) and vitamin C transporter ($SVCT2^{+/+}$, $SVCT2^{+/-}$) as the between-group variables.

A P value of <0.05 was considered statistically significant. Significant results from the omnibus ANOVA were followed by the Bonferroni-corrected pairwise comparisons and are reported in the text with interaction effects denoted as factor × factor. Due to the limitations of the statistical tests performed, in each experiment direct comparisons between the control groups and the double deficient group (VC+VE+ and VC−VE− or $Gulo^{+/+}$/$SVCT2^{+/+}$ and $Gulo^{-/-}$/ $SVCT2^{+/-}$) could not be assessed. Figures indicate significant differences between-group variables noted in their respective figure legend. All statistical analyses were performed by IBM SPSS Statistics Software (Version 19).

RESULTS

Experiment 1 – Combined vitamin C and E deficiency in $Gulo^{-/-}$ mice

Body weight and general appearance—As expected, the weights of all mice increased with age. During vitamin C deprivation all the groups except for the control decreased slightly (P<0.01); however, there were no significant interaction effects with time for the groups (P=0.56) (Fig. 3A). There were no physical signs of early scurvy in any of the mice following vitamin C removal (hair loss, hunched posture, or lethargy).

Behavioral assessment—Gait analysis for changes in stride length showed no main effects of time, VE or VC status, and no VE × VC, or time × VE interactions (P > 0.062). There were, however, significant interaction effects of time×VC and of time×VC×VE (Ps<0.038) (Fig. 3B). Follow-up analyses showed that the VE+VC− group was the only group to show altered gait with time, with a decreased stride length between Tests 1 and 2 (P < 0.01). At test 1 VE+VC− mice had a slightly longer gait than VE+VC+ mice (P < 0.05), but at Test 2 the direction of this difference was reversed (P < 0.05).

As expected, the distance traveled during locomotor activity testing decreased at least 35% in Test 2 in all groups indicating normal habituation to the chamber as supported by the main effect of time (P < 0.01) (Fig. 3C). Both VC-deficient groups (indicated by the dashed lines) explored less than the VC-supplemented groups at Test 2 (P< 0.01) (Fig. 3C). Compared to the control group (VE+VC+), all of the mice maintained similar locomotor velocity during both test sessions except for the VE+VC− group, which decreased in Test 2 as evident from an interaction effect of time × VC × VE (P < 0.01) (Fig. 3D). Follow-up analyses confirmed a significant effect of time in the VE+VC− group (P < 0.01) but not in any of the other groups (P > 0.181).

On the inverted screen, both of the single and double vitamin-deficient groups had shorter latencies to fall than the VE+VC+ group. There was a main effect of VC status (P < 0.01) (Fig. 3E). Results in Fig. 3F are from the Roto-rod probe trial only (day 2 of each 2-day test session). Vitamin E deprivation alone did not lead to any differences among the groups in latency to fall from the rod (Test 1). All of the groups maintained their performance over time; however, the vitamin C-deficient groups had lower baseline level of performance as shown by a shorter latency to fall at both testing points compared to VC supplemented mice (P < 0.01) (Fig. 3F).

Vitamin C and α-tocopherol measurements of brain cortex and liver—Vitamin C and α-tocopherol were measured in both brain and liver to determine the effectiveness of deprivation treatments. The liver was studied to reflect whole body vitamin C status, since it receives dietary vitamin C from the portal system and does not synthesize the vitamin in gulo$^{-/-}$ mice. As expected, vitamin C deprivation for 3 weeks regardless of vitamin E treatment lowered the vitamin C contents of cortex and liver. In cortex, vitamin C levels decreased 86% in VE+VC− and VE−VC− groups compared to the VE+VC+ group ($P < 0.01$) (Fig. 4A). Liver vitamin C decreased 73 and 78% in the VE+VC− and VE−VC− groups, respectively (Fig. 4B). In addition to the main effect of VC status ($P < 0.01$) and VE status ($P < 0.05$), there was also interaction between the two factors ($P = 0.038$) in liver. Pairwise comparisons revealed that in addition to the decreased VC in liver of both groups of VC- deprived mice ($P < 0.05$), VE deprivation without VC deprivation lowered VC in VE −VC+ compared to VE+VC+ ($P < 0.05$).

As expected, Vitamin E deprivation for 16 weeks lowered α-tocopherol in cortex (Fig. 4C) and liver (Fig. 4D). In cortex, there were main effects of both VC ($P < 0.05$) and VE ($P < 0.01$) status, with no significant VC × VE status interaction ($P = 0.356$). In liver, there were main effects of VE and VC deprivation ($P < 0.01$). A significant VC × VE status interaction in liver ($P = 0.01$) indicated that although deprivation of either vitamin led to decreases in liverα-tocopherol levels compared to control (P's < 0.01), no differences were found in VE− groups according to VC status or in VC− groups according to VE status ($P > 0.27$). Thus, deprivation of either vitamin led to a significant decrease in α-tocopherol levels in liver. In both tissues, VE was decreased in mice deficient of vitamin C by at least 50% ($P < 0.01$). VE−VC− decreased 88 and 97% in cortex and liver tissue, respectively.

Measures of oxidative stress—Diets did not affect glutathione (GSH) contents in cortex or liver ($P > 0.448$) (Fig. 4E and 4F). Malondialdehyde (MDA) levels in cortex and liver were increased in the VE−VC− group compared tothe groups with normal vitamin E intakes (Fig. 5A and 5B). Vitamin C deprivation increased MDA in the cortex ($P < 0.05$). In liver there was an increase in MDA following removal of both VC ($P < 0.01$) and VE ($P < 0.01$), with no interaction between the groups ($P = 0.547$). F_2-Isoprostanes, derived from non-enzymatic peroxidation of arachidonic acid, were increased almost 3-fold in cortex in groups deprived of vitamin E compared to groups on adequate vitamin E ($P < 0.01$; Fig. 5C). F_2-isoprostanes were also increased in cortex to a lesser extent with vitamin C deprivation, leading to a main effect of VC status ($P < 0.05$). A similar result was observed in liver with VE−VC+ and VE−VC− group levels7-fold higher than controls. This was reflected in a main effect of VE ($P < 0.01$). However, there was no main effect of VC status (Fig. 5D). F_4-Neuroprostanes, derived from peroxidation of docosahexaenoic acid, were about 30-fold higher than those of F2-isoprostanes (compare Fig. 5C–5E). Similar to F2-isoprostanes, vitamin E deprivation increased F_4-neuroprostanes in cortex ($P < 0.05$) (Fig. 5E).

Experiment 2 – combined vitamin C and E deficiency in gulo$^{-/-}$/SVCT2$^{+/-}$ mice

In Experiment 2, a cellular vitamin C deficiency was added to the systemic vitamin C deficiency, using gulo$^{-/-}$ mice that had been crossed with mice heterozygous for the vitamin C transporter, SVCT2 (SVCT2$^{+/-}$). All mice were on the same Western diet and were deprived of vitamin E for 16 weeks, followed by vitamin C-deficient intakes as described for Experiment 1. This experiment was designed to confirm the effects observed in the vitamin C- and E-depleted gulo$^{-/-}$ mice in Experiment 1 and to expand motor testing by adding other coordination tests (wire hang, horizontal beam), an anxiety task, and also biochemical assessments of cerebellum to relate the behavioral phenotype to a brain region most associated with motor coordination.

Body weight and general appearance—Weights of all mice increased with age at the time points measured and there were no differencesbetween the groups (Fig. 6A). None of the mice had overt signs of severe scurvy, although gulo$^{-/-}$/ SVCT2$^{+/-}$ mice lost weight following 3 weeks of vitamin C deficiency ($P < 0.01$).

Behavioral assessment—Changes in behavioral phenotype were observed during the last 3 weeks of the diet treatments. Micewere assessed both under normal vitamin C supplementation (Test 1) and after 2 (Test 2) and 3 (Test 3) weeks of vitamin C deprivation (Experimental Scheme 2, Fig. 2).

There was a significant main effect of time on gait as stride length of all the groups tended to decrease by week 3 compared to weeks 1 and 2 ($P < 0.01$) (Fig. 6B). There was a main effect of SVCT2 genotype ($P < 0.05$), but not of gulo genotype ($P = 0.17$), and also a Gulo × SVCT2 interaction ($P < 0.05$). Therefore, stride length was shorter in gulo$^{-/-}$/SVCT2$^{+/-}$ than in gulo$^{+/+}$/SVCT2$^{+/-}$ mice, but there was no effect of gulo genotype in SVCT2$^{+/+}$ mice. Similarly, lacking one allele for SVCT2 shortened stride length in gulo$^{-/-}$ mice but did not affect gait in gulo$^{+/+}$ mice. The greatest changes in stride length were thus seen in gulo$^{-/-}$/ SVCT2$^{+/-}$ mice. There were no other interactions among the factors of Time, Gulo and SVCT2 ($P > 0.38$).

Analysis of locomotor activity revealed significant main effects of time, both gulo and SVCT2 genotypes, and a time × Gulo interaction ($P < 0.05$) (Fig. 6C). However, these effects were all subsumed by a significant three-way interaction among the factors (time × Gulo × SVCT2, $P < 0.01$). All mice explored less across test sessions ($P < 0.01$) except the gulo$^{+/+}$ SVCT2$^{+/-}$ mice ($P = 0.32$). There was no clear pattern of differences among the groups across the first two testing sessions. At Test 3, when the greatest differences were expected, the gulo$^{-/-}$SVCT2$^{+/-}$ genotype combination again had the greatest effect on behavior as these mice explored less than the other groups ($P < 0.05$). The time × Gulo × SVCT2 interaction for locomotor velocity ($P < 0.05$) showed only that the gulo$^{-/-}$SVCT2$^{+/-}$ were faster than the other groups during Test 1 (supporting the greater distance traveled at this time point). There were no other velocity differences among the groups.

On the inverted screen test, there was a time× Gulo × SVCT2 interaction ($P < 0.05$), although there were no main effects of any of the individual factors of time, or gulo or SVCT2 genotype ($P > 0.1$) (Fig. 6D). Across the test sessions, Gulo$^{-/-}$/SVCT2$^{+/-}$ mice showed progressively shorter fall latencies ($P < 0.01$), whereas the other groups either improved or showed no change.

As expected, the wild-type, gulo$^{-/-}$, and SVCT2$^{+/-}$ groups improved or maintained latency to fall or first rotation with training on the Rota-rod from Tests 1 to 2 despite vitamin C deprivation in the gulo$^{-/-}$ group (Fig. 6E). During this period a decrease in fall latency was observed in the gulo$^{-/-}$/SVCT2$^{+/-}$ group. There was interaction of time × Gulo × SVCT2 ($P < 0.05$). The gulo$^{-/-}$/SVCT2$^{+/-}$ group was the only group that performed more poorly on the Rota-rod across 3 weeks of vitamin C deprivation ($P < 0.01$). This group also demonstrated shorter fall latencies compared to other test groups at the final session ($P < 0.05$). Gulo$^{+/+}$/ SVCT2$^{+/-}$ also performed more poorly than Gulo$^{+/+}$/SVCT2$^{+/+}$ control mice on day 3 ($P < 0.05$) supporting the role of SVCT2 genotype in performance of the Rota-rod task under these conditions.

Horizontal beam results produced a similar pattern (Fig. 6F), with a time × Gulo × SVCT2 interaction ($P < 0.01$) for test score. Again, the gulo$^{-/-}$/ SVCT2$^{+/-}$ mice were the only group to show diminishing performance across test sessions ($P < 0.01$), these mice also performed more poorly than the other three groups at Test 3 ($P < 0.01$). Although a similar trend was

observed in the wire hang test (Fig. 6G), the differences did not reach statistical significance. There were main effects of SVCT2 genotype ($P < 0.01$) because the $SVCT2^{+/-}$ mice performed more poorly than wild-type mice, and of time ($P < 0.05$) because overall scores diminished over the test session. Although this latter result was likely driven by the poorer performance of the $gulo^{-/-}/SVCT2^{+/-}$ mice, all other interactions between the factors were non-significant ($P > 0.062$) (Fig. 6G). Together, these results point to consistent deficits in motor and coordination skills in the $gulo^{-/-}/SVCT2^{+/-}$ mice.

A decrease in locomotor activity could also indicate increased anxiety. However, there were no significant differences in the percent of time mice in each group spent in the open arm of the zero maze indicating no evidence of increased anxiety (Fig. 6H). Alternation behavior in the Y-maze was normal, and did not differ according to genotype (60–65% alternation for each group; $P > 0.05$), indicating intact spatial working memory on this task in all groups of mice (results not shown). However, the total number of arm entries was lower in the $gulo^{-/-}/SVCT2^{+/-}$ group, consistent with their decreased tendency to explore (Fig. 6C).

Vitamins C and E content of brain cortex, cerebellum, and liver—The vitamin C content of cortex, cerebellum, and liver tissues was decreased in $gulo^{+/+}/SVCT2^{+/-}$ mice compared to wild-type ($gulo^{+/+}/SVCT2^{+/+}$) mice, despite the fact that these mice can synthesize their own vitamin C. As in Experiment 1, placing $gulo^{-/-}$ mice on a vitamin C-free diet for 3 weeks decreased vitamin C in each tissue type by 95, 85, and 87%, respectively. In the cortex, overall vitamin C was lower in $gulo^{-/-}$ compared to $gulo^{+/+}$ mice ($P < 0.01$), and lower in $SVCT2^{+/-}$ compared to $SVCT2^{+/+}$ mice ($P < 0.05$). There was also an interaction between Gulo × SVCT2 ($P < 0.05$) because vitamin C was decreased in all $SVCT2^{+/-}$ mice. Similarly, in $gulo^{-/-}$ mice, there was no further effect of SVCT2 genotype on vitamin C level ($P = 0.93$), but being heterozygous for SVCT2 did lead to lower vitamin C in $gulo^{+/+}$ cortex ($P<0.05$) (Fig. 7A). Further, in cerebellum, there was a main effect of both gulo ($P < 0.01$), and SVCT2 status ($P < 0.01$), while the interaction effect between the two genotypes was not significant ($P = 0.073$) (Fig. 7B). In liver, lower vitamin C was seen in $gulo^{-/-}$ mice than in $gulo^{+/+}$ controls ($P<0.01$) (Fig. 7C).

As expected, α-tocopherol levels in both cortex and liver were markedly decreased in all groups in this experiment, since all groups were on the vitamin E-deficient diet (compare Fig. 4D and 4E to Fig. 7D and 7F). In addition to this lowering due to vitamin E-deficient diets alone, α-tocopherol levels were significantly decreased in cortex (but not cerebellum and liver) in the three experimental groups compared to wild-type mice. In cortex, there was a main effect of both gulo and SVCT2 genotypes ($P < 0.01$), while in cerebellum there was only a main effect of gulo status ($P < 0.05$). Vitamin E appears to be retained in the cerebellum slightly better than the other tissues with the $gulo^{-/-}/SVCT2^{+/-}$ group having less than a 50% decrease in content compared to the control mice (Fig. 7D–7F), however, overall levels were lower in the cerebellum than the cortex. Liver vitamin E levels were often undetectable and no different between the groups (Fig. 7F).

Measures of oxidative stress—Cortex GSH levels in were increased in $gulo^{-/-}/SVCT2^{+/-}$ mice deprived of vitamin C compared to wild type and to $SVCT2^{+/-}$ mice (Fig. 8A). There was a main effect of both gulo and SVCT2 genotypes ($P < 0.01$). A similar trend was observed in cerebellum with the highest levels in the $gulo^{-/-}/SVCT2^{+/-}$ mice; however, the interaction between Gulo × SVCT2 was not significant ($P = 0.070$) (Fig. 8B). The same effect was not observed in liver (Fig. 8C). MDA levels were increased in cortex in all of the $gulo^{-/-}$ mice ($P < 0.01$) mice after vitamin C deprivation compared to the other two $gulo^{+/+}$ groups (Fig. 8D). There were no additional effects of SVCT2 genotype. MDA levels were not different in cerebellum among the genotypes, consistent with the slight changes in vitamin E content. In liver, MDA was increased compared to wild type in the two vitamin

C-deprived groups (Fig. 8F). There was a main effect of both gulo and SVCT2 (P's<0.01). Together, these results show that vitamin C deprivation, especially in the gulo$^{-/-}$/SVCT2$^{+/-}$ mice, causes a modest increase in oxidative stress in cortex and liver.

DISCUSSION

In Experiment 1, we investigated the effects of a combined vitamin E- and C-deficient diet on the neurobehavioral phenotype and oxidative stress measures in mice unable to synthesize their own vitamin C. In contrast to guinea pigs, mice were more tolerant of combined vitamins E and C deprivation in terms of neurological deficits and survival. Vitamin E deficiency takes approximately 10 months to cause motor symptoms in mice compared to just 4–5 weeks in guinea pigs.[20] In guinea pigs deprived of vitamin E for only 2 weeks and vitamin C for only 5–6 days, an ascending paralysis leading to death was observed[6] whereas as long as 10 months of vitamin E deprivation in normal mice does not shorten stride or impair Rota-rod performance, despite an 80% decrease in vitamin E levels in cortex and liver.[21] Thus, it was not surprising that in the present study a vitamin E-deficient diet alone for 16 weeks had no discernible effect on mouse gait or coordination, although it did decrease the latency to fall in the inverted screen test.

Vitamin C deprivation alone in Experiment 1 decreased locomotor velocity and impaired inverted screen test performance. Normal habituation to the testing chamber, as evidenced by decreased exploration, was accentuated by vitamin C deficiency alone in the current study. Although this change could be attributable to increased habituation in this group, it is more likely to reflect decreased voluntary locomotor activity due to physical weakness. Both vitamin C-deprived groups showed poor performance on the Rota-rod. However, this difference was already apparent following the vitamin E deprivation period, suggesting that it occurred by chance and was unrelated to the vitamin C deficiency. Thus, although there were motor performance defects in this experiment, they did not confirm that the combined deficiency at this level of deprivation worsened strength or coordination compared to the single deficiencies.

In contrast, vitamin E deprivation alone did increase both cortex and liver F_2-isoprostanes, an established and sensitive marker of lipid peroxidation due to oxidative stress.[22] Cortex F_4-neuroprostanes were also modestly increased by vitamin E deprivation independently of vitamin C status. For both markers, this could relate to the location of vitamin E in the lipid bilayer of cellular membranes, where it can readily convert lipid peroxide radicals to the less damaging hydroperoxides. Other markers of oxidative stress, including MDA, and GSH were variably affected by dietary deprivation of either vitamin. Although these findings support the contention that vitamin E deprivation in this manner causes a modest increase in brain lipid peroxidation, motor skills defects induced by short-term vitamin C deprivation were not worsened by vitamin E deficiency. Vitamin C deprivation consistently decreased vitamin E content in both the cortex and liver. These results support those of in vitro studies showing that vitamin C can recycle oxidized vitamin E as α-tocopherol in lipid bilayers.[5] Despite this role, vitamin C deficiency in mice did not increase F_2-iso- or F_4-neuroprostanes, the most specific markers of lipid peroxidation,[22] irrespective of vitamin E depletion.

Since deficiency of vitamin C rather than E was related to defects in motor performance in Experiment 1, to accentuate this a cellular deficit in vitamin C was added to the systemic deficiency of both vitamins in Experiment 2 by crossing gulo$^{-/-}$ mice with SVCT2$^{+/-}$ mice. These mice on the doubly deficient diet had clear and consistent defects in all of the motor coordination skills tests. Onlystride length and locomotor velocity remained unaffected. Decreased performance following vitamin C deprivation on the horizontal beam and Rota-

rod suggest that the defects were not simply due to decreased strength, but rather to a motor coordination defect reflecting neurologic impairment. Although an increase in anxiety could have confounded the coordination tests and accounted for the decrease in locomotor activity observed,[23,24] data from the zero- maze test failed to show this, since the gulo$^{-/-}$/ SVCT2$^{+/-}$ mice spent a similar percentage of time in the closed area to the other groups. α-Tocopherol levels were substantially decreased in all mice in Experiment 2 compared to the vitamin E-supplemented groups in Experiment 1, so it is possible that this exacerbated the motor skills defects. However, since vitamin E deprivation did not affect motor performance independently of vitamin C in Experiment 1, the contribution of vitamin E deficiency to the observed motor skills defects would seem to be minimal.

Vitamin C levels in cortex, cerebellum, and liver were decreased by about 50% in the gulo$^{+/+}$/ SVCT2$^{+/-}$ mice. This shows that this transporter is rate-limiting for vitamin C uptake and content in both brain and liver. This is surprising (and unexplained) for liver, since these mice can synthesize their own vitamin C in this organ. Vitamin C levels were more severely decreased in both brain areas and liver when the SVCT2$^{+/-}$ genotype was combined with the gulo$^{-/-}$ mice that were deprived of vitamin C. However, vitamin C contents of cortex and liver, although low, were no different than in gulo$^{-/-}$ mice deprived of vitamin C. Considering that the defects in motor performance observed might arise in the cerebellum, we measured levels of both vitamins, GSH, and MDA in cerebellum. Although levels of both vitamins were decreased as expected, both GSH and MDA were increased in cortex and not cerebellum, indicative of a greater oxidative stress in cortex. In previous studies with Gulo$^{-/-}$ mice deprived of vitamin C, the cerebellum had better retention of vitamin C and fewer changes in F2-isoprostane levels compared to the cortex.[11] Data in the present study support the idea that the cerebellum is better protected against vitamin C depletion and subsequent increased oxidative stress.

The present results suggest that although vitamin E deficiency for more than 16 weeks modestly increased lipid peroxidation in mouse brain and liver, it did not worsen the small and inconsistent motor skills deficits due to vitamin C deficiency. Motor skills deficits became more extensive when cellular vitamin C deficiency due to a decrease in cellular vitamin C uptake was superimposed on vitamin E deprivation and then followed by an acute deprivation of vitamin C. Even though the mice in this study had no overt physical signs of scurvy beyond weight loss, it is likely that cellular deficiency of vitamin C due to partial loss of the SVCT2 in the gulo$^{-/-}$/ SVCT2$^{+/-}$ mice hastened deficits in motor and coordination skills. The latter are likely to be early manifestations of acute scurvy. A recent study of whole-body vitamin C deficiency in zebrafish showed altered cellular energy metabolism homeostasis by activation of the purine nucleotide cycle.[25] This suggests that vitamin C deficiency may lead to inadequate energy production which could also con- tribute to the behavioral deficits observed in this study. Further studies will have to be performed to determine to role of energy homeostasis in this behavioral model.

Although adult gulo$^{-/-}$ mice require significant systemic and cellular vitamin C deficiency to manifest motor and coordination skills defects, developing mice appear to be more sensitive. In fact, gulo$^{-/-}$ dams that have been mated with gulo$^{-/-}$ sires have progeny that manifest similar motor skills defects as young adults, with increases in oxidative stress markers.[11] In this study, all gulo$^{-/-}$ mice were derived from such matings. Although these mice may not be as capable as wild-type mice would be in completing the motor skills tasks, the dietary and genetic modifications in adult gulo$^{-/-}$ mice in the present study clearly worsened any existing defects.

In conclusion, in regard to our original question of whether there is a neurological phenotype that is associated with oxidative stress and/ or vitamin C deficiency, our results show that a

16-week deficiency of vitamin E modestly increased oxidative stress markers, but did not affect mouse motor skills or coordination. Vitamin C deficiency in addition to vitamin E deficiency only minimally increased oxidative stress markers compared to the single-deficiency model. However, vitamin C deficiency did begin to impair motor and coordination skills, deficits in which become more evident when the cellular uptake of vitamin C is decreased. Whereas the latter may be an early manifestation of scurvy, its mechanism does not appear to be due to an increase in lipid peroxidation and remains to be determined.

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ABBREVIATIONS

SVCT2	sodium-dependent vitamin C transporter 2
Gulo	Gulonolactone oxidase

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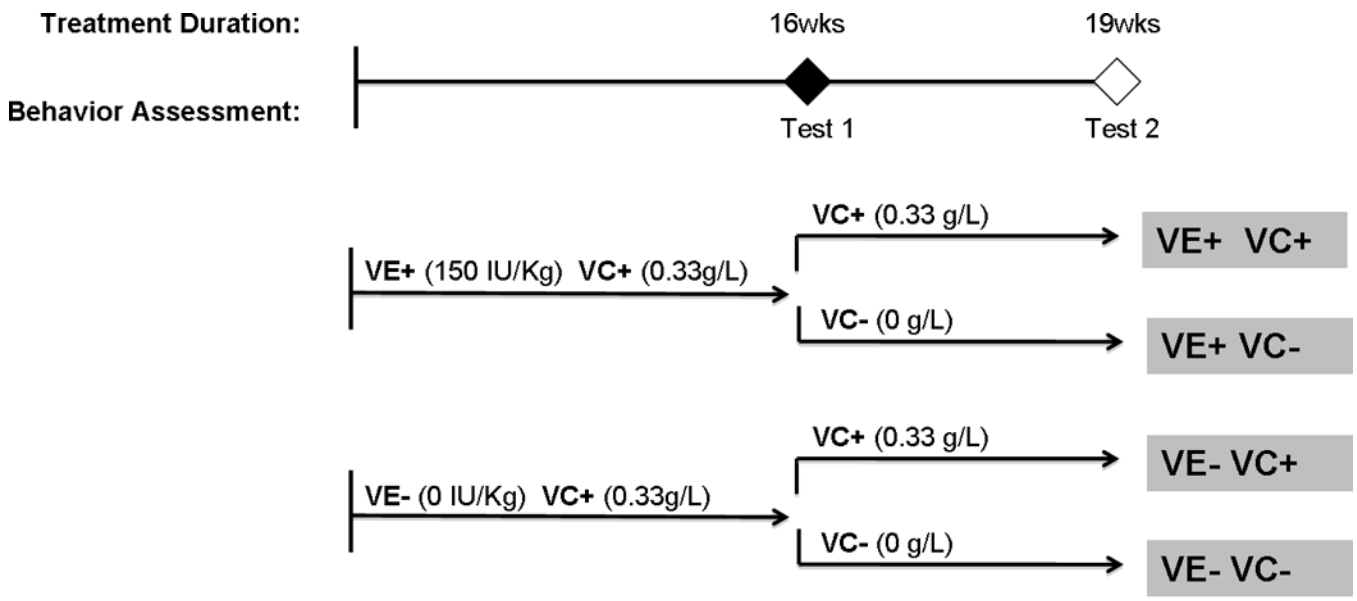

* Western diet for all groups included 16% Tocopherol-Stripped Lard; 0.2% Cholesterol; 34% Sucrose

** All mice were Gulo$^{-/-}$ genotype

Figure 1. Experimental Scheme 1: combined vitamins C- and E-deficient diets

Mice started vitamin E treatments at 4–5 weeks of age with continued vitamin C supplementation. After 16 weeks of vitamin E treatments mice were tested (Test 1) to observe changes in behavior. Vitamin C treatment lasted 3 weeks, during which another set of behavior tests were performed (Test 2). Mice were then sacrificed for biochemical analysis.

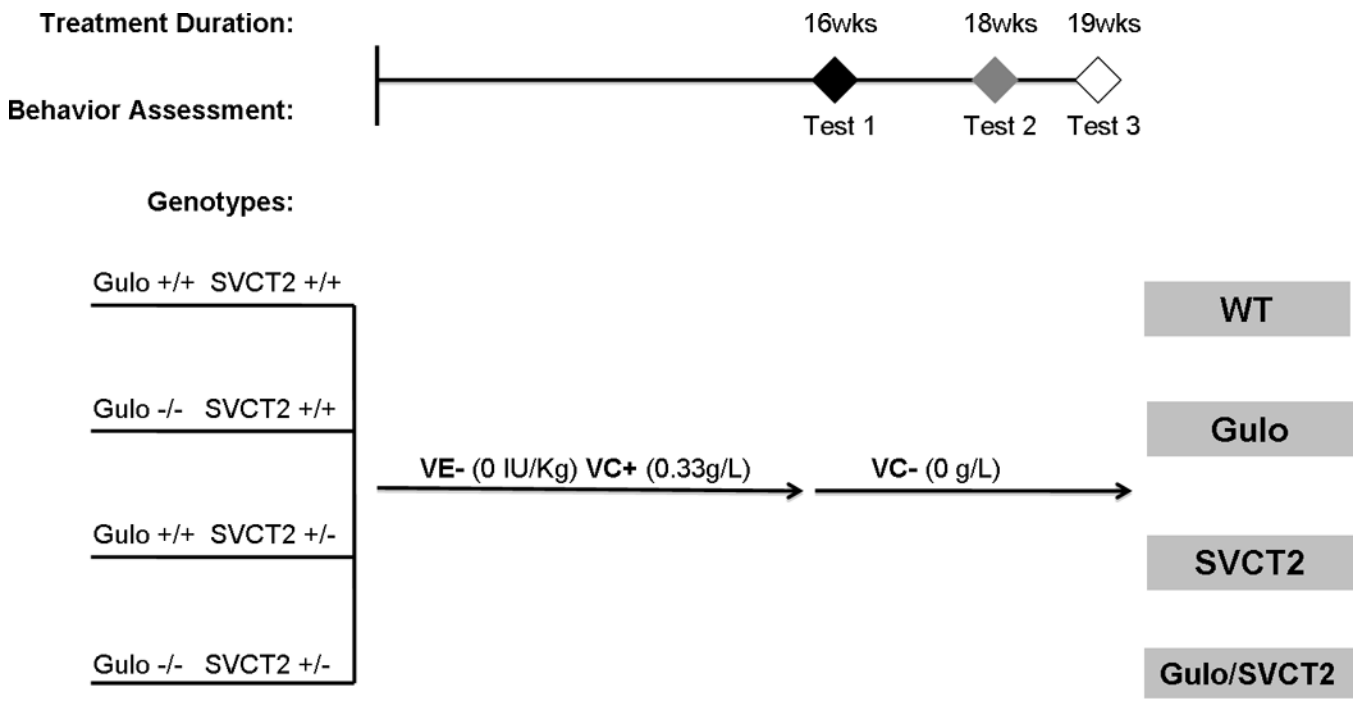

Figure 2. Experimental Scheme 2: combined vitamin deficiency effects on gulo$^{-/-}$ SVCT2$^{+/-}$ mice

Mice started vitamin E deprivation at 4–5 weeks of age with continued vitamin C supplementation. After 16 weeks mice were tested (Test 1) to observe changes in behavior. Vitamin C treatment lasted 3 weeks, after which another set of behavior tests were performed after 2 weeks and followed-up with another at 3 weeks (Tests 2 and 3, respectively). Mice were then sacrificed for biochemical analysis.

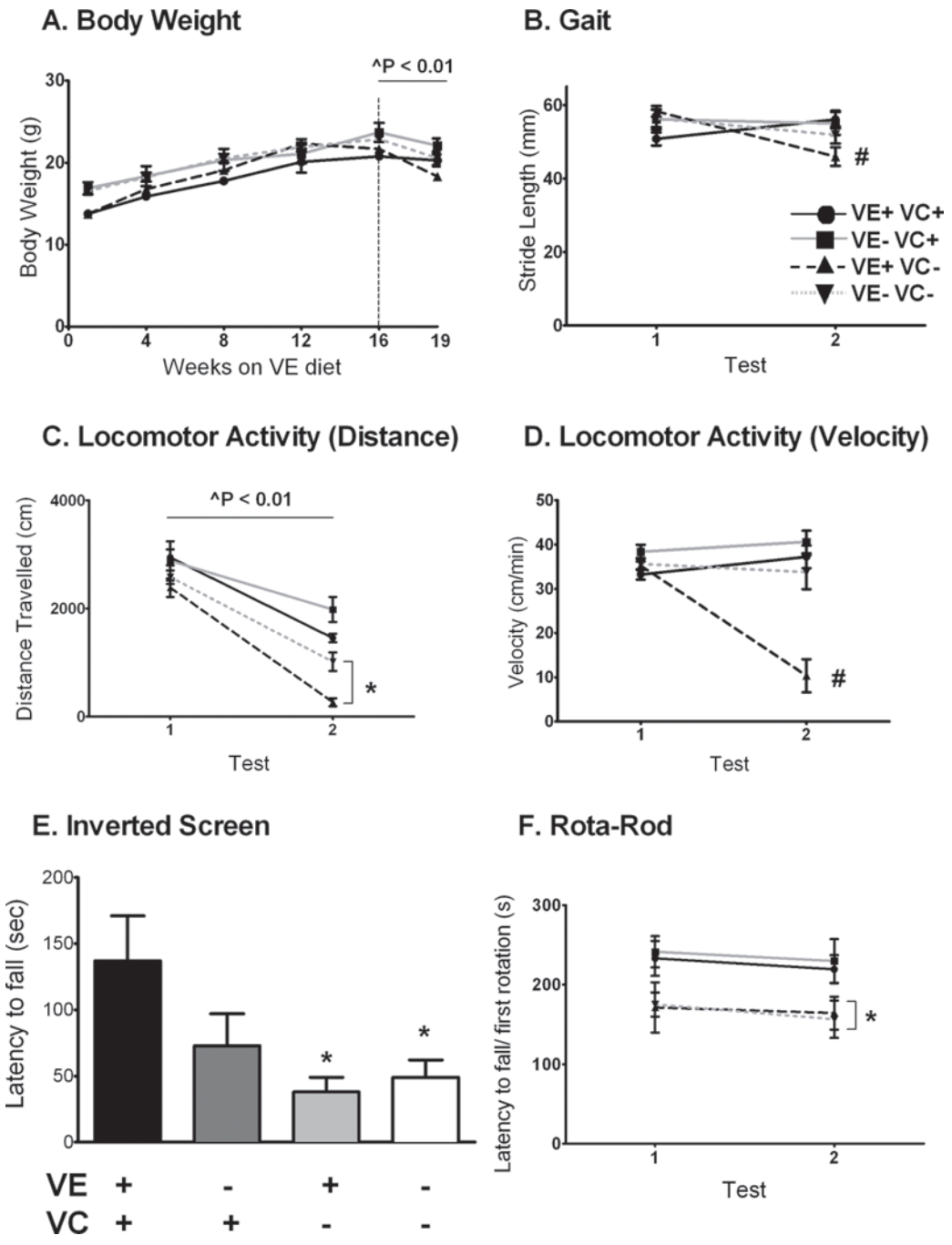

Figure 3. Behavioral phenotype of combined vitamin E and C deficiency

Behavioral assessments included changes in body weight (A) stride length (B) locomotor activity (C), and motor coordination on the Rota-rod (D). Displayed are group means ± standard error of means. ° Denotes a significant difference between Tests 1 and 2.

* Denotes a significant difference between the respective VC+ and VC− groups for Test 2 ($P < 0.05$).

# Denotes a significant difference between VC+ and VC− groups over time ($P < 0.05$).

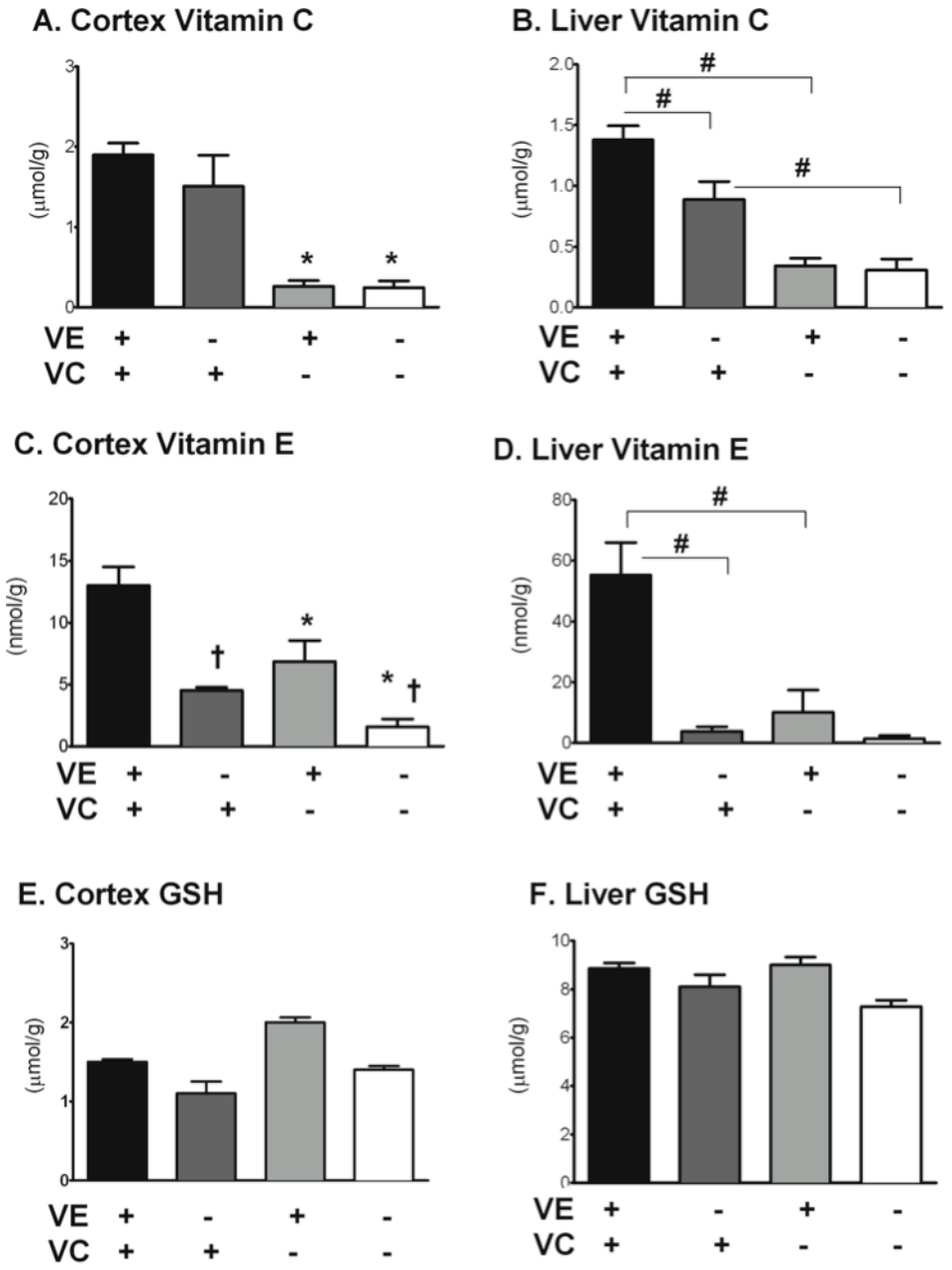

Figure 4. Combined vitamins C and E deficiency decreases antioxidant levels in cortex and liver

Biochemical assessment included measurements of vitamin C in cortex (A) and liver (B) and GSH in cortex (C), and liver (D).

* Denotes a significant difference between the respective VC+ and VC− groups ($P < 0.05$).

† Denotes a significant difference between VE+ and VE− groups ($P < 0.05$).

# Denotes a significant difference between the two diet groups indicated with brackets ($P < 0.05$).

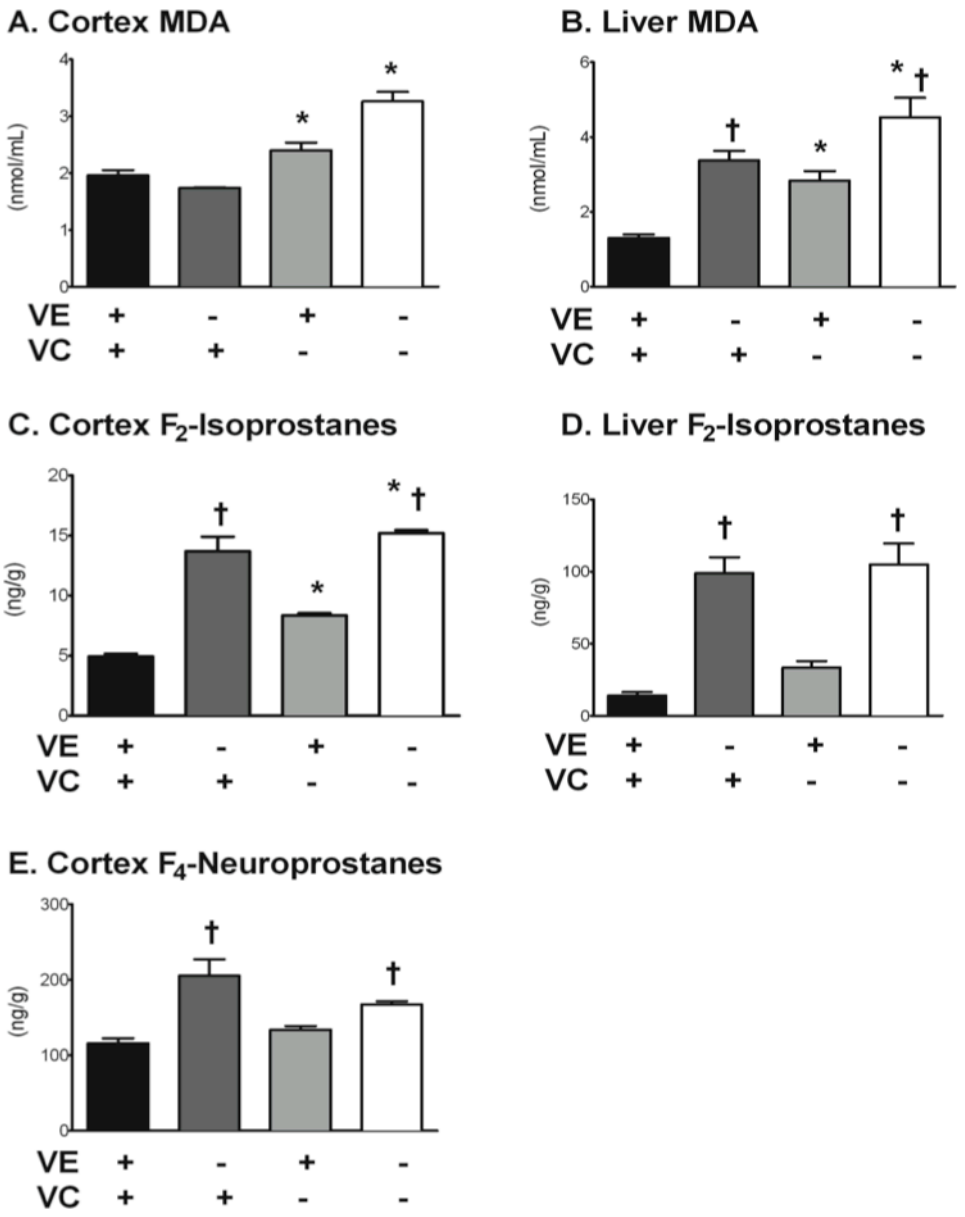

Figure 5. Vitamin E deficiency induces oxidative stress

Biochemical assessment included measurements of malondialdehyde in cortex (A) and liver (B); F2-isoprostanes in cortex (C) and liver (D); and F4-neuroprostanes in cortex (E).

* Denotes a significant difference between VC+ and VC− groups ($P < 0.05$).

† Denotes a significant difference between VE+ and VE− groups ($P < 0.05$).

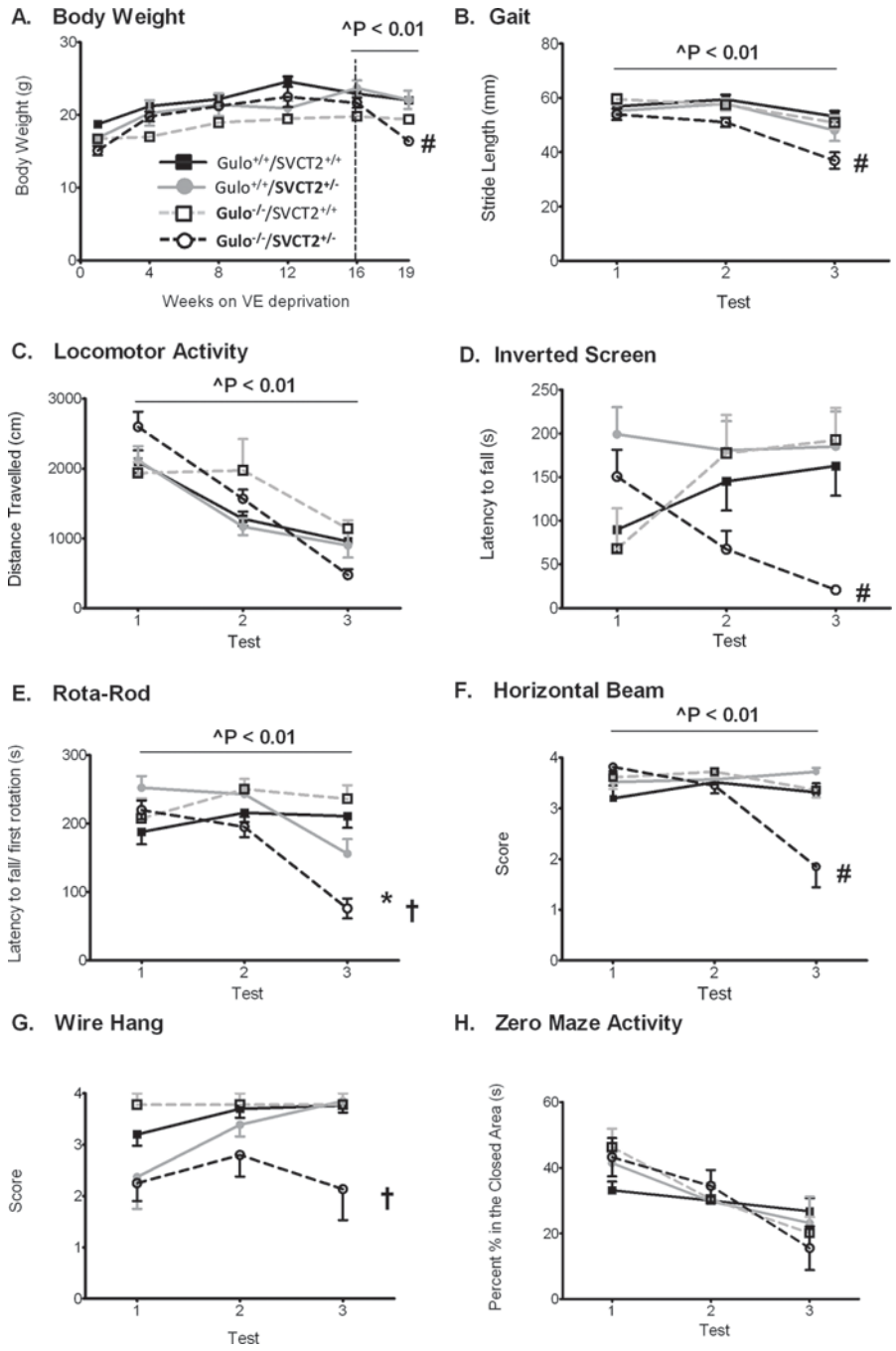

Figure 6. Genetic modification of gulo$^{-/-}$/SVCT2$^{+/-}$ alters behavioral phenotype

Behavioral assessments included changes in body weight (A) stride length (B) locomotor activity (C) and motor coordination including inverted screen (D) Rota-rod (E) horizontal beam (F) wire hang (G), and zero-maze (H).

* Denotes a significant difference between gulo$^{+/+}$ and gulo$^{-/-}$ groups ($P < 0.05$).

† Denotes a significant difference between SVCT2$^{+/+}$ and SVCT2$^{+/-}$ groups ($P < 0.05$).

# Denotes a gulo × SVCT2 interaction effect ($P < 0.05$).

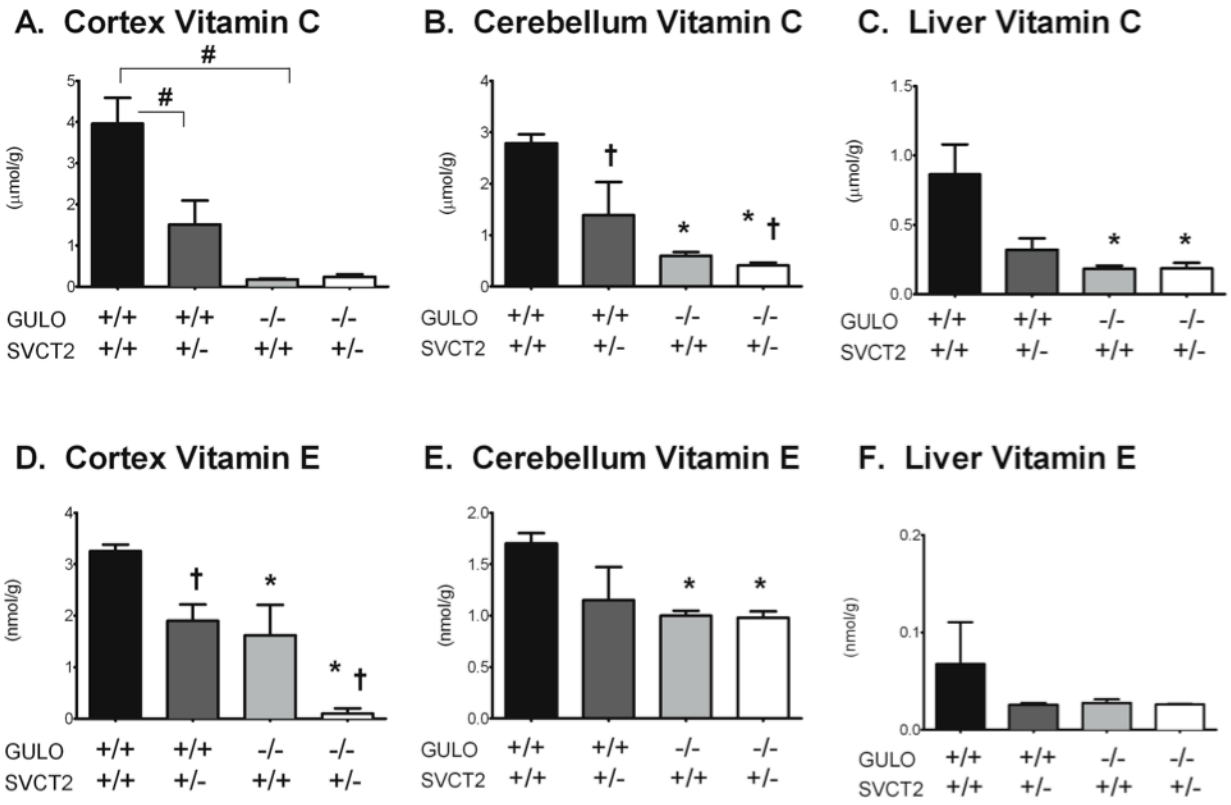

Figure 7. Combined vitamin C and E deficiency decreases antioxidants in cortex, cerebellum, and liver in gulo$^{-/-}$/SVCT2$^{+/-}$

Biochemical assessment included measurements of vitamin C in cortex (A) cerebellum (B), and liver (C); vitamin E in cortex (D) cerebellum (E), and liver (F).

* Denotes a significant difference between gulo$^{+/+}$ and gulo$^{-/-}$ groups ($P < 0.05$).

† Denotes a significant difference between SVCT2$^{+/+}$ and SVCT2$^{+/-}$ groups ($P < 0.05$).

# Denotes a significant difference between the two genotype groups indicated with brackets ($P < 0.05$).

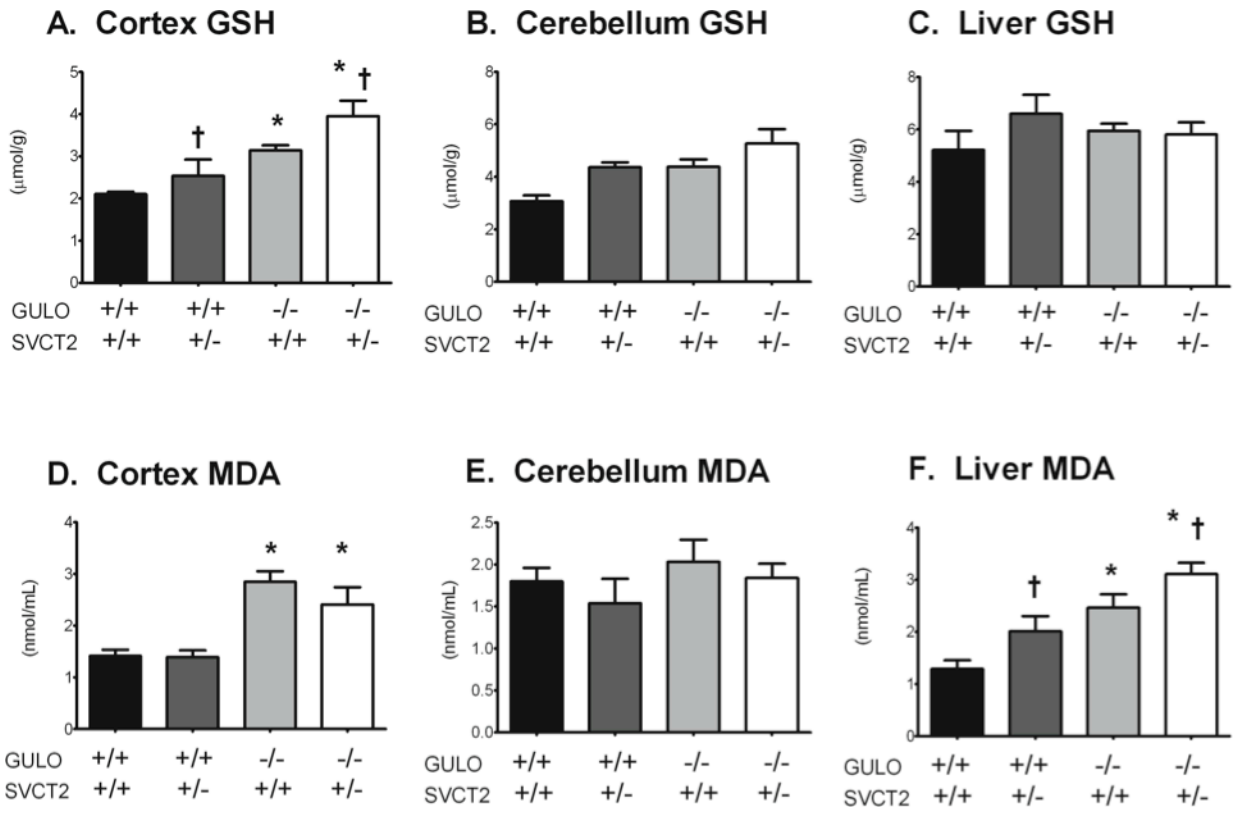

Figure 8. Combined vitamin C and E deficiency increases oxidative stress in cortex and liver in gulo$^{-/-}$/SVCT2$^{+/-}$

Oxidative stress assessment included measurements of GSH in cortex (A) cerebellum (B), and liver (C); malondialdehyde in cortex (D) cerebellum (E), and liver (F). Displayed are group means ± standard errors of means.

* Denotes a significant difference between gulo$^{+/+}$ and gulo$^{-/-}$ groups ($P < 0.05$).

† Denotes a significant difference between SVCT2$^{+/+}$ and SVCT2$^{+/-}$ groups ($P < 0.05$).